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The contents in this manual are being continuously updated

RESEARCH MANUAL

ORISOD ENZYME®

Enhance Endogenous Antioxidant Enzymes Complex

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SUMMARY

CLINICAL TRIALS AND TESTINGS

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1. INTRODUCTION

1.1. ORISOD ENZYME® SCIENCE

ORISOD Enzyme® is a fermented ingredient formulated to enhance body antioxidant status and to prevent aging-related diseases. It formula combines a mixture of bioactives from 2 edible Mediterranean plants: **olive and rosemary**.

The complex is fermented using a green technology based on vegetal ferments.This fermentation offers a highly effective bioactive compared to raw extract.

Mediterranean diet

Interest in the health benefits of Mediterranean diet has increased tremendously, because of its link with greater longevity and lower cardiovascular disease rate, cancer, and type II diabetes.

The Orisod project was developed in considering 3 importants factors:

(1) Safety

Product safety is a priority and **ORISOD Enzyme®** was developed with ingredients: Olive leaf and rosemary which are 100% plants origin, grown in farms with strict quality control. Pharmaceutical testing are realized for all our products to choose the optimum efficacy while having always the safety under control.

(2) Bioavailability

Bioavailability is a key step in ensuring the efficacy of bioactive compounds. Sometimes, bioactives are poorly absorbed from the intestine, highly metabolized, or rapidly eliminated.

ORISOD Enzyme® uses the ADS (Advanced Delivery System) technology which helps delivering the actives to the researched target. The ingredients lead directly to the site where they are really effective in the body: the interior of cells and mitochondria.

(3) Efficacy

ORISOD Enzyme® extracts are mixed in an innovative way that maintains stability and offer better results at the long term.

The complex rich in metabolites, obtained after the fermentation process, enhances the efficacy of endogenous enzymes at the cellular level (SOD, Catalase and Gpx) and reduces lipid peroxidation, supports brain energy and supports liver detoxification.

1.2. COMPOSITION

The efficacy of the product in the claimed effect is centred on the active ingredients of the formula:

Ingredients	Active Substances
Olive extract	Oleuropein Oleacein Hydroxytyrosol
Rosemary extract	Carnosic Acid & Carnosol Rosemarinic Acid

Characteristics of the food ingredient ORISOD Enzyme®

The recommended dosage of ORISOD Enzyme® is 500 mg/day (2 times 250mg, best taken at lunch and dinner time), for a total 200 mg of active ingredient and 300 mg of excipient.

1.2.1 Olive Leaf

Olive leaf *(Olea europaea)* contains several potentially bioactive compounds that may have various beneficial health properties. However, two of the main components of olive leaves thought to be responsible for much of its beneficial effects are oleuropein and oleacein.

Studies have shown these components to exert a range of antioxidant, antihypertensive, anti-inflammatory, hypoglycemic, and hypocholesterolemic properties.

1.2.2 Rosemary

Rosemary *(Rosmarinus officinalis)* leaves possess a variety of bioactive agents, including antioxidants anti-tumor and anti-inflammatory.

The main relevant constituents are composed of vast numbers of polyphenolics, including carnosic acid, carnosol, rosemarinic acid, ursolic acid, etc.

Among these, carnosic acid (CA) (a phenolic diterpene compound), and carnosol are the most potent antioxidant constituents (about 90% of antioxidant activity).

1.2.3 Fermentation issued metabolites

The complex contains hundreds of several metabolites and trace elements. The main amino acids present are :

L-glutamine is one of 20 amino acids in the body that is encoded by the standard genetic code and is the most abundant amino acid in the body.

Serine is an amino acid derived from the amino acid glycine. It exits in L-serine and D-serine forms. It is important to overall good health, both physical and mental.

Methionine is a protein-based amino acid which assists with metabolic function, breaks down fat, and is the primary source of sulfur in the body.

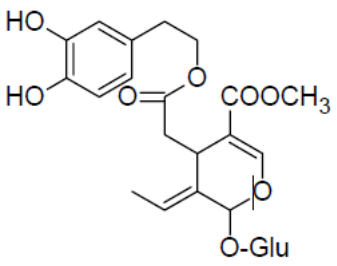
2. BIOACTIVE COMPOUNDS

2.1. OLIVE LEAVES

2.1.1. Oleuropein

Oleuropein is the major phenolic constituent of the olive (Olea europaea) and is present throughout the different parts of the olive tree: fruit, leaves and bark.

Is is the most abundant polyphenol and the ester of elenoic acid with 3,4'-dihydroxyphenylethanol (hydroxytyrosol). This secondary metabolite responsible for the characteristic bitter, pungent taste of the olive oil.



Oleuropein Chemical structure

Molecular formula: $C_{25}H_{32}O_{13}$

Molecular weight: 540.51 g/mol

CAS Registry Number: 32619-42-4.

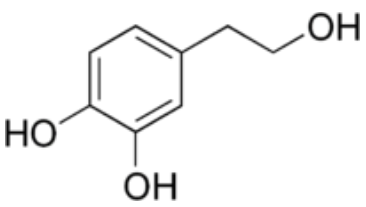
Oleuropein is represented by the chemical structure on the left.

Oleuropein has a powerful anti-bacterial and anti-viral effects. It has proven to be useful in cases of yeast and fungal infections, herpes, chronic fatigue, allergies, psoriasis and many other pathogens. In addition, it has been shown to lower blood sugar, normalize arrhythmias, inhibit oxidation of LDL (the bad cholesterol), and relax arterial walls, thereby helping to lower blood pressure. Other benefits are that it boosts energy and helps increase the body's immune response..

2.1.2. Hydroxytyrosol

Hydroxytyrosol (3, 4-dihydroxyphenylethanol; DOPET) is a phytochemical with antioxidant properties present naturally in olives. It is responsible together with other phenolic compounds as oleuropein for its bitter taste.

Hydroxytyrosol is a metabolite obtained from oleuropein hydrolysis. It is incorporated in the aglycon of oleuropein and is thought to be released from this glycoside owing to the action of cellular esterase or acidic catalysis.



Hydroxytyrosol Chemical structure

Molecular formula: $C_8H_{10}O_3$

Molecular weight: 154.16 g/mol

CAS Registry Number: 10597-60-1

Hydroxytyrosol is represented by the chemical structure on the left.

Hydroxytyrosol has a number of health benefits in humans which main is fighting harmful free radicals thanks to its action as potent inhibitor of metal-induced oxidation of low density lipoprotein. Metal-independent oxidation is also significantly retarded by hydroxytyrosol. The antioxidant activities of hydroxytyrosol, which has been proven to be more effective than BHT or vitamin E, were further confirmed, by the use of stable free radicals, such as DPPH

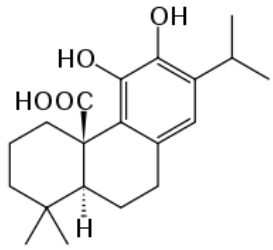
It also acts as reducing risk of cancer, reducing risk of diabetes, and slowing ageing process. It is also acts as an antibacterial and can strengthen the immune system.

The safety profile of hydroxytyrosol appears to be excellent: no untoward effects have been demonstrated even at very high doses.

2.2. ROSEMARY

2.2.1. Carnosic Acid & Carnosol

Rosemary extracts contain several compounds which have been shown to present antioxidative functions.



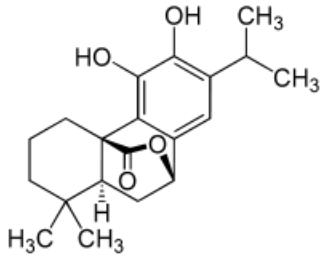
Carnosic Acid chemical structure

Molecular formula: $C_{20}H_{28}O_4$

Molecular weight: 332.42 g/mol

CAS Registry Number: 3650-09-7

Carnosic Acid is represented by the chemical structure on the left.



Carnosol chemical structure

Molecular formula: $C_{20}H_{26}O_4$

Molecular weight: 330.42 g/mol

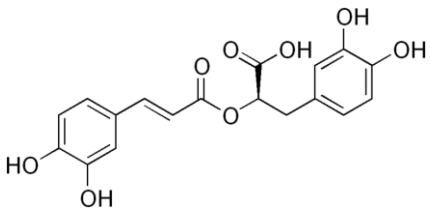
CAS Registry Number: 5957-80-2

Carnosol is represented by the chemical structure on the left.

These compounds belong mainly to the classes of phenolic acids, flavonoids, diterpenoids and triterpenes. The principal antioxidative components of the extracts are the phenolic diterpenes carnosol and carnosic acid.

2.2.2. Rosemarinic Acid

Rosmarinic acid is a natural phenol antioxidant carboxylic acid and a fundamental compound of *Rosmarinus officinalis*. Chemically, rosmarinic acid is an ester of caffeic acid with 3,4-dihydroxyphenyl lactic acid.



Rosmarinic acid chemical structure

Molecular formula: $C_{18}H_{16}O_8$

Molecular weight: 360.31 g/mol

CAS Registry Number: 20283-92-5

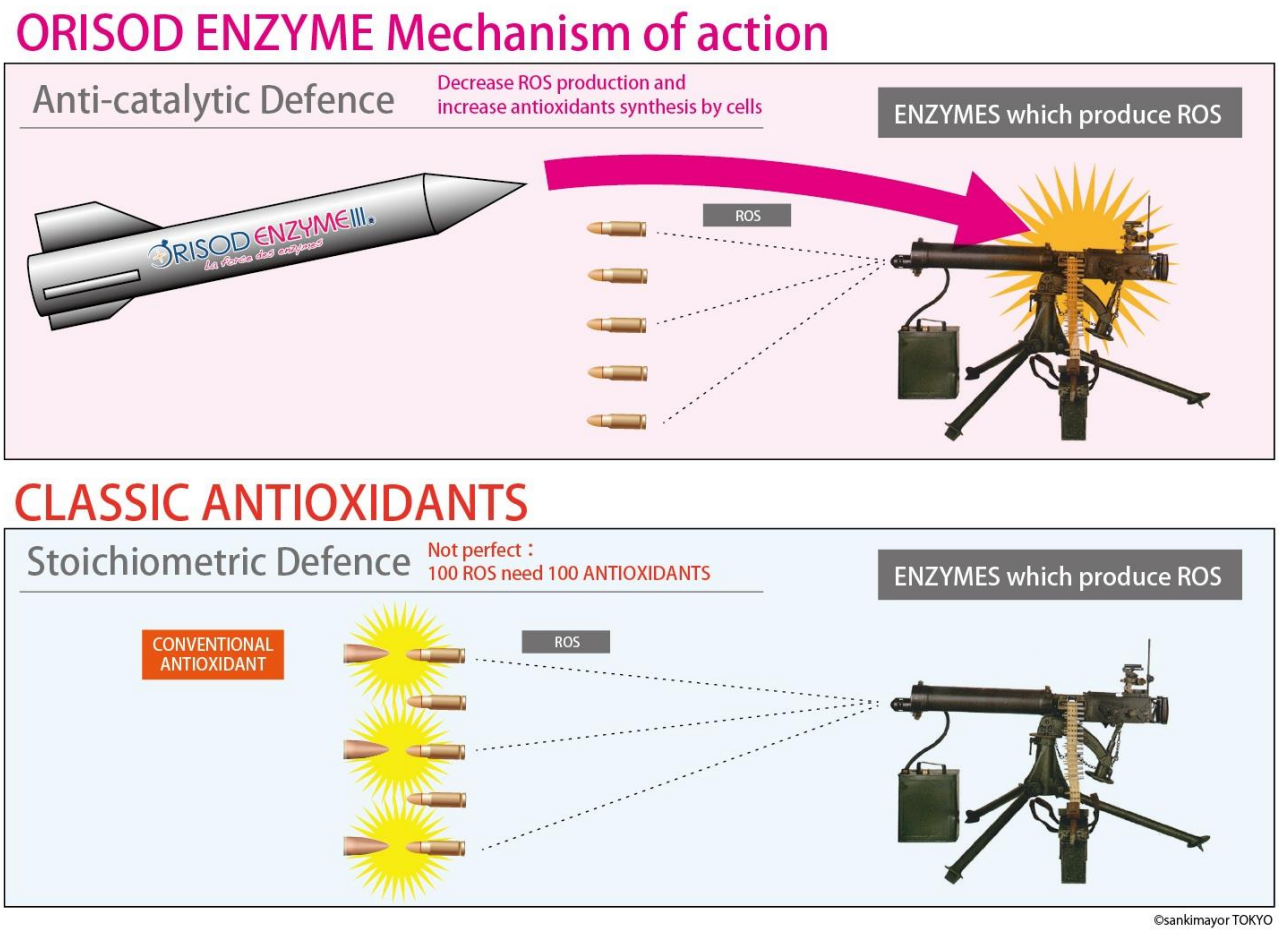
Rosemarinic Acid is represented by the chemical structure on the left.

The biosynthesis of rosmarinic acid starts with the amino acids l-phenylalanine and l-tyrosine. All eight enzymes involved in the biosynthesis are known and characterised and cDNAs of several of the involved genes have been isolated.

Rosmarinic acid has a number of interesting biological activities: anti-microbial, anti-inflammatory and antioxidant. The anti-inflammatory properties of rosmarinic acid are based on the inhibition of lipoxygenase and cyclooxygenases, and on the interference of rosmarinic acid with the expression of inflammatory cytokines. Rosmarinic acid has also antioxidant properties and can act as scavenger of free radicals in biological systems.

3. MECHANISMS OF ACTION

Rather than classical antioxidants like vitamins, **ORISOD Enzyme®** mechanims consist of enhancing endogenous enzymes, which is more effective and faster for improving body antioxidants status.



3.1. ANTIOXIDANT EFFECT

One of most famous health benefit recognized by scientists about olive leaf and rosemary is the antioxidant effect. Olive leaf has antioxidant properties associated with Oleuropein, hydroxytyrosol, and extracts of Olea europaea leaf (containing 19% oleuropein, 1.8% flavonoid glycosides, and 3,4-dihydroxy-phenethyl esters). Rosemary has antioxidant effect properties associated with carnosic acid and carnosol.

Free radicals and ROS

A free radical is an atom, molecule, or compound that is highly unstable because of its atomic or molecular structure (i.e., the distribution of electrons within the molecule). As a result, free radicals are very reactive as they attempt to pair up with other molecules, atoms, or even individual electrons to create a stable compound. To

achieve a more stable state, free radicals can “steal” a hydrogen atom from another molecule, bind to another molecule, or interact in various ways with other free radicals.

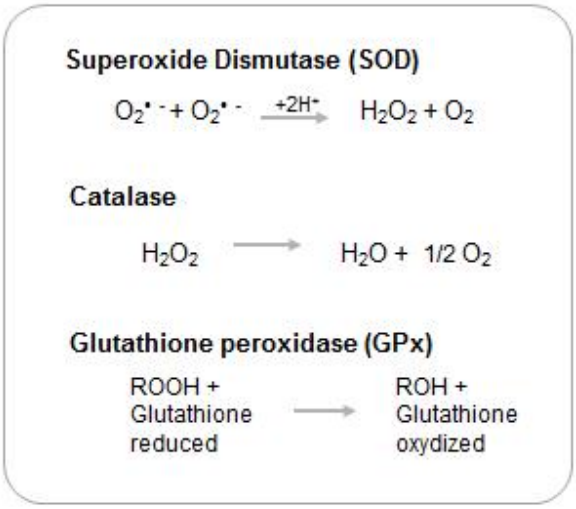
One chemical element frequently involved in free radical formation is oxygen. Molecular oxygen (O_2) is essential for cell function because it plays a pivotal role in a series of biochemical reactions occurring in the respiratory chain, which is responsible for most of the production of adenosine triphosphate (ATP), which provides the energy required for a multitude of cellular reactions and functions.

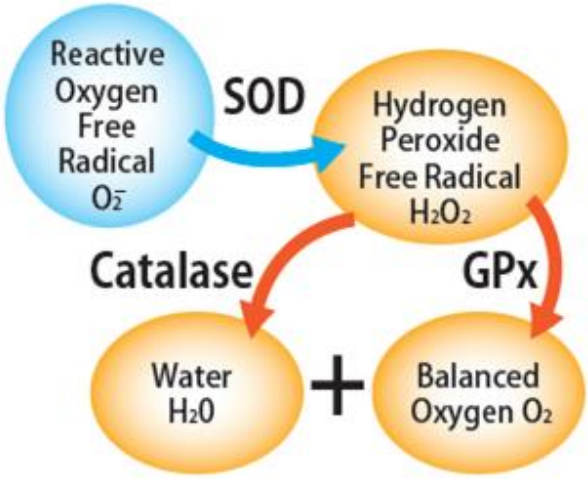
Molecular oxygen can accept a total of four electrons, one at a time, and the corresponding number of protons to generate two molecules of water. During this process, different oxygen radicals are successively formed as intermediate products, including superoxide (O2); peroxide (O2), which normally exists in cells as hydrogen peroxide (H2O2); and the hydroxyl radical (·OH). Superoxide, peroxide, and the hydroxyl radical are considered the primary ROS and have sparked major research on the role of free radicals in biology and medicine. However, because they are unstable and rapidly react with additional electrons and protons, most of these ROS are converted to water before they can damage cells. It has been estimated that only about 2 to 3 % of the O2 consumed by the respiratory chain is converted to ROS. Nevertheless, the toxic effects of oxygen in biological systems—such as the breakdown (i.e., oxidation) of lipids, inactivation of enzymes, introduction of changes (i.e., mutations) in the DNA, and destruction of cell membranes and, ultimately, cells—are attributable to the reduction of O2 to ROS.

Oxidative stress

Because ROS form naturally during many metabolic processes, cells have developed several protective mechanisms to prevent ROS formation or to detoxify the ROS. These mechanisms employ molecules called antioxidants. Under certain conditions, ROS production is enhanced and/or the level or activity of antioxidants is reduced. The resulting state— which is characterized by a disturbance in the balance between ROS production on one hand and ROS removal and repair of damaged complex molecules (such as proteins or DNA) on the other—is called oxidative stress.

In humans, oxidative stress is involved in many diseases, such as atherosclerosis, Parkinson's disease, heart failure, myocardial infarction, Alzheimer's disease, and chronic fatigue syndrome, but short-term oxidative stress may also be important in prevention of aging by induction of a process named mitohormesis. Reactive oxygen species can be beneficial, as they are used by the immune system as a way to attack and kill pathogens.





Antioxidant fight off oxidative stress

Adequate amounts of antioxidants are needed to fight off damaging free radicals and oxidative stress.

ORISOD Enzyme® enhances the activity of two main endogenous antioxidant enzymes: SOD, and GPx, and improves antioxidant defense. Furthermore, **ORISOD Enzyme**® significantly decreases lipid peroxidation level and oxLDL levels: which also shows less formation of free radicals. **ORISOD Enzyme**® supplies the free radical with replacement for its missing electrons.

3.2. ALDH ENZYMES AND ENERGY PRODUCTION IN MITOCHONDRIA

Aldehyde dehydrogenases (ALDHs) are important enzymes that eliminate toxic aldehydes by catalysing their oxidation to non-reactive acids.

ALDH2 is expressed abundantly in the liver and lung, and is also present in organs that require high mitochondrial capacity for oxidative ATP generation such as heart and brain.

Alcohol detoxification ALDH2 Enzyme

ALDH2 is best known for its ability as a detoxifying enzyme of acetaldehyde, an intermediate of ethanol metabolism.

When you consume alcohol, your body detoxifies it and then extracts calories from it. It's a complex process that involves many different enzymes and multiple organs, although most of the process takes place in the liver. First, an enzyme called alcohol dehydrogenase converts the alcohol into another chemical called acetaldehyde; another

enzyme—cleverly called acetaldehyde dehydrogenase—converts the acetaldehyde into acetate. And a third enzyme converts that into fat, carbon dioxide, and water. (The calories synthesized from alcohol are generally stored as fat—beer bellies really do come from beer.)

Asian population ADLH2*2 mutant allele

More than 40% of the East Asians population carries a common ALDH2*2 mutant allele, which results in a dramatic reduction in the enzymatic activity when compared with the ALDH2*1, wild-type allele carrier of this mutant ALDH2 has a characteristic acetaldehyde-induced facial flushing when drinking alcohol.

The affected world population of ALDH2*2 is estimated to be at least 540 million or 8% of the world population, it is warranted that health risk for cardiac diseases be re-evaluated in ALDH2*2 carriers.

ORISOD Enzyme® action on aldehydes load reduction

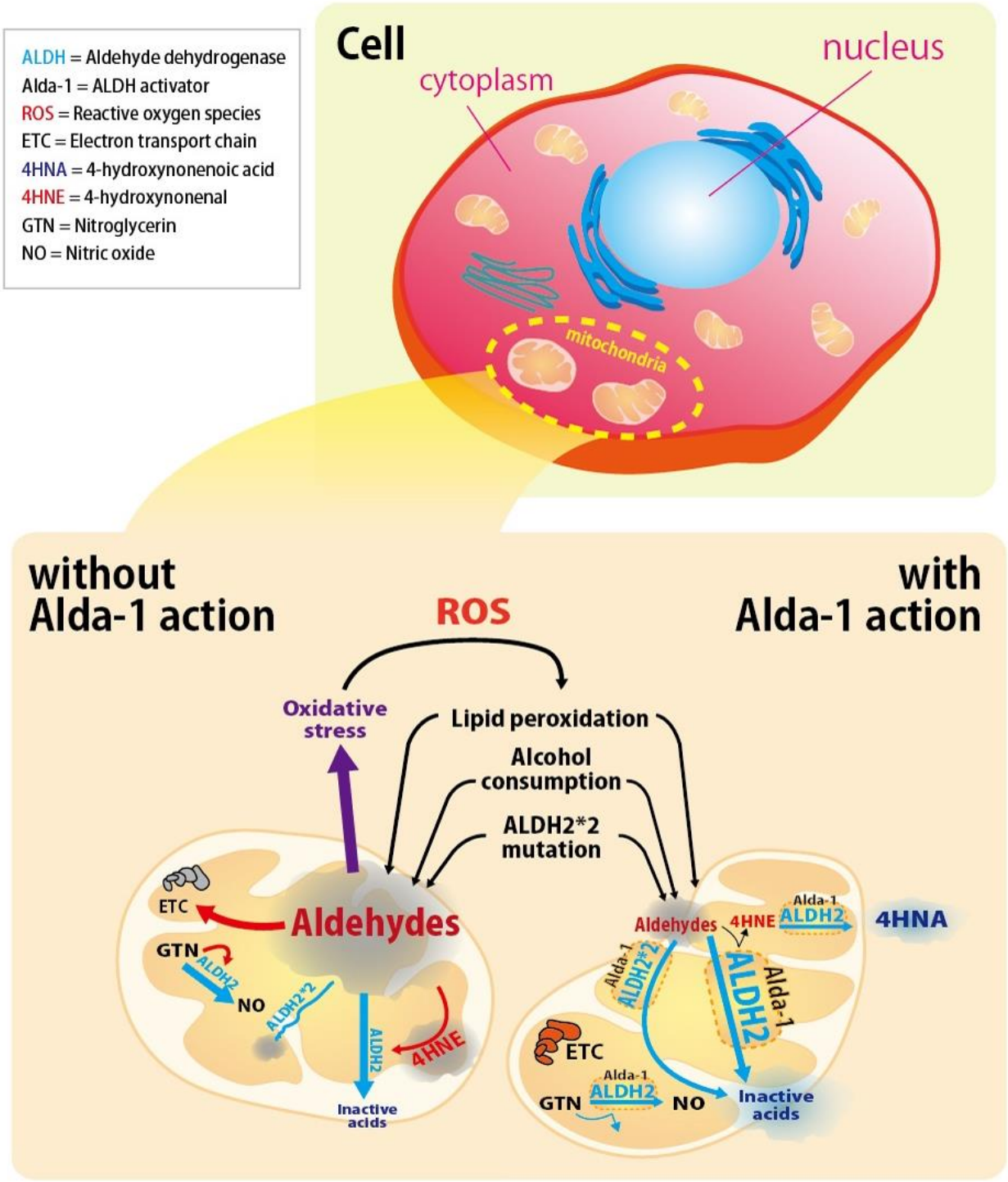
In the mitochondria, the aldehydes load, increased by environment, alcohol and food, induces the inactivation of some macromolecules and increases oxidative stress.

ORISOD Enzyme® enhances the activity of the ALDH2 agent Alda-1 which will decrease the aldehydic load by enhancing the conversion of aldehydes to non-reactive acid.

Alda-1 blocks ALDH2 inactivation by both aldehydes and GTN, and increases the cell's natural ability to protect from oxidative stress.

Reduction in aldehydic load increases ATP generation to regulate cellular metabolism. Mitochondria are regenerated; the fat and sugar are absorbed for energy production.

ALDH2 reduces aldehydic toxicity in mitochondria



3.3. LIVER DETOXIFICATION

Internal detoxification is an on-going process that our bodies perform on a daily basis. Our metabolic processes continuously encounter and dispose of a variety of toxins and poisons.

The liver is the largest gland in the body and is an important organ that performs many functions necessary for survival. It liver breaks nutrients down and builds up body tissue. The liver also acts as a storage site for vitamins and minerals. The liver is vital when it comes to metabolic processes and how it affects other organs and the body, including hormonal concentration levels and disposal of toxins.

Liver role in body detoxification

The liver is one of the four major organs that eliminate toxins from the body. The other three organs involved are the kidneys, intestinal tract and skin.

The liver detoxifies harmful substances whether they come from internal sources such as burning sugars, fats, protein, or from external sources like medications, drugs, hormone enhancers, food additives, preservatives, food colorings, sweeteners, flavor enhancers, chemicals used in agriculture, alcohols, volatile organic compounds, fumes, air pollution and many other factors.

Many of the toxins that enter the body are fat soluble which means they dissolve only in fatty or oily solutions and not it water. They all must travel through the body and the first step in the detoxification process they will encounter is the liver.

Detoxification mechanisms

The detoxification process starts in the liver. Its function is to convert fat soluble toxins into water soluble substances that can be excreted from the body. The enzymatic process to dispose of toxins occurs in two phases:

Phase 1 – Oxidation : Converts toxic chemicals into a less harmful chemical through oxidation, reduction and hydrolysis reactions.

During this process, free radicals are produced and if there are too many it can damage the liver cells. With the help of antioxidant, it reduces the damage caused by free radicals. Phase 1 of detoxification involves a group of 50 to 100 enzymes collectively named cytochrome P450. ORISOD Enzyme® olive leaf extracts help the liver to eliminate toxins during the first phase.

Phase 2 – Conjugation : The liver cells add another substance such as cysteine, glycine, or a sulphur molecule to a toxic chemical to make it less harmful. As a result it makes the toxin water-soluble so that it may then be excreted from the body via watery fluids such as bile or urine.

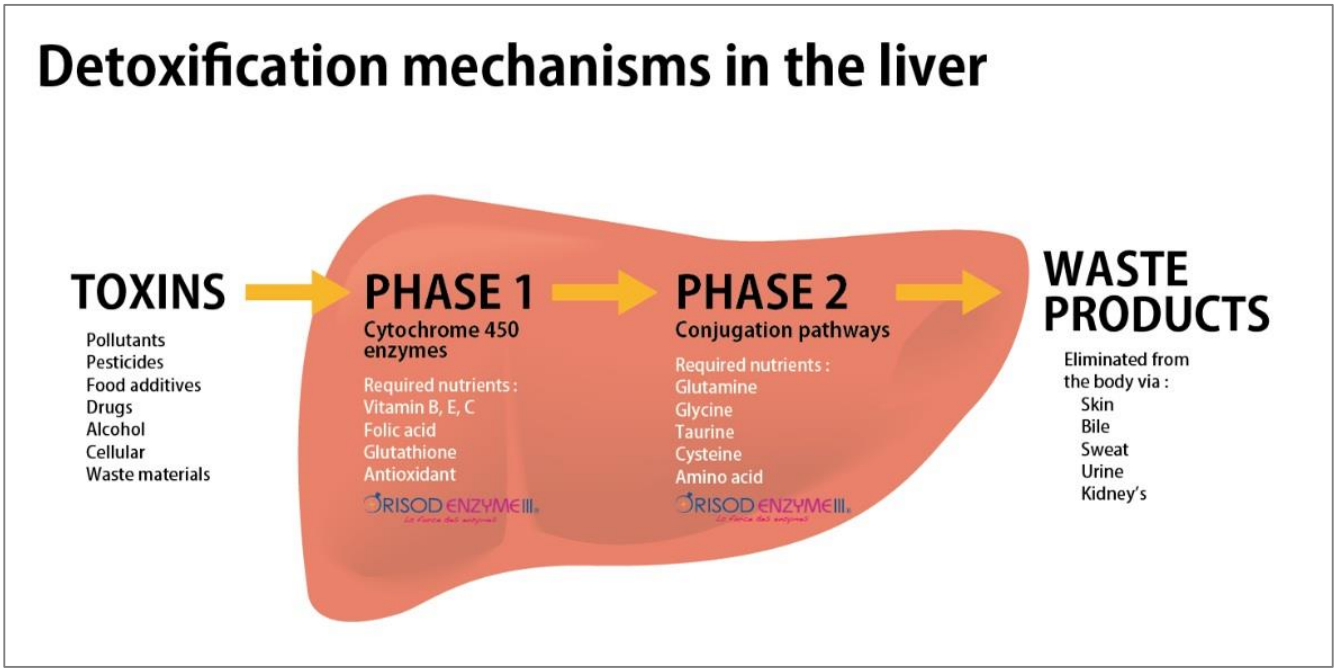
There are six phase 2 detoxification pathways:

1. Glutathione conjugation
2. Amino acid conjugation
3. Sulfation
4. Acetylation
5. Glucuronidation

These conjugation molecules join with specific enzymes to catalyze the reaction process. The liver is then able to turn drugs, hormones, and other various toxins into substances that are secreted from the body.

The key in the liver detoxification process is to keep the activity of Phase I and Phase II in balance. Since Phase I often involve oxidation, we need to supply our body with sufficient antioxidants to prevent our liver to be overexposed to oxidative stress. More the body is exposed to toxins, more it produce free radicals that raise the risk of oxidative damage.

Hydroxytyrosol contained in **ORISOD Enzyme®** olive leaves active Nrf2 (a key genetic regulator) and directly increase activity of phase 2 enzymes.



L-Glutathione enzyme enhance toxins elimination

L-Glutathione is a water soluble enzyme involved in detoxification mechanisms. It is the reduced form of Glutathione, and is an antioxidant that he body produces from tree basic amino acids (L-glutamine, L-cysteine, and glycine) which are found in our diet sources.

L-Glutathione is involved through conjugation reactions via the enzyme glutathione transferase. Heavy metals such as mercury are removed from the body by conjugation with glutathione.

Increased free radical metabolite production during phase I reactions can result in depleted glutathione levels and glutathione conjugation in phase II detoxification

Action in phase 1 : Without adequate free-radical defences, every time the liver neutralizes a toxin, it is damaged. Glutathione the most important antioxidant for neutralizing the free radical/intermediates during detoxification phase 1.

Action in phase 2: L-Glutathione is also required for the key Phase II detoxification processes: Depletion halts Phase II which is glutathione dependent

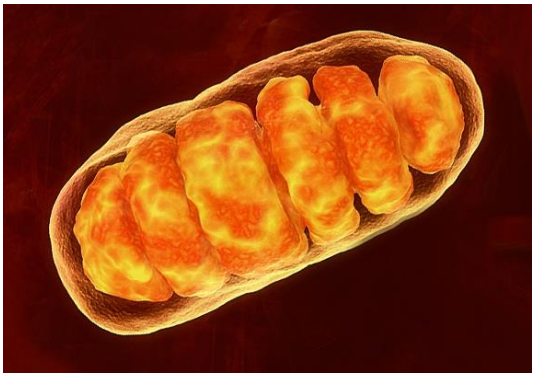
3.4. MITOCHONDRIAL BIOGENESIS: NRf2 activation

Mitochondria are the power centers of the cell. They provide the energy a cell needs to move, divide, produce secretory products, and contract.

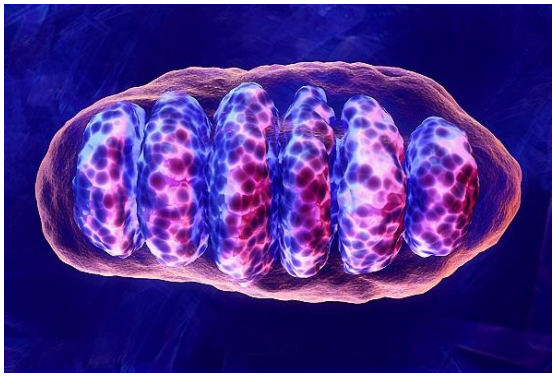
Mitochondrial dysfunction

Mitochondrial dysfunction plays a central role in a wide range of age-associated disorders and various forms of cancer, as well as type 2 diabetes.

Increasing evidence shows that mitochondrial metabolism and ATP synthesis decline in concert with a reduction of key factors regulating mitochondrial biogenesis in patients with insulin resistance, type 2 diabetes and obesity.



Healthy mitochondria



Damaged mitochondria

Key factors regulating this process include peroxisome proliferator-activated receptor (PPAR) coactivator 1 alpha (PPARGC1 α) and the nuclear respiratory factors (Nrfs)

Hydroxytyrosol and Nrf2 activation

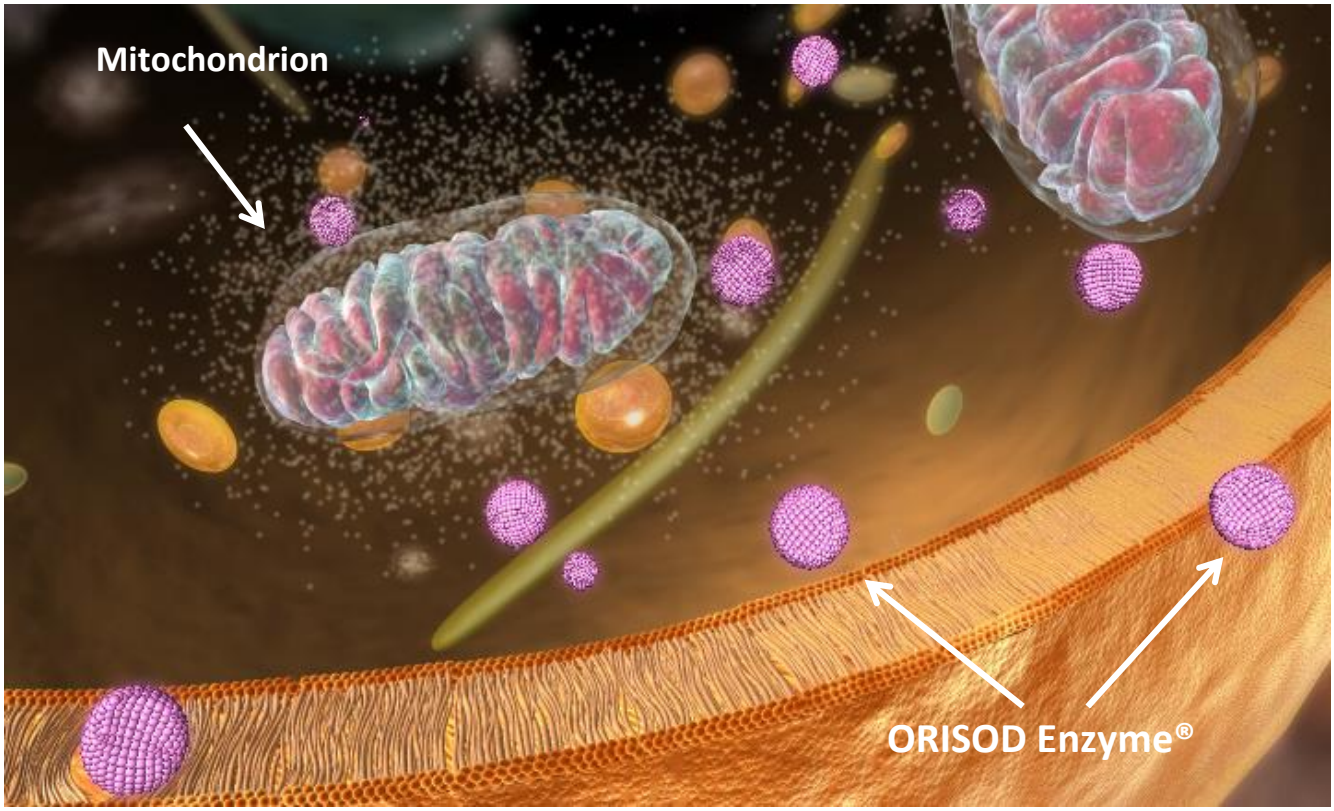
The Mediterranean diet has been associated with a lower incidence of certain cancers and of cardiovascular disease, which is the most common and serious complication of diabetes.

Olive oil is the principal source of fats in the Mediterranean diet, and hydroxytyrosol, a polyphenolic constituent of extra-virgin olive oil, is considered to be one of the most potent determinants of its efficacy.

Hydroxytyrosol increases significantly the activation of Nrf2, a key genetic regulator (or transcription factor) that protects cells and tissues from oxidative stress by activating protective antioxidant and detoxifying enzymes.

Two important proteins involved in Nrf2 (erythroid 2p45-related factor) translocation, the protein kinase B and the extracellular regulated kinases, were also activated by Hydroxytyrosol.

Nrf2 is considered as powerful regulator of antioxidant and cellular defenses and is a critical activator required for genetic expression of key genes related to cellular defenses, for balancing oxidative stress, and for enzymatic detoxification

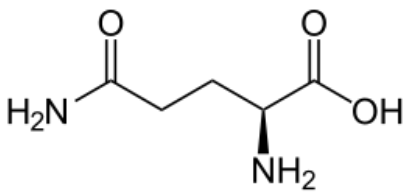


ORISOD Enzyme® cross the cell membrane to reach the mitochondria

3.5. AMINO ACID METABOLITES

3.5.1. L-Glutamine

L-glutamine is one of 20 amino acids in the body that is encoded by the standard genetic code. Amino acids are the building blocks of proteins in the body. L-glutamine is the most abundant amino acid in the body. Sixty percent of our glutamine is found in our skeletal muscle, with the rest being in the lung, liver, brain and stomach tissue.



Glutamine Chemical structure

Molecular Formula : $C_5H_{10}N_2O_3$

Molecular Weight : 146.14 g mol^{-1}

CAS Registry Number: 56-85-9

Other names : 2-Amino-4-carbamoylbutanoic acid; (levo)glutamide; L-glutamine

Glutamine is represented by the chemical structure on the left.

In times of stress or increased metabolic demand, glutamine is an especially important source of energy for the gastrointestinal health tract. It also supports muscle health and immune system function.

Glutamine improves brain functioning

L-Glutamine is the most abundant free amino acid in brain. It plays a critical role in synaptic maintenance and plasticity. It also stimulates mental alertness, improves intelligence, normalizes physical equilibrium, detoxifies ammonia from the brain.

Due to its ability to cross the blood brain barrier, L-glutamine is a wonderful way of providing glutamic acid to the brain.

Glutamine, Glutamate, & GABA are all neurotransmitters which means they are chemical messengers in the brain without which the brain could not function. GABA is a calming neurotransmitter, Glutamate is a stimulating neurotransmitter, while Glutamine is a modulator of the inhibitory & excitatory activity of the other two.

Glutamine is highly concentrated in the brain, being 10-15 times more concentrated in the cerebro-spinal fluid than in the blood. It is an important fuel for the brain, & can provide adequate energy in the absence of glucose (the other major brain fuel). For this reason it is helpful with focus, concentration, memory, intellectual performance, alertness, attentiveness, improving mood, & eliminating brain fog & cloudiness. It is one of the first nutrients I prescribe when any of these symptoms are present.

Research suggests Glutamine may protect the brain cells in situations of decreased oxygen supply. It also helps in the brain to detoxify ammonia.

Gastrointestinal Health

In the gut, glutamine maintains healthy integrity of the intestinal tract and enhances the protective mucosal lining. This helps to ensure proper nutrient utilization and absorption while limiting the amount of toxins that pass through the intestinal barrier.

L-Glutamine is needed for glutathione formation and glutathione is vital for liver detoxification.

Muscle Support and Recovery

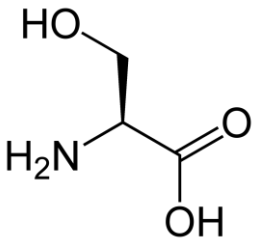
L-Glutamine provides potential support for muscle function and glycogen replenishment following exercise. In addition, its effects on immune function may support postexercise recovery and resistance.

L-Glutathione antioxidant effect

L- Glutathione is a sulfur- containing, water soluble enzyme that is absorbed mostly in the liver. It naturally occurs in the body as a combination of the amino acids L-Glycine, L- Glutamine, and L- Glycine. Since these three amino acids are known to have protective antioxidant properties, it tripled the antioxidant potency of L- Glutathione plus the added benefits you can get from each of the three amino acids.

3.5.2. Serine

Serine is a non-essential amino acid derived from the amino acid glycine. It is synthesized in the body. It exits in L-serine and D-serine forms. It is important to overall good health, both physical and mental.



Serine Chemical structure

Molecular Formula : $C_3H_7NO_3$

Molecular Weight : 105.09 g mol^{-1}

CAS Registry Number: 302-84-1

Other names : 2-Amino-3-hydroxypropanoic acid

Serine is represented by the chemical structure on the left.

It plays roles in protein, fatty acid, genetic code carriers (DNA and RNA) synthesis, and muscle build-up Serine is a constituent in the brain and protective covers of nerves. Therefore, serine is an important amino acid for the proper functioning of the brain and central nervous system.

Additional serine's health benefits come indirectly through its effect on other biochemicals. For example, serine is a precursor for the production of amino acids such as glycine, cystein, and tryptophan. Tryptophan, necessary for the synthesis of serotonin and functioning of neurotransmitters, is known to help relieve stress, anxiety and depression.

Brains health benefits

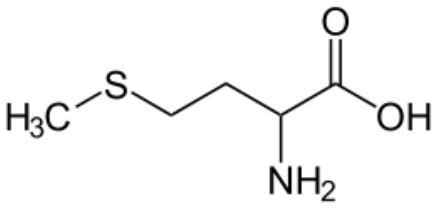
The proteins used to form the brain, as well as the protective myelin sheaths that cover the nerves, contain serine. Without serine, the myelin sheaths could fray and become less efficient at delivering messages between the brain and nerve endings in the body, essentially short circuiting mental function.

Serine is also needed to produce tryptophan, an amino acid that is used to make serotonin, a mood-determining brain chemical. Both serotonin and tryptophan shortages have been linked to depression, insomnia, confusion, and anxiety. Research suggests that low levels of serine may contribute to chronic fatigue syndrome (CFS) and fibromyalgia (FM).

Serine helps produce immunoglobulins and antibodies for a strong immune system, and also aids in the absorption of creatine, a substance made from amino acids that helps build and maintain all the muscles in the body, including the heart.

3.5.3. Methionine

Methionine is a protein-based amino acid which assists with metabolic function, breaks down fat, and is the primary source of sulfur in the body.



Methionine Chemical structure

Molecular Formula : $C_5H_{11}NO_2S$

Molecular Weight : 149.21 g mol^{-1}

CAS Registry Number: 59-51-8

Other names : 2-amino-4-(methylthio)butanoic acid

Methionine is represented by the chemical structure on the left.

Methionine assists in the breakdown of fats and thereby prevents the build-up of fat in the arteries, as well as assisting with the digestive system and removing heavy metals from the body since it can be converted to cysteine, which is a precursor to gluthione, which is of prime importance in detoxifying the liver.

The human body does not naturally produce methionine; therefore it must be ingested in food, via supplement like **ORISOD Enzyme®.**

Methionine residues as antigeneous antioxidant

Methionine, like cysteine, functions as an antioxidant and as a key component of a system for regulation of cellular metabolism.

Methionine is readily oxidized tomethionine sulfoxide by many reactive species. The oxidation of surface exposed methionines thus serves to protect other functionally essential residues from oxidative damage. Methionine sulfoxide reductases have the potential to reduce the residue back to methionine, increasing the scavenging efficiency of the system.

Reversible covalent modification of amino acids in proteins provides the mechanistic basis for most systems of cellular regulation. Interconversion of methionine and methionine sulfoxide can function to regulate the biological activity of proteins, through alteration in catalytic efficiency and through modulation of the surface hydrophobicity of the protein.

4. ADS: ADVANCED DELIVERY SYSTEM

Bio availability is a very important factor to allow bioactives to cross the cells double membrane and act at the cellular level. A green carrier allowed us to create a complex with the ability to cross hydrophilic and hydrophobic barriers.

4.1. CONTROLLED DELIVERY OF BIOACTIVE COMPOUNDS

Formulate an active ingredients is important, but to deliver the actives to the researched target has the same importance; otherwise the ingredients are useless to the body.

The main idea of the protection is to create a carrier for the actives so they won't be in contact with the external environment until the release phase. The shell will act as a vessel and will navigate to the researched locations in the body.

We have developed and created ADS® – Advanced Delivery System for a targeted, controlled delivery of bioactive compounds.

Background

The active ingredients are given into the body, go through various membranes and arrive to the points of action. The movement of active ingredients depends on the efficiency of amount of the ingredient and time, which is Bioavailability.

Since 1995, our researchers began research on Fenugreek plant.

Why Fenugreek?

The main research was focusing on the anti-diabetic activity of Fenugreek. While trying to understand the mechanism of action of this plant, our researchers noticed that the Fenugreek has the particularity to present an exceptional system of delivery of molecules.

Fenugreek properties have been analysed and our scientist team discovered that the plant has the ability to facilitate and guide the circulation of actives for acting on defined points of the skin and cell.

Thanks to this discovery, we decided to use similar Fenugreek receptors to deliver the active ingredients and created a carrier encapsulation.

Created by amino phospholipid, ADS® carrier allows a targeted and innovative controlled delivery of actives. ADS® protects the activity of the ingredients, and allows their delivery on zones never reached with classical ingredient.

Advantages:

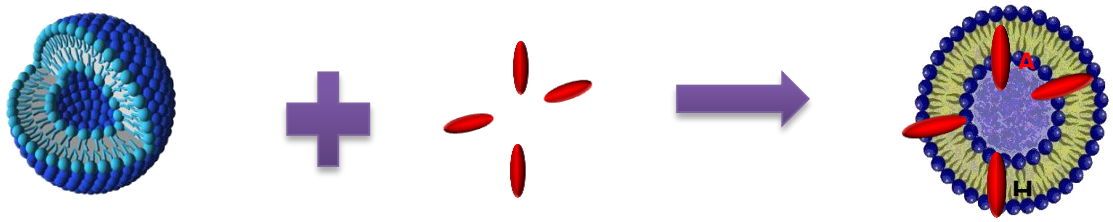
- Quick and visible effect of the treatment (both cosmetics & nutraceuticals)
- Little quantity of ingredient is enough, as no waste
- No need to intake big amount of actives to feel the results, thus reducing side effect of overtaking of actives
- Reducing the size of end products (capsules)
- 100% safe, no animal origin ingredients, only natural vegetable origin

4.2. LOCALISATION OF ORISOD Enzyme® IN CELLULAR SYSTEM

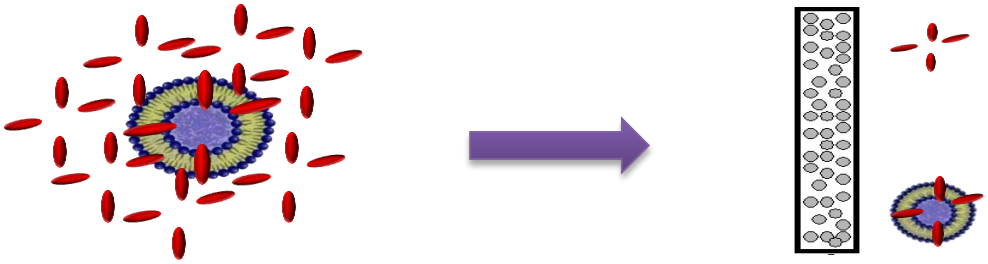
Intracellular localisation of ORISOD Enzyme® by fluorescence microscopy using lipophilic probes in mitochondria.

Labelling of ORISOD Enzyme® with a fluorescent lipophilic probe

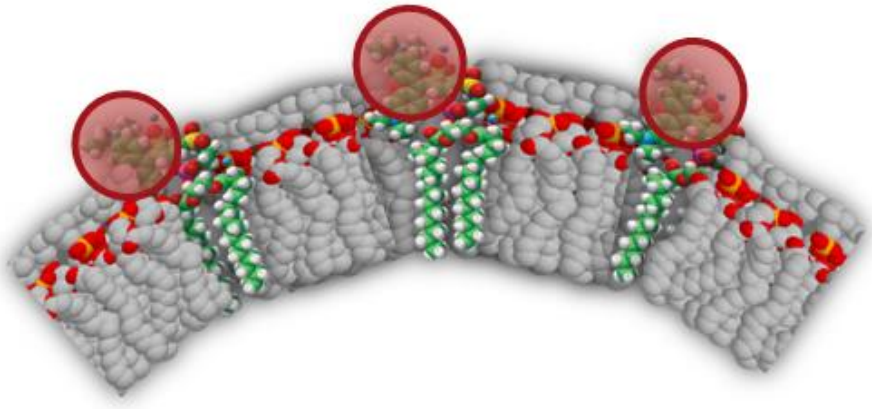
Labelling of ORISOD Enzyme® carrier



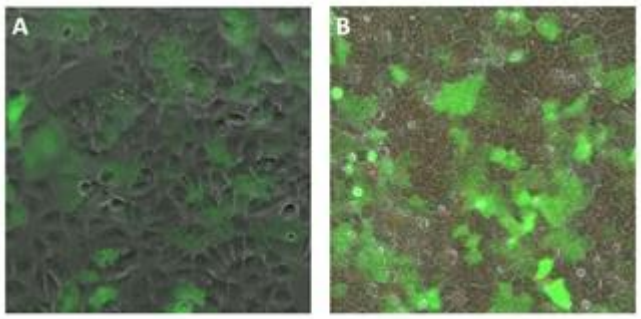
Separation of labelled ORISOD Enzyme® from free probe



Labelling of ORISOD Enzyme® with a fluorescent lipophilic probe



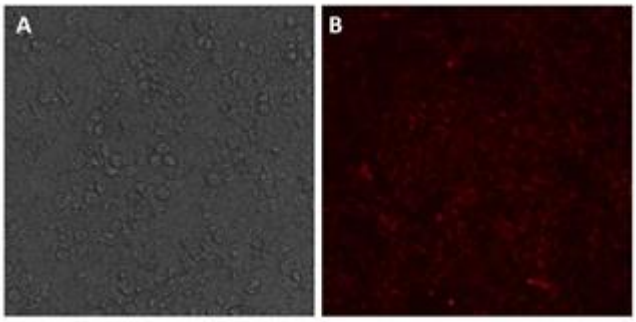
Incubation of human cells with labeled ORISOD Enzyme® (fluorescence microscopy)



A - Non-labeled MCF-7 cells (green cells express GFP, green fluorescent protein)

B - MCF-7 cells incubated with labeled ORISOD Enzyme® (red labelling is ORISOD Enzyme®)

Incubation of human cells with labeled ORISOD Enzyme® (fluorescence microscopy)



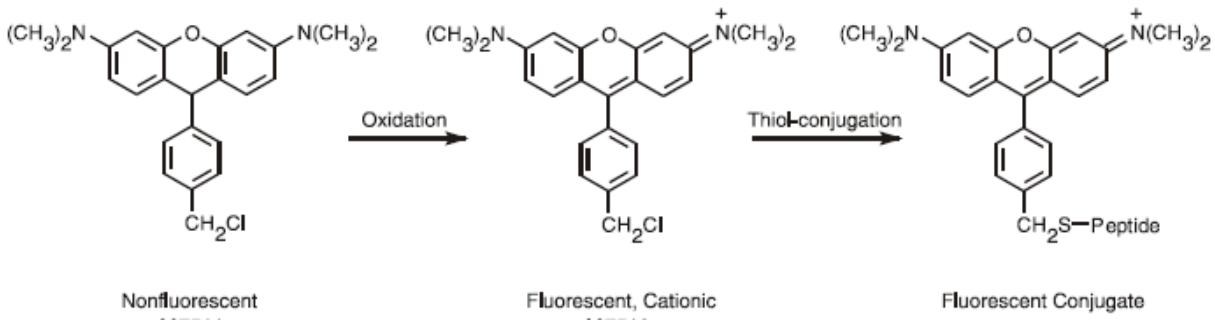
A- Non-labeled MCF-7 cells observed by phase contrast microscocopy.

B- MCF-7 cells incubated with labeled ORISOD Enzyme® observed by fluorescence microscopy (red labelling is ORISOD Enzyme®)

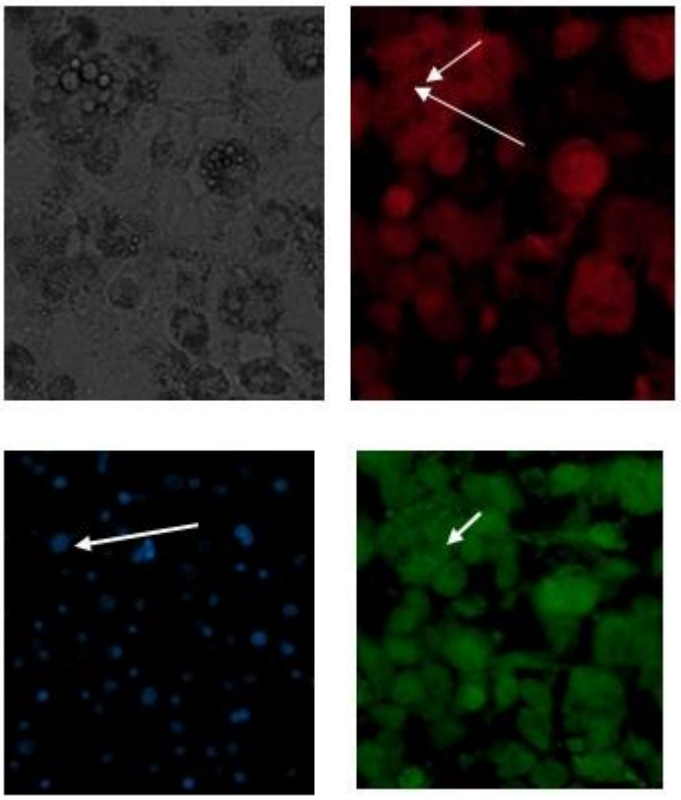
Colocalization of ORISOD Enzyme® and mitochondria in adipocytes

MitoTracker Green FM probe is essentially nonfluorescent in aqueous solutions and only becomes fluorescent once it accumulates in the lipid environment of mitochondria. (InvitroGen).

MitoTracker Green FM probe preferentially accumulates in mitochondria regardless of mitochondrial membrane potential (InvitroGen).



Co-staining of ORISOD Enzyme® phospholipids and adipocyte mitochondria



Red: Phospholipid (ORISOD Enzyme®) / **Green:** Mitotracker Green / **Blue:** Hoesch dye (nuclei)

5. CLINICAL TRIALS

5.1. ANTIOXIDANT CAPACITY

5.1.1. Evaluate the antioxidant activity of ORISOD ENZYME® (TBARS Test)

Customer: SANKI MAYOR

Laboratory: MEDICA TOKYO, study managed by Dr Taro Hirata

Objective: Evaluate the antioxidant activity of ORISOD ENZYME® (TBARS test).

Introduction

Many articles have been published reporting the neuroprotective effect and vascular protection of fruits and vegetables, connected to the Mediterranean diet: supporting the hypothesis that dietary antioxidant and anti-inflammatory compounds could directly exert a neuroprotective effect.

During the last few years, emerging evidence has suggested that dietary flavonoids (representing a diverse range of polyphenolic compounds) that occur naturally in plants and foods may exert beneficial effects on the central nervous system due to their efficacy in protecting neurons against oxidative stress-induced injury, suppressing neuro-inflammation and in ameliorating cardiovascular risk factor control.

Olive (*Olea europaea*) and rosemary (*Rosemary officinalis*) are vegetable species rich in phenolic compounds bearing strong antioxidative activity, and have been also a part of the Mediterranean diet for centuries. Hydroxytyrosol and oleuropein from olives, have been shown as potent radical scavengers. In addition, carnosic acid and rosmarinic from rosemary, contributes to the bioactivity of **ORISOD Enzyme®**.

Proved efficacy of ORISOD Enzyme® against oxidative damage

The antioxidant capacity of **ORISOD Enzyme®** was evaluated through its capacity to decrease the level of thiobarbituric acid reactive substances (TBARS) derived from the lipid peroxidation of EYPC liposomes induced by AAPH.

There is strong correlation between thiobarbituric acid-reactive substances (TBARS) as a marker of lipid peroxidation and products that reflect oxidative damage to DNA.

ORISOD Enzyme® had a significant effect on the prevention of lipid peroxidation by TBARS assay in a dose dependent manner.

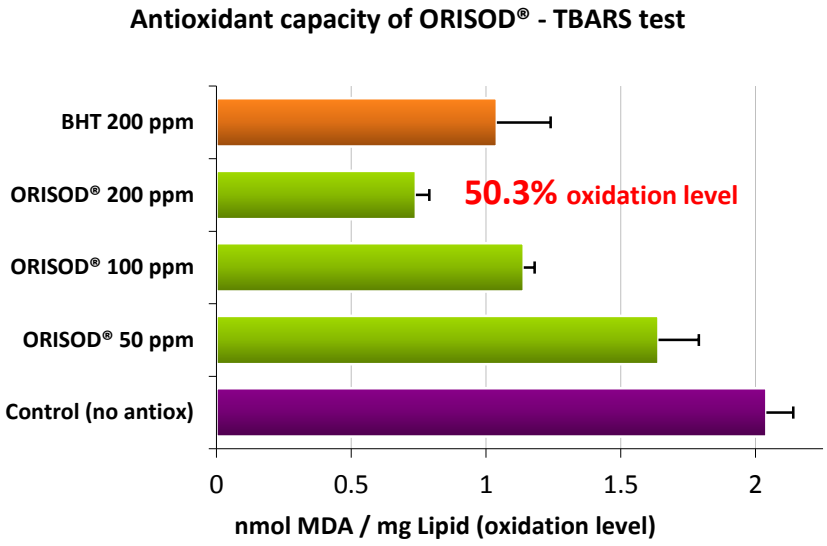


Figure1. Antioxidant capacity of ORISOD® (50, 100 and 200 ppm), compared to BHT (200 ppm), to prevent lipid peroxidation measured by TBARS assay in EYPC liposomes. Level of oxidation was expressed as nmole malondialdehyde / mg phospolipid, measured by fluorescence and phosphorous determination assay respectively, compared to a control in the absence of antioxidants. Each bar represents the mean of three independent experiments performed in triplicate.

Several concentrations of **ORISOD Enzyme®** were tested by this assay, 50 ppm, 100 ppm and 200 ppm obtaining levels of the lipid oxidation of 81.23%, 58.10% and 37.67% respectively.

ORISOD Enzyme® also exhibited a higher antioxidant capacity than BHT (butylated hydroxytoluene), which yielded a 50.3% of oxidation when used at an identical concentration, i.e. 200 ppm.

BHT is a powerful synthetic antioxidant agent used as preservatives in many fat-containing foods, in edible fats and oils.

5.1.2. ORISOD Enzyme®, endogenous antioxidant enzymes complex enhancer

TITLE CLINICAL TRIAL

ORISOD Enzyme®, endogenous antioxidant enzymes complex enhancer

TYPE OF STUDY

A clinical randomized double-blind trial comparing ORISOD Enzyme® vs Placebo

SPONSOR

SANKI MAYOR

LABORATORY

MEDICA TOKYO Co.LTD - 20-1, 3Chome Nishi-Shinjuku, Shinjuku-ku Tokyo JAPAN

Managed by Dr Taro Hirata

PRODUCT INFORMATION

Product name: ORISOD Enzyme®

Appearance: Powder

Color: Pale green

Taste: Characteristic

STUDY OBJECTIVES

The objective is to verify that the activity of endogenous enzymes (SOD and GPx) is enhanced by ORISOD Enzyme®. Lipid peroxidation and LDL Oxidation reduction was also controlled.

VARIABLE VALUED

Endogenous enzymes (SOD and GPx) as well as lipid peroxidation and LDL oxidation have been monitored.

TOTAL NUMBER OF PATIENTS

30 subjects (men and women) aged 35 and 55 years participated in a double-blind, placebo-controlled trial testing the efficacy of the oral supplement ORISOD Enzyme®.

DURATION OF TREATMENT AND DOSAGE

Ingestion of ORISOD Enzyme® supplement and placebo during 90 days of treatment with a dosage of 500mg/day (each tablet contains 200mg of active ingredients and 300mg of excipient).

Introduction

ORISOD Enzyme® is a fermented bio-active formulated to enhance body antioxidant status and to prevent aging-related diseases. It formula combines a mixture of bioactives from 2 Mediterranean plants **olive and rosemary**. The complex is fermented using a green fermentation technology based on vegetal ferments.

Olive (*Olea europaea*) and rosemary (*Rosemary officinalis*) are known to be rich in phenolic compounds. Olive leaves antioxidant properties are associated with Oleuropein, hydroxytyrosol, and extracts of Olea europaea leaf (containing 19% oleuropein, 1.8% flavonoid glycosides, and 3,4-dihydroxy-phenethyl esters). Rosemary has antioxidant effect properties associated with carnosic acid and carnosol.

This study proves that the ingestion of ORISOD Enzyme® enhances the efficacy of endogenous enzymes at the cellular level (SOD, Catalase and Gpx) and reduces lipid peroxidation, supports brain energy and support liver detoxification.

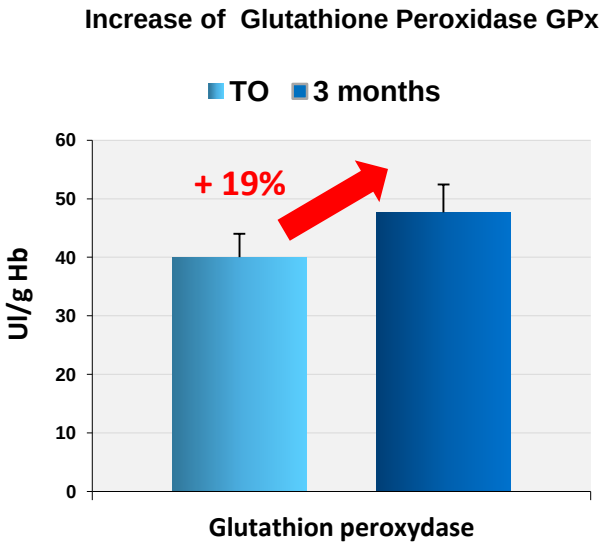
Protocol

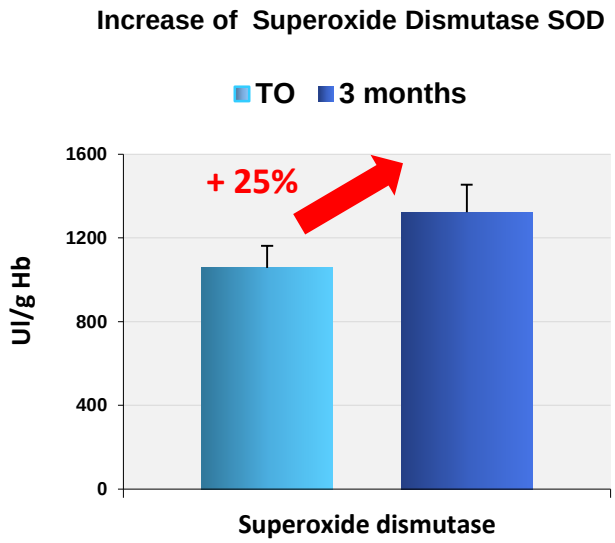
Several bio-markers of oxidative stress were analysed to measure the antioxidant activity of ORISOD Enzyme®. 30 subjects (men and women) aged 35 and 55 years participated in this double-blind, placebo-controlled trial. They ingested of ORISOD Enzyme® supplement and placebo during 90 days of treatment.

Results

- **Increase of two main endogenous enzymes SOD and CAT**

The activity of 2 main endogenous antioxidant enzymes: SOD (Superoxide Dismutase) and GPx (Glutathione Peroxidase) was significantly increased after 3 months. SOD enzyme increased by 25 % while GPx enzyme increased by 19%.





Mechanisms of action

A free radical is an atom, molecule, or compound that is highly unstable because of its atomic or molecular structure (i.e., the distribution of electrons within the molecule). One chemical element frequently involved in free radical formation is oxygen. Molecular oxygen can accept a total of four electrons, one at a time, and the corresponding number of protons to generate two molecules of water.

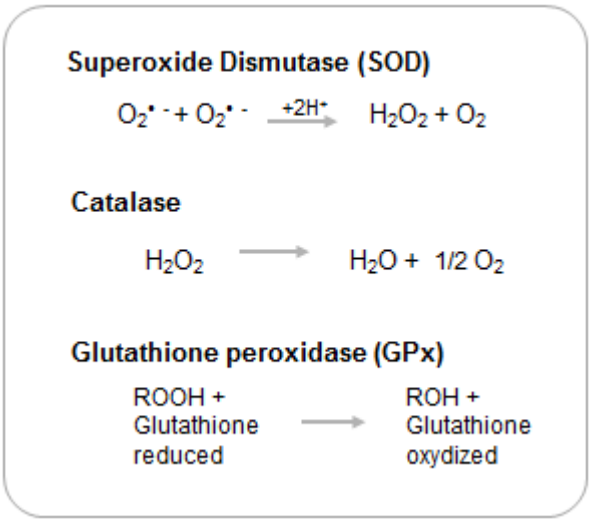
During this process, different oxygen radicals are successively formed as intermediate products, including superoxide (O2); peroxide (O2), which normally exists in cells as hydrogen peroxide (H2O2); and the hydroxyl radical (·OH). Superoxide, peroxide, and the hydroxyl radical are unstable and rapidly react with additional electrons and protons, most of these ROS are converted to water before they can damage cells. Nevertheless, the toxic effects of oxygen in biological systems—such as the breakdown (i.e., oxidation) of lipids, inactivation of enzymes, introduction of changes (i.e., mutations) in the DNA, and destruction of cell membranes and, ultimately, cells—are attributable to the reduction of O2 to ROS.

Because ROS form naturally during many metabolic processes, cells have developed several protective mechanisms to prevent ROS formation or to detoxify the ROS. These mechanisms employ molecules called antioxidants. Under certain conditions, ROS production is enhanced and/or the level or activity of antioxidants is reduced. The resulting state is called oxidative stress and is characterized by a disturbance in the balance between ROS production on one hand and ROS removal and repair of damaged complex molecules (such as proteins or DNA) on the other.

In humans, oxidative stress is involved in many diseases, such as atherosclerosis, heart failure, myocardial infarction, chronic fatigue syndrome, cancers etc.

ORISOD Enzyme® unique mode of action

Rather than classical antioxidants like vitamins, ORISOD Enzyme® mechanisms consist of enhancing endogenous enzymes SOD and GPx, which is more effective and faster for improving body antioxidants status.



The antioxidant defence is activated by the SOD enzymes which reduce the production of ROS, not just fight against the ROS already created. The enzyme superoxide dismutase (SOD) catalyzes the dismutation of superoxide into oxygen and hydrogen peroxide. As such, it is an important antioxidant defence in all cells exposed to oxygen.

ORISOD Enzyme® unique mode of action

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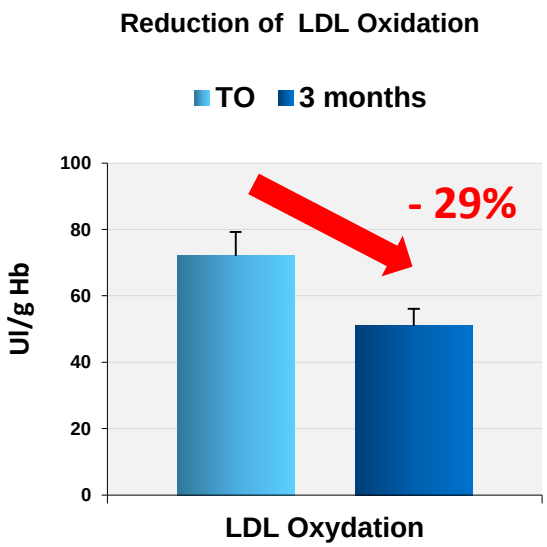
The antioxidant defence is activated by the SOD enzymes which reduce the production of ROS, not just fight against the ROS already created. The enzyme superoxide dismutase (SOD) catalyzes the dismutation of superoxide into oxygen and hydrogen peroxide. As such, it is an important antioxidant defence in all cells exposed to oxygen.

Glutathione peroxidase (GPx) provides a mechanism for detoxification of peroxides in living cells. This reaction plays a crucial role in protecting cells from damage by free radicals, which are formed by peroxide decomposition.

- **Reduction of LDL Oxidation**

The oxidation of LDL occurs when the LDL cholesterol particles in the body react with free radicals. The oxidized LDL itself then becomes more reactive with the surrounding tissues, which can produce tissue damage.

Once LDL becomes oxidized, it goes directly within the inner-lining of artery in the body. Once there, it encourages the accumulation of inflammatory cells, such as macrophages, at the site of the vessel and promotes their adhesion to the damaged area.

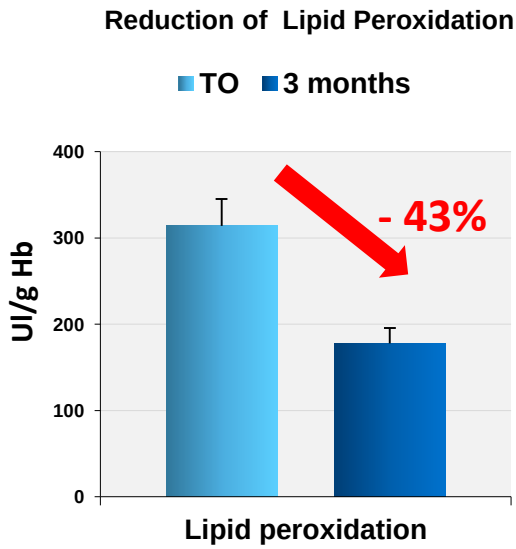


Orisod Enzyme® treatment exhibited significant decreases of oxLDL levels (-29%), protecting the cells and blood vessels against inflammation.

- **Reduction of Lipid peroxidation**

Lipid peroxidation to the process whereby free radicals “steal” electrons from the lipids in cell membranes, resulting in cell damage and increased production of free radicals.

The results show that ORISOD Enzyme® supplement reduced the lipid peroxidation by 43%, decreasing also the level of oxidative stress.



Conclusion

The results of this study show that **ORISOD Enzyme®** ingestion stimulates the antioxidant enzymes activity in the cells and protects them against risks resulting from oxidative stress: cardiovascular diseases, diabetes, etc.

The activity of endogenous antioxidant enzymes SOS and GPx increased significantly after 3 months of intake. SOD increased by 25% and GPx increased by 19%.These results are confirmed by the significant decrease of Lipid peroxidation (-43%) and oxLDL levels (-29%).

ORISOD Enzyme® is a new generation of antioxidant aimed reduce oxidation and restore body's natural antioxidant system. The fermentation technology allows producing a complex rich in metabolites, which support in a synergic way body antioxidant enzymes. When the body's natural defences are weakened: age, exposure to sun, stress, bad eating habits… ORISOD Enzyme® helps restore metabolism and provide energy for every day.

5.2. FIBROBLAST & KERATONOCYTES

5.2.1. Fibroblast & keratonocytes from superoxide toxicity

Purpose of the study

We study whether **ORISOD Enzyme®** can protect against free radicals generated by the hypoxanthine-xanthine oxidase system in vitro.

Method

On Day 1

Inoculation of 12-well plates with 60.000 cell per well, in MEM culture medium (for fibroblasts) or in DMEM (keratinocytes) enriched with 10% fetal calf serum.

On Day 3

Treatment with the hypoxanthine-xanthine oxidase system, with or without **ORISOD Enzyme®**, Hypoxanthine: 160 µg/ml, Xanthine oxidase :

- fibroblasts: 4 mU/ml
- Keratinocytes: 2 mU/ml

The product to test was diluted to 20 units in the Hypoxanthine solution.

- Contact time: 120 minutes at 37°C in a 5% CO2 atmosphere.
- Plates were rinsed with HBSS (Hanks balanced salt solution) buffer.
- Incubation with a solution of neutral red (50µg/ml) for 3 hours at 37°C.
- Development of color with acetic acid/absolute ethanol/water (1/49/50).
- Reading at 540 nm.

Results

The results are expressed as a percent of anti-radical activity:

$$\% \text{ Activity} = \frac{A - B}{A} \times 100$$

$$\text{With A: Control toxicity} = \frac{\text{A non-treated control} - \text{A treated control}}{\text{A non-treated control}}$$

$$\text{With B: Toxicity test} = \frac{\text{A non-treated} - \text{A treated test}}{\text{A non-treated test}}$$

At 0.5 % **ORISOD Enzyme®** protects cells from free radical toxicity. These protective effects are confirmed at higher concentration. Compared to SOD from bovine (SIGMA) France, **ORISOD Enzyme®** is a more powerful antioxidant than SOD.

These results are listed in the table below.

	% Activity	
	Keratinocytes	**Fibroblasts**
ORISOD Enzyme® (0.5 %)	**95%**	**100%**
ORISOD Enzyme® (1 %)	**97%**	**100%**
ORISOD Enzyme® (1.5 %)	**100%**	**100%**
Bovine SOD (20 U)	**85%**	**90%**

5.2.2. Effects of Orisod Enzyme® on Fibroblast & Keratonocytes viability

Purpose of the study

We study whether **ORISOD Enzyme®** is cytotoxic in two models:

Using **NCTC KERATINOCYTES**

NCTC keratinocytes were grown in 96 – well plates (inoculation by 20,000 cells per well) in DMEM medium containing 10% fetal calf serum. Cultures were grown for 24 hours in the presence of increasing quantities of **ORISOD Enzyme®**

The medium was then replaced by medium containing M.T.T. at 0.5 mg/ml.

Cell viability was determined by reading at 540 nm with a plate reader (IEMS, Labsystem).

Using **HUMAN FIBROBLAST**

The cells were grown as above in MEM medium containing M.T.T. at 0.5 mg/ml.

Cell viability was determined by reading the plate at 540 nm with a plate reader

RESULTS Cell viability (%)		
ORISOD Enzyme®	**Keratinocytes**	**Fibroblasts**
0.5 %	**100 %**	**100 %**
1 %	**97 %**	**100 %**
3 %	**96 %**	**99 %**
10 %	**87 %**	**84 %**

After 24 hours of contact with **ORISOD Enzyme®**, there is no cytotoxity observed.

5.3. DNA DAMAGE DETECTION

5.3.1. Evaluate the efficacy of Orisod Enzyme® on DNA damage reduction

PURPOSE OF THE STUDY

The purpose of this investigation is to evaluate the antioxidant efficacy of **ORISOD Enzyme®** on DNA plasmid. The 3D TEST (DNA Damage Detection) is a fast and sensitive microplate screening system adapted to detect protective effects of samples as shown below.

GENERAL PRESENTATION

The 3D TEST allows the detection of genotoxic agents at the early stage of the process damage - mutation and cancerogenisis. Its principle is the detection of damage by means of *in vitro* reconstituted cell-free repair system of the DNA damage repair process.

Genotoxic agents can be either of exogenous (UV irradiation, ionizing radiations, chemical substances...) or of endogenous origin (free radicals produced by the cellular metabolism). DNA is the ultimate target of natural or synthesized genotoxic agents.

The biological consequences of the DNA damage in normal mammalian cells are DNA damage checkpoints operating in the G2 phase of cell cycle block entry mitosis and then either allowing DNA damage repair process or programmed cell death (apoptosis). Much of current understanding of genotoxic agents induced DNA damage and repair show that mammals defect in DNA repair systems or their different defense mechanism failing to function correctly could contribute to genetic instability and cancer.

Generation of reactive oxygen species

The hydroxyl radical formation is generated by homolytic scission of hydrogen peroxide. ($^{1}O_{2}$) is produced by photoactivation of methylene blue.

These ROS are powerful electrophiles, which have a very short half-life (around 10^{-9} second). However, they react very quickly with DNA bases and induce various oxidative damages such as the modifications or loss of bases. The bases alterations induced by ROS are mainly recognized and removed by the pathway of base excision repair (BER).

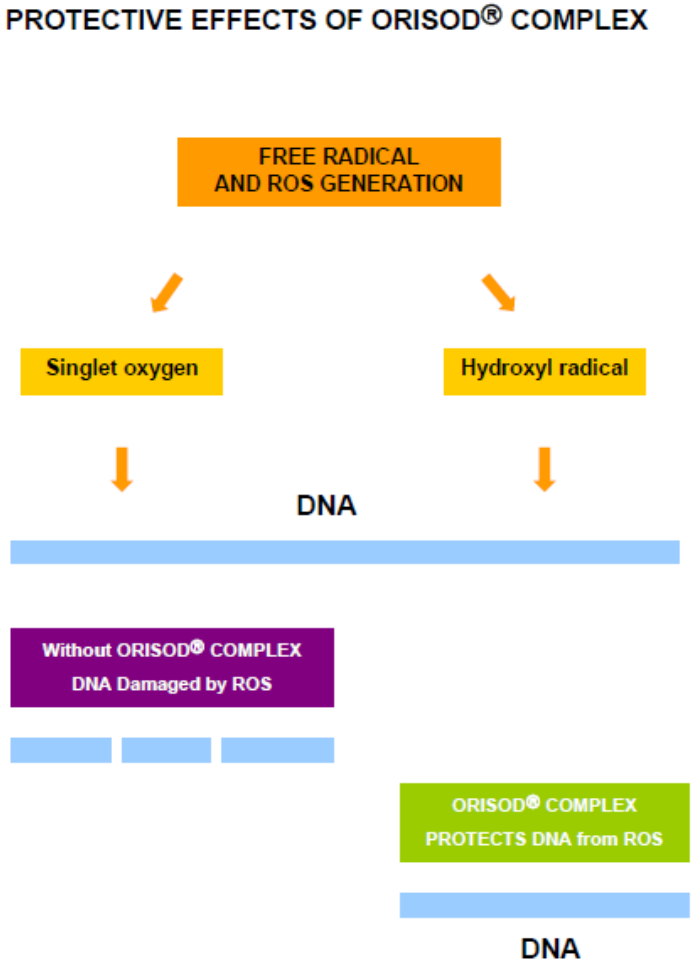
Generation of ROS by homolytic scission of hydrogen peroxide

The hydrogen peroxide is diluted at 10mM with ultrapure water (quality MilliQ of Millipore).This oxidant solution is mixed with equal volume of different sample dilutions, and 50µl of the mixture are added in the wells containing

the absorbed plasmid DNA. The wells are then irradiated for 1 minute and 30 seconds under a UVB lamp (312 nm) which corresponds to a quantity of 700 Joules/m².

Generation of ROS by photoactivation of methylene blue

A methylene blue solution is prepared at 4 ng/ml in ultrapure water, (quality MilliQ of Millipore). This methylene blue solution is mixed with the equal volume of different sample dilutions of, and 50µl of the mixture are added in the wells containing the absorbed plasmid DNA. The wells are then irradiated with visible light for 20 minutes under two 100 Watts lamps, 30 cm apart.



3D TEST METHOD ON PLASMID DNA

<u>Dilutions of test samples</u>

The stock solution of test samples are adjusted at concentration of 100 mg/ml in pure ethanol. Then, a serial sub-dilutions of samples are prepared twice as more concentrated in 40% of ethanol with ultrapure water in order to obtain the concentrations required after addition of a volume of peroxide hydrogen solution or methylene blue solution.

Silymarine is used as control for antioxidant characterization.

RESULTS: EVALUATION OF PROTECTIVE EFFECTS ON DNA

According to the antioxidant action mechanisms and the general principle of its detection by means of 3D TEST, any compound exhibiting an antioxidant effect in response to the oxidative damage induced by ROS (OH°, $^{1}O_{2}$) should lead to a dose-depend inhibition of oxidative damage formation on DNA under oxidative actions.

The protective effects of test sample on DNA are expressed as the percentage of repair signal inhibition in the presence of ROS via its protection DNA from oxidative damage. The 0% value corresponds to the repair signal of DNA damaged by the oxidant alone.

The percentage of **inhibition in the presence of ROS** is calculated as the relative decrease of the oxidative damaging effect resulted from the oxidant exposure (OH° or $^{1}O_{2}$, i.e.):

$$\frac{[\text{RLU oxidant}] - [\text{RLU (oxidant + sample)}]}{[\text{RLU oxidant}]} \times 100$$

The DNA repair inhibition given by a test compound in the presence of ROS can be results of:

- A real protection against oxidative damaging.

- A decrease in the repair activity on damaged DNA due to a direct interaction between the molecule to be tested and DNA or treated microwells.

- The simultaneous appearance of these two phenomena.

Protective effects against singlet oxygen on plasmid DNA

The values of this table have been calculated from values in RLU.

Table 1 : Antioxidant activity detected by 3D TEST in vitro on plasmid DNA

	Dilution / Concentration	% of protection with presence of EOR ($^1O_2$)	% Non specific Inhibition	% Specific Protection	Concentration giving 50% of protective activity
ORISOD® Food supplements	10 %	64 %	2 %	**62 %**	**1.23%**
	1 %	49 %	5 %	**49 %**	
	0.1 %	33 %	3 %	**30 %**	
	0.01 %	25 %	4 %	**21 %**	
ORISOD® Cosmetic	10 %	99 %	30 %	**69 %**	**0.65%**
	1 %	85 %	33 %	**52 %**	
	0.10 %	51%	12 %	**39 %**	
	0.01 %	36 %	7 %	**29 %**	
Silymarine	1mg/ml	81 %	2 %	**79 %**	**0.04 mg/ml**
	0.1 mg/ml	68 %	5 %	**63 %**	
	0.01mg/ml	29 %	0 %	**29 %**	
	0.001mg/ml	11 %	1 %	**11 %**	

The antioxidant activity is measured as the percentage of specific protection against oxidative DNA damage and the concentration giving 50% protective activity. The values given in this table have been calculated from repair signal detected as RLU.

Silymarine used as a reference has good antioxidant ability. This product prevents the oxidative damage induced by singlet oxygen exposure.

A good antioxidant efficacy is detected with 3D TEST. As shown in the table 1, the concentration of product giving 50% inhibition of the DNA damage formation is between 0.65 % and 1.23 %.

Protective effects against hydroxyl radical

The values of this table have been calculated from values in RLU.

*Table 2: Anti-free radical activity of **ORISOD Enzyme®** and reference antioxidant tested by 3D TEST on plasmid DNA.*

	Dilution / Concentration	% of protection with presence of EOR ($^1O_2$)	% Non specific Inhibition	% Specific Protection	Concentration giving 50% of protective activity
ORISOD® **Food supplements**	10 %	78 %	1 %	**79 %**	**0.45 %**
	1 %	65 %	5 %	**60 %**	
	0.1 %	43 %	4 %	**39 %**	
	0.01 %	28 %	6 %	**22 %**	
ORISOD® **Cosmetic**	10 %	95 %	17 %	**78 %**	**0.18 %**
	1 %	76 %	11 %	**65 %**	
	0.1 %	47 %	2 %	**45 %**	
	0.01 %	19 %	5 %	**14 %**	
Silymarine	1mg/ml	77 %	7 %	**70 %**	**0.046 mg/ml**
	0.1 mg/ml	63 %	4 %	**59 %**	
	0.01mg/ml	40 %	8 %	**32 %**	
	0.001mg/ml	9 %	1 %	**8 %**	

The values have been calculated from values of repair signal detected as RLU. Silymarine is found to have a significant antiradical ability, it is a good ROS scavenger.

The anti-free radical activity of ORISOD Enzyme® as radical scavenger is determined. ORISOD Enzyme® has a protective effect on DNA against free radical. The concentration of sample giving 50% inhibition of the DNA damage formation is to 0.18 % for ORISOD Enzyme® for food supplements.

CONCLUSION

In this study, we investigated the antioxidative effects of **ORISOD Enzyme®** by means of 3D TEST *in vitro*.

Using the 3D TEST on plasmid DNA: The aim of this investigation was to evaluate the possible protective effects of **ORISOD Enzyme®** against DNA oxidation induced by ROS exposure using the 3D TEST *in vitro*.

A protective activity of samples on DNA against the formation of oxidative damage on plasmid DNA by reactive oxygen species has been observed and characterized. The antioxidant activity of samples against free radical OH° scavenging efficacy are summarized in the table 3.

Protection %	**Protection %**

	in the presence of OH°	in the presence of $^{1}O_{2}$
ORISOD Enzyme® Cosmetics	**0.18%**	**0.65%**
ORISOD Enzyme® Food supplements	**0.45%**	**1.23%**

Table 3

Strong ROS scavenger activities are observed in vitro for ORISOD Enzyme® both for cosmetic and food supplements. The scavenger activity of ORISOD Enzyme® for cosmetic has the same level as polyphenols tested with the “3D TEST”: myricetin or ellagic acid.

5.4. ANTI-INFLAMMATORY ACTION

5.4.1. Anti-inflammatory activity of ORISOD® supplementation during chronic exercice

TITLE CLINICAL TRIAL

Anti-inflammatory activity of ORISOD® supplementation during chronic exercise

TYPE OF STUDY

Randomized and double-blind, placebo-controlled procedure comparing ORISOD® and Placebo

SPONSOR

Sanki Mayor

LABORATORY

INNOVATION LABO

Kanaya Bldg 5F, 4-11-3 Hatchobori Chuo-ku, Tokyo 104-0032 JAPAN

Managed by Dr Ikeda Yuki

PRODUCT INFORMATION

Product name: ORISOD®

Appearance: Powder

Colour: Pale green

Taste: Characteristic

STUDY OBJECTIVES

The aim of this study is to determine the efficacy of ORISOD® on cytokines' profile.

VARIABLE VALUED

Inflammatory cytokines levels were determined in blood samples, respectively collected at Day0 and at Day30.

TOTAL NUMBER OF PATIENTS

20 healthy male subjects (around 20 years old), 10 subjects in ORISOD® group and 10 subjects in placebo group.

DURATION OF TREATMENT AND DOSAGE

Ingestion of 500mg/day of ORISOD® supplement and placebo for a period of 30 days (2 tablets of 250mg, one before lunch and one before dinner). Each tablet was specified to contain 100 mg of actives and 150mg of excipient for a total daily amount of 200 mg of active ingredient).

Introduction

Exercise can have both positive and negative effects on inflammatory. Moderate activity can enhance immune function, as the contrary, excessive and intense exercise can impair immune function.

Intense physical activity increases oxygen consumption and activate the formation of ROS, leading to an acute phase immune response similar to infection, including signs of inflammation such as cytokines release.

The aim of this study was to determine the efficacy of ORISOD® to minimize the actions of free radicals resulting from exercise and decrease inflammatory markers.

Materials and methods

The study was conducted on 20 healthy athletic young men during 30 day. The exclusion criteria included chronic disease as hypertension, diabetes, cardiovascular disease, alcohol, drug dependence, etc... or other abnormal or unhealthy food habits. Diet and exercise routine were monitored with weekly meetings. Subjects were asked to avoid the intake of any other supplementation during the duration of the trial.

The 20 participants were assigned either to ORISOD® group (10 subjects) or placebo group (10 subjects). The participants took 250mg of ORISOD® at breakfast and 250mg at dinner (500 mg/day) or placebo during 30 days.

In order to assure the homogeneity of the subjects, a strict selection was performed based on similar physical characteristics.

Table 1 – Physical characteristics of 20 participants at D0

Parameters	Placebo Group	ORISOD® Group
Age	21.6	22.1
Height	178	181
Weight	73	78
BMI	22	24

During the study, the subjects performed 1h30min running sessions (around 15 km) three times a week during 30 days. All blood samples were collected between 9 and 11 am, at Day0 and at Day30. Blood samples were taken more than 24 h after any physical activity.

Statistical analysis

Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS). The results were expressed as mean and standard errors of the mean (mean ± SEM). All the data were tested for normality.

Results

IL-6 and TNF-a Cytokines levels

The results obtained in the present study derived from whole blood stimulation experiments, which is a system reported to be closer to in vivo conditions.

As shown in Figure1, IL-6 decreased in both groups, moreover, a more significant decrease was observed for the ORISOD® group than for the placebo group when the final values of the two groups were compared (-58% ORISOD® vs -22% PLB).

Regarding TNF-a concentration (Figure 2) a significant decrease of the level of this cytokine was observed in ORISOD® group (-54%). The placebo group only exhibited a weak decrease (-14%).

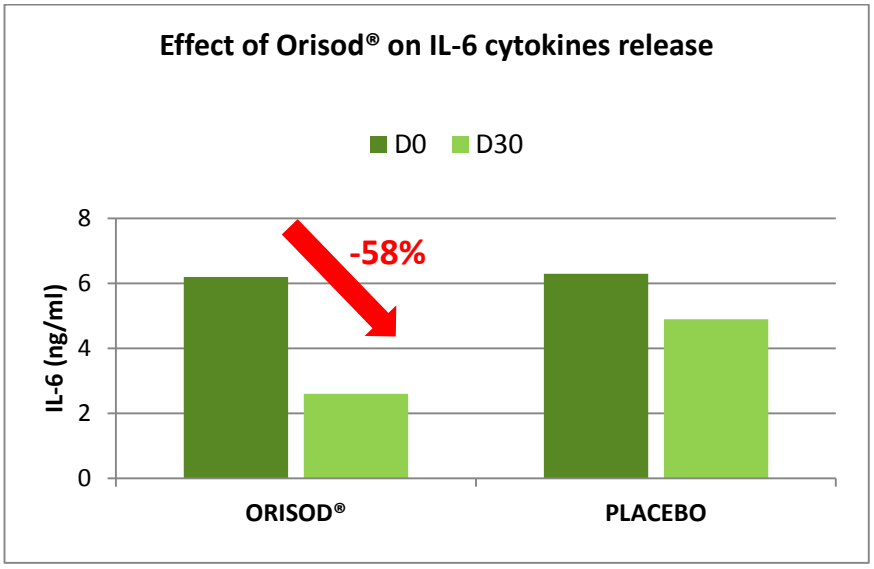


Figure 1: Effects of Orisod supplementation on IL-6 cytokines release.

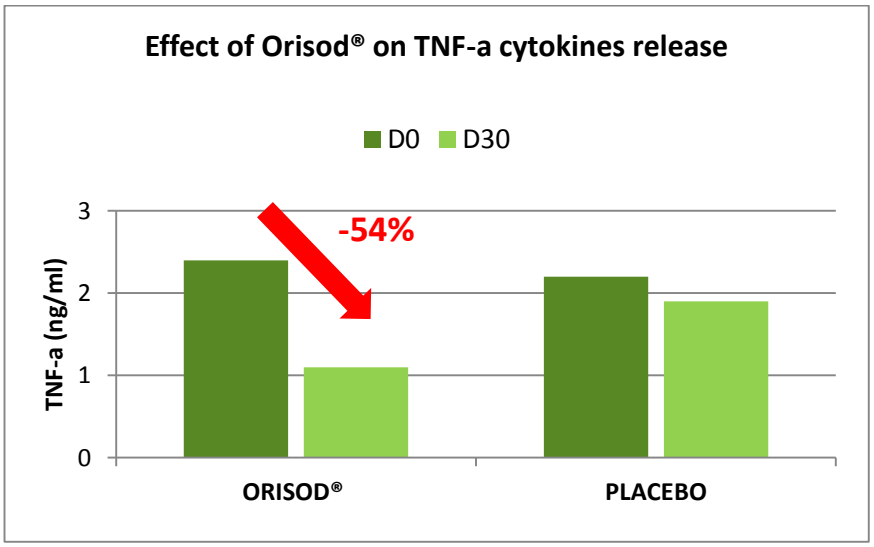


Figure 2: Effects of Orisod supplementation on TNF-a cytokines release.

Discussion

In the present study the effect of ORISOD® formulated with Mediterranean natural plants olive and rosemary on cytokines levels was exanimated.

Physical exercise induces immunological acute changes in the cytokines level. An increase in IL-6 levels, which seems to derive from skeletal muscle, is observed as well as an increase in TNF-a levels after intense exercises.

In the present study, a significant decrease of cytokines levels IL-6 and TNF-a after 30 days was measured. These decreases were clearly influenced by the ORISOD® supplementation (as the placebo group didn't show any significant decrease).

Conclusion

In conclusion, ORISOD® intake during 30 days by young healthy male submitted to regular intense physical training induced a decrease of IL-6 and TNF-a cytokines levels. ORISOD® antioxidants contained in olive leaf and rosemary extracts have shown a protective effect against oxidative damage, resulting in decreasing the signs of inflammation.

6. CONCLUSION

ORISOD Enzyme® is a new generation of antioxidant formulated to support body's natural antioxidant system and improve body's regeneration, including age-related damages. Olive and Rosemary, grown in Mediterranean land in the south of France, are used to activate cells self-healing processes that naturally exist in the living organism.

The unique fermentation process behind **ORISOD Enzyme®** allows to produce a complex rich in metabolites which support in a synergic way body metabolism by enhancing extracts digestibility, maintains stability and offer better results at the long term.

Rather than classical antioxidants like vitamins, **ORISOD Enzyme®** mechanims consist of enhancing endogenous enzymes, the first line of intracellular defence. This approach is more effective and faster for improving cells health than classical antioxidant.

ORISOD Enzyme®also supports the liver detoxification by eliminating the toxins that can come from internal or external factors: pollution, stress, smoking, alcohol etc. and helps restoring mitochondrial biogenesis for cell generation and energy production.

The results of clinical trials performed on **ORISOD Enzyme®** have demonstrated significant results:

- **ORISOD Enzyme®** exhibited a higher antioxidant capacity than BHT (a powerful synthetic antioxidant) when used at identical concentration during TBARS assay (- 33% in oxidation level)
- **ORISOD Enzyme®** improves the activity of 2 main endogenous antioxidant enzymes: Superoxyde Dismutase SOD (+25%) and Glutathione Peroxidase GPx (+19%).
- The treatment also exhibited significant decreases of lipid peroxidation (-43%) and LDL Oxidation (-29%) after 3 months.
- **ORISOD Enzyme®** antioxidants contained in olive leaf and rosemary extracts have shown a protective effect against oxidative damage, resulting in reducing the signs of inflammation: decrease of IL-6 cytokines levels (-58%) and TNF-a cytokines levels (-54%).

ORISOD Enzyme® is a new generation of antioxidant acting in speed mode to reduce oxidation and restore body's natural antioxidant system, providing best efficacy on chronic deseases.

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Mitochondrial aldehyde dehydrogenase and cardiac diseases

Che-Hong Chen, Lihan Sun, and Daria Mochly-Rosen

Abstract

Numerous conditions promote oxidative stress, leading to the build-up of reactive aldehydes that cause cell damage and contribute to cardiac diseases. Aldehyde dehydrogenases (ALDHs) are important enzymes that eliminate toxic aldehydes by catalysing their oxidation to non-reactive acids. The review will discuss evidence indicating a role for a specific ALDH enzyme, the mitochondrial ALDH2, in combating oxidative stress by reducing the cellular 'aldehydic load'. Epidemiological studies in humans carrying an inactive ALDH2, genetic models in mice with altered ALDH2 levels, and small molecule activators of ALDH2 all highlight the role of ALDH2 in cardioprotection and suggest a promising new direction in cardiovascular research and the development of new treatments for cardiovascular diseases.

Keywords: ALDH2, Mitochondria, Ischaemia, Nitroglycerin, Alda-1

1. Introduction: oxidative stress and aldehyde toxicity

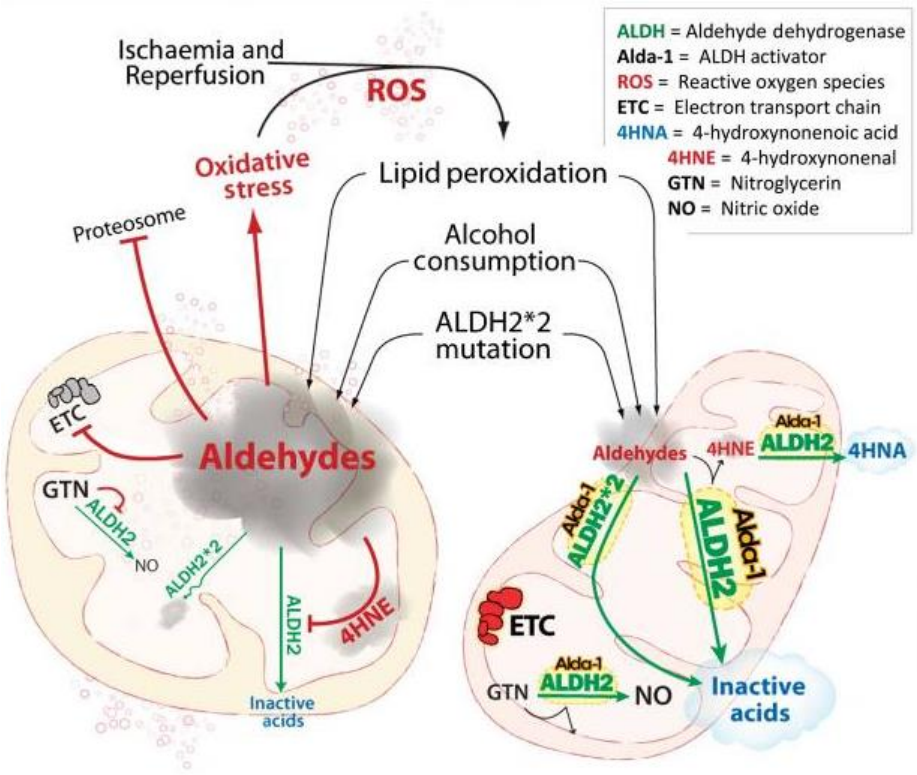
Ischaemia and reperfusion as well as mismatch between cardiac demand and cardiac function result in oxidative stress in the myocardium.[1,2] Oxidative stress is a state in which excessive reactive oxygen species (ROS), such as O_2^- and H_2O_2, accumulate and result in cellular toxicity, due to imbalance between production and removal of ROS.[3,4] In the myocardium, a significant ROS production occurs in the mitochondria and when it is excessive, it contributes to oxidative stress and mitochondrial dysfunction.[5,6] The need to reduce oxidative stress to protect the heart led to both preclinical and clinical research. Although early research efforts focused on the deleterious effects of ROS in the myocardium,[1,7] more recent work revealed that accumulation of cytotoxic and reactive aldehydes derived from ROS-induced stress[8–10] or from direct insults of exogenous aldehydes[11] can also severely impair cardiac functions. Although low levels of ROS and aldehydes may be regarded as second messengers that trigger stress-activated anti-oxidant mechanisms (review by Poli *et al*.[12]), it is nevertheless critical that the cells maintain a defensive capacity to prevent acute or chronic build-up of excessive oxidative stress and toxic aldehydes, as these cause irreversible injuries.

Aldehydes are generated during numerous physiological processes including catabolism of amino acids, transmitters such as GABA, serotonin, noradrenaline, adrenaline, and dopamine.[13–15] Further, more than 200 different aldehydes are generated through lipid metabolism[16] and metabolism of carbohydrates also generates many aldehydic intermediates.[13] In addition to these endogenous aldehydes, aldehydes are ubiquitously present in the environment in smog, cigarette smoke, motor vehicle exhaust, and in a variety of industrial processes including the production of polyurethane, polyester plastics (these include formaldehyde, acetaldehyde, and acrolein). Although some dietary and aromatic aldehydes (e.g. citral, cinnamaldehyde, benzaldehyde, and retinal) are approved additives in various foods and cosmetics where they impart flavour and odour, many others are cytotoxic. A mechanism for a rapid clearance of aldehydes is essential to protect the human body, in general, and the myocardium and the brain, in particular, from the harmful effects of these aldehydes.

Aldehydes are diffusible and highly reactive agents in cells; they form adducts with lipids, proteins, and DNA, which affect the function of these macromolecules and can lead to their inactivation.[16] Because of DNA damage induced by aldehyde adducts, a wide range of aldehydes have been classified as mutagenic and carcinogenic.

These include even acetaldehyde, which is derived from ethanol drinking; excessive ethanol consumption has been linked to alcoholic liver diseases and various upper aerodigestive and gastrointestinal cancers.[17] 2,4-Dihydroxy-phenyacetaldehyde, another highly potent neurotoxin, is an intermediate of dopamine metabolism and has been implicated in neurodegenerative diseases.[18,19] Methylglyoxal, an aldehyde accumulated in hyperglycaemia, is a key intermediate for the adduction formation in advanced glycation end-products found in various organs of diabetic patients.[20] Many other reactive and cytotoxic aldehydes have been shown to be associated with other diseases.

Of particular interest to cardiovascular diseases are reactive aldehydes such as 4-hydroxy-2-nonenal (4-HNE) and malondialdehyde (MDA).[21] In conditions like ischaemia and reperfusion, ROS-induced peroxidation of polyunsaturated fatty acids like linoleic acid and arachidonic acid present in the plasma membrane leads to 4-HNE production[16,22] (*Figure 1*). 4-HNE is a highly reactive carbonyl compound due to the presence of α,β-unsaturated carbons.[23] 4-HNE readily reacts with cysteine, histidine, and lysine residues, and thus forms protein adducts via the Michaels addition.[23,24] 4-HNE adduct formation in the myocardium has been shown to lead to the inhibition of key metabolic enzymes such as glyceraldehyde 3-phosphate dehydrogenase[24] and the 26S proteosome,[25,26] thus leading to further accumulation of damaged proteins in the cell. 4-HNE also impairs ATP-generating ability in the mitochondria,[27] induces opening of mitochondria permeability transition pore, and impairs mitochondrial integrity in a concentration-dependant and calcium-mediated manner[28,29] (*Figure 1*), thus reducing the cell's ability to repair the damaged macromolecules. As a potent cardiac cytotoxin, 4-HNE has been shown to directly inhibit contractility,[30] induce pro-arrhythmic effects in isolated cardiac myocytes,[31] and cause tissue damage after cardiac ischaemia.[32]



A scheme depicting aldehyde-induced mitochondrial damage and how ALDH2 reduces this aldehydic toxicity. (Left) Ischaemia and reperfusion and other oxidative stress in the heart increase ROS production, which triggers lipid peroxidation and the accumulation of reactive aldehydes, such as 4-HNE. Other xenogenic aldehydes from the environment, from ethanol metabolism, and from food additives can also increase the cellular 'aldehydic load' (depicted as a grey cloud in the figure). Aldehydes induce inactivation of a number of macromolecules including the proteasome, the electron transport chain (ETC) in the mitochondria, as well as inactivation of ALDH2 itself. This aldehyde-induced macromolecule inactivation contributes to mitochondrial impairment and increases in oxidative stress, thus leading to cell damage. Particularly relevant to cardiac disease, the use of nitroglycerin (GTN) can further contribute to ALDH2 inactivation, thus decreasing the cell's natural ability to reduce ROS-induced aldehydic load and cytotoxicity. Finally, a common mutation in ALDH2 (ALDH2*2) in humans further impairs the ability to reduce the aldehydic load under oxidative stress conditions. (Right) Agents that increase ALDH2 activity and protect ALDH2 from inactivation by aldehydes and by GTN will decrease the aldehydic load by enhancing the conversion of aldehydes to non-reactive acid (blue cloud in the scheme), thus leading to cytoprotection. Such one potential agent is Alda-1, an aldehyde dehydrogenase 2 activator. Alda-1 increases ALDH2 activity by about two-folds, blocks ALDH2 inactivation by both aldehydes and GTN, and thus increases the cell's natural ability to protect from oxidative stress, leading to 60% reduction from cardiac damage in an animal model of AMI. The ability of Alda 1 to increase the activity of the mutant ALDH2*2 may be of particular importance for over >0.5 billion humans who carry this mutation. (See text for details.) Reduction in aldehydic load decreases mitochondrial structural and functional damages and increases ATP generation, thus leading to cardiac protection from oxidative stress.

In another study of aldehyde-induced cardiac toxicity, Wang *et al*.[11] showed that acrolein, an exogenous reactive aldehyde that is ubiquitously present in many food sources, can exacerbate ischaemic damage to the heart. In a model of acute myocardial infarction (AMI), oral delivery of acrolein (5 mg/kg) to mice 30 min before coronary artery occlusion and 24 h reperfusion produced a significantly increased myocardial infarct size when compared with the water-treated control group.[11] Interestingly, acrolein also abolished the late preconditioning cardioprotective effect offered by the pre-treatment of an NO donor, diethylenetriamine/NO. The levels of acrolein–εPKC adducts and acrolein adducts in other proteins in the mitochondrial fraction increased in an acrolein dose-dependent manner.[11] High cardiovascular toxicity and statistically significant association between aldehyde-containing components of polluted air or cigarette smoke and incidents of ischaemic heart disease, arrhythmias, and heart failure have also been reported.[31] These findings heightened the importance and awareness that both endogenous reactive aldehydes such as 4-HNE and aldehydes present as environmental pollutants might be contributors of these harmful cardiovascular effects and that a mechanism that protects from these aldehydes and accelerates their removal is essential to protect the myocardium as well as other organs from oxidative stress.

2. Aldehyde dehydrogenases

There is a class of detoxifying enzymes that catalyse the removal of aldehydes in the body. These are the $NAD(P)^+$-dependent aldehyde dehydrogenase (ALDH) super gene family. Nineteen ALDH genes have been mapped in the human genome, of which 17 ALDH isozymes are expressed with different tissue distributions.[15] All the ALDH genes are nuclear encoded, but at least five ALDH isozymes reside and function in the mitochondria.[13] We have focused this review on the role of the mitochondrial ALDH2 in cardiovascular disease, since this enzyme has emerged as a key enzyme of cardioprotection.[8,9,33–35]

ALDH2 is a tetrameric enzyme and is expressed abundantly in the liver and lung, and is also present in organs that require high mitochondrial capacity for oxidative ATP generation such as heart and brain.[36] ALDH2 is important in

the oxidization of aldehydic substrates, such as 4-HNE, acrolein, and short chain, aromatic or polycyclic carbons.[23,37] In addition to its dehydrogenase activity, ALDH2 can function as an esterase and reductase, depending on the substrates. Much current attention has also been drawn to ALDH2 for its role in the biotransformation of nitroglycerin, by its reductase activity, to 1,2-glyceryl dinitrate for the production of nitric oxide, a critical vasodilator.[38,39]

ALDH2 is best known for its ability as a detoxifying enzyme of acetaldehyde, an intermediate of ethanol metabolism. Importantly, more than 40% of the East Asians population carries a common ALDH2*2 mutant allele, which results in a dramatic reduction in the enzymatic activity when compared with the ALDH2*1, wild-type allele;[40] carrier of this mutant ALDH2 has a characteristic acetaldehyde-induced facial flushing when drinking alcohol. The mutation is caused by a single-nucleotide substitution (G to A) in exon 12, leading to a change from glutamate to lysine at position 487.[41] The biochemical characterization, molecular structure, and physiological consequences of ALDH2*2 have been extensively studied.[41,42] The E487K amino acid substitution at the dimer interface of the tetrameric enzyme resulted in disruption of the co-enzyme NAD binding and reduced catalytic activity of ALDH2*2.[43] Heterozygous ALDH2*1/*2 individuals retain only 10–45% of the enzymatic activity and homozygous ALDH*2/*2 individuals have 1–5% of wild-type ALDH activity, due to this single amino acid polymorphism.[44] The ALDH2*2 allele has been linked to an increased risk of oesophageal and other upper aerodigestive tract cancers among alcohol drinkers.[45–47] But, higher incidences of insensitivity to nitroglycerin treatment for angina,[48] MI,[49,50] hypertension,[51,52] and other oxidative-related neurodegenerative diseases[14] have also been associated with ALDH2*2 mutation in recent years (see further discussion in the following). Since the affected world population of ALDH2*2 is estimated to be at least 540 million or ∼8% of the world population,[45] it is warranted that health risk for cardiac diseases be re-evaluated in ALDH2*2 carriers.

3. Mitochondrial ALDH2 and cardioprotection against ischaemia and reperfusion injury

AMI is one of the leading causes of disability and death in the USA.[53] Clinical interventions such as angioplasty or thrombolytic agents have been effective in re-establishing the coronary flow. However, treatments to further reduce the injuries incurred during the ischaemic period or by reperfusion are not available. A large body of research has identified an endogenous process of cytoprotection from ischaemia/reperfusion injury.[54] Cytoprotection can be induced by subjecting an organ to short bouts of ischaemia prior to the prolonged ischaemia, a process termed ischaemic preconditioning.[55] Preconditioning can also be induced and/or enhanced by select hormones and neurotransmitters[56] and is dependent on the activation of the diacylglycerol-dependent protein kinase C epsilon (εPKC[57,58]).

One pharmacological agent that mimics ischaemic-preconditioning-induced cardioprotection is ethanol.[59,60] This effect may be different from the cardioprotective effect of moderate habitual consumption of alcohol;[61] we found that a brief exposure to 10 mM ethanol 10–20 min prior to ischaemia can protect rat heart from prolonged ischaemic damage in both cultured cardiac myocytes and adult whole heart.[60] Using an unbiased proteomic approach, we then identified the mitochondrial ALDH2 as a key enzyme downstream of ethanol-induced εPKC-dependent cardioprotection.[9] ALDH2 is phosphorylated under conditions that lead to cardiac cytoprotection and its enzymatic activity is inversely correlated with the severity of the damage from cardiac ischaemia ($R^2 = 0.95$[9]). High-throughput screening of libraries of compounds identified *N*-(1,3-benzodioxol-5-ylmethyl)-2,6-dichlorobenzamide (ALDH activator-1 or Alda-1, MW = 324) and its halogen analogues as selective agonists of ALDH2 (*Figure 1*).

The identification of a selective activator for ALDH2 confirmed that ALDH2 activation is not only required, but sufficient to induce cardioprotection; treatment by Alda-1 increased ALDH2 activity by two-fold and reduced infarct size by 60% in an in vivo model of myocardial infarction in rats. It is likely that the benefit of ALDH2

activation is to facilitate the removal of cytotoxic aldehydes, such as 4-HNE and others that accumulate during ischaemia and reperfusion.[9,32,62,63] 4-HNE has been shown to be a substrate as well as a potent inhibitor of ALDH2 (due to 4-HNE adduct formation on ALDH2[64,65]). Using a human recombinant ALDH2 enzyme, at 100 μM, 4-HNE completely inactivated ALDH2 activity in vitro. However, the 4-HNE-induced inhibition of ALDH2 activity was completely prevented in the presence of Alda-1.[9] As anticipated, following ischaemia and reperfusion, the accumulation of 4-HNE–protein adducts was lower in hearts treated with Alda-1, relative to vehicle-treated controls.[9] The ability of Alda-1 to reduce cardiac damage is therefore likely due to a combination of direct enzyme activation of ALDH2 and prevention of ALDH2 inactivation by its reactive substrate, 4-HNE, which is formed and accumulates under oxidative stress (Figure 1). Using an open-chest model of AMI in vivo, Churchill et al.[33] further demonstrated that under cardioprotective conditions, εPKC translocates directly into the mitochondria where it interacts with ALDH2, increases its enzymatic activity, diminishes the pro-apoptotic signalling activity of JNK1/2 and ERK1/2 and reduced 4-HNE–protein adduct formation. Further, in εPKC knockout mice, direct activation of mitochondrial ALDH2 by an Alda-1 analogue, Alda-44, protects the heart against ischaemia/reperfusion injuries similar to what was achieved by ethanol preconditioning in the wild-type mice.[66] These results indicate that ALDH2 is a direct downstream substrate of εPKC and suggest that the activation of ALDH2 is necessary and sufficient to confer. Hill et al.[67] have shown that inhibition of 4-HNE oxidation during ischaemia may be due to a decrease in NAD/NADH ratio, which renders ALDH2 less effective. On the other hand, excessive production of 4-HNE via lipid peroxidation occurs most likely during the reperfusion period when NAD levels are rapidly restored. It is during that reperfusion period that ALDH2 may play a critical role in cardioprotection by enhancing the removal of ROS-generated 4-HNE. We found that Alda-1 increases the removal of 4-HNE to levels closer to basal levels[9] by increasing the productive substrate–enzyme interaction.[68] Furthermore, Beretta et al.[69] have recently demonstrated that Alda-1 also increases the affinity of ALDH2 for NAD, which should result in increased ALDH2 activity even if NAD levels have not yet recovered back to basal levels, and cardioprotection by enhancing the detoxifying capability of the cells against reactive aldehydes.

Recent epidemiological studies indicated that ALDH2 is critical in cardiovascular diseases. A study conducted in Korea showed an association of higher risk of MI with the E487K mutation carried by both ALDH*1/*2 heterozygous and ALDH2*2/*2 homozygous individuals in older Korean men.[49] An independent study conducted in Japan also identified ALDH2*2/*2 genotype as a risk factor for MI incidents in Japanese me.[50] An important feature of Alda-1 is its ability to increase the activity of the inactive ALDH2*2 mutant.[9] Alda-1 increased the enzymatic activity of ALDH2*2 homotetramers and ALDH2*2 heterotetramers by 11- and two-fold, respectively. The ability of Alda-1 to partially complement or restore the activity of the mutant, ALDH2*2, is striking, as it is rare to find a small molecule that can specifically rescue a mutation in humans. This enhancement of catalytic activity translates to a possibility of rescuing some enzymatic activity for the ALDH2*2/*2 individuals and restoring the activity of ALDH2*1/*2 individuals to almost the basal level in the wild-type ALDH2*1/*1 individual.[9] Co-crystal structures of Alda-1 with both ALDH2*1 wild-type and ALDH2*2 mutant enzymes have recently been resolved and offered detailed mechanistic explanations on how Alda-1 can enhance the enzymatic activity, protect ALDH2 against 4-HNE inactivation, and restore the function of ALDH2*2 mutation.[68] Structural comparison revealed that Alda-1 interacts with ALDH2 at the substrate-binding tunnel and kinetically increased the rate of catalysis by reducing the probability of non-productive substrate hydrolysis. The unique binding site of Alda-1 also positions this compound as a shield to the key sulfhydryl amino acid, Cys302, at the catalytic centre and likely protects the ALDH2 molecule against enzyme inactivation by its reactive aldehyde, 4-HNE. Finally, even though Alda-1 made no direct contact with the mutated E487K residue, it nevertheless functioned as a molecular chaperone and restored the electron density and structural abnormality of the co-enzyme-binding site within ALDH2*2. Alda-1 therefore represents as a new pharmacological agonist and opens the possibility that

modulation of ALDH2 by small molecule enzyme activators should have therapeutic value for cardiovascular and other ALDH2-related diseases.

4. Angina, nitroglycerin bioactivation, tolerance, and ALDH2

Since its discovery in 1860s, nitroglycerin has become one of the most widely used drugs, treating patients with stable and unstable angina and with AMI and heart failure. About 9.8 million Americans experience angina annually and many of them are given nitroglycerin for both acute and chronic symptom relief.[70] The beneficial effect of nitroglycerin is achieved due to its ability to increase blood flow to the heart by dilating coronary arteries and decreasing cardiac preload due to venodilation.[71] Although acute treatment including sublingual regimen is immediate and effective, the benefit of nitroglycerin has been limited because of *in vivo* tolerance that rapidly develops on continuous treatment.[72] (Nitroglycerin tolerance is manifested as a reduced vasodilatation effect and requirement of high doses of the drug after continuous treatment.)

Previous experimental and clinical investigations have uncovered several critical mechanisms of nitroglycerin tolerance, including oxidative stress, endothelial dysfunction, and increased sensitivity to vasocontrictors.[72,73] Recently, Chen *et al*. reported a pathway involving the mitochondrial enzyme, ALDH2, that provides a novel mechanistic insight into the development of nitroglycerin tolerance.[38,39,74] In their study measuring conversion of nitroglycerin to nitric oxide, *ex vivo*, they found that bioactivation of nitroglycerin into 1,2-glyceral dinitrate, the physiologically relevant metabolite of nitroglycerin, was significantly diminished in aortas from ALDH2 knockout mice. Similar results were observed using ALDH2-selective inhibitors in animals as well as in humans.[75,76] These studies have defined an unequivocal dependence of nitroglycerin bioactivation on ALDH2. Further studies in murine and human tissues have demonstrated a more targeted role for ALDH2 that explains nitroglycerin tolerance[77,78] (*Figure 1*). In rabbit aorta made tolerant by large doses of nitroglycerin, ALDH2 dehydrogenase activity was inhibited by ∼50%.[74] Similar inhibition was observed in studies using rat hearts, *ex vivo*.[9] Although it remains to be determined how prolonged treatment with nitroglycerin leads to inactivation of ALDH2, these studies have opened a new avenue for examining the role of ALDH2 in the myocardium.

As described above, activation of ALDH2 reduced cardiac damage caused by ischaemia insult, indicating a cardioprotective role for ALDH2.[9] That study also demonstrated that inactivation of ALDH2 associated with nitroglycerin tolerance resulted in an increase in infarct size.[9] These data suggest a potential risk to patients who experience an AMI while on continuous nitroglycerin treatment (*Figure 1*). Whereas clinical treatments such as the intermittent use of organic nitrates have proven effective in reducing tolerance, other consequences of nitroglycerin tolerance have been poorly understood. Correlation studies analysing existing patients have yielded useful but incomplete data. In a 5-year follow-up study examining the long-term efficacy of nitrate therapy for the treatment of AMI in Japan, Yamauchi *et al*. found higher mortality rate in nitrate-treated group (18.9%), compared with the control patients (11.0%). However, the author acknowledged the limitation of the study, as the difference may be attributed to, among other uncontrolled factors, an increased frequency of nitrate use in patients with more severe conditions.[79] Information on the consequence of nitroglycerin tolerance will be valuable for a better clinical use of nitroglycerin and other nitrates in the treatment of patients with angina-related cardiovascular diseases.

In the light of the ALDH2-dependent bioactivation of nitroglycerin in Asians carrying the E487K mutation, it is predicted that the substantially diminished ALDH2 activity would lead to a decreased response to nitroglycerin treatment. Indeed, *in vitro* experiments showed that the E487K mutant enzyme was ∼10-fold slower in catalysing nitroglycerin conversion to 1,2-glyceral dinitrate.[69] Moreover, previous clinical studies confirmed a marked decrease in nitroglycerin efficacy in patients carrying the mutant ALDH2*2 relatively to carriers of the wild-type enzyme.[48,75] Not surprisingly, larger doses of nitroglycerin were required to achieve sufficient vasodilatation in

subjects with the ALDH2*2 form. Because the Asian ALDH2*2 mutation may be associated with a higher risk of various diseases including ischaemic damage[9,13] due to a significant loss of ALDH2 activity, the consequence of nitroglycerin tolerance in the background of E487K polymorphism needs to be further investigated.

Since Alda-1 activates both the dehydrogenase activity and esterase activity of ALDH2,[9,68] Beretta *et al*. evaluated the effect of Alda-1 on bioactivation of nitroglyercin.[69] Surprisingly, Alda-1 failed to increase GTN denitration and bioactivation in these assays using either the ALDH2 wild-type and ALDH2*2 recombinant enzyme *in vitro*. A drug that can increase the potency of nitroglycerin either by enhancing its bioconversion to NO and/or by preventing the inhibitory effect of nitroglycerin on ALDH2 will clearly be beneficial, especially for the ALDH2*2 human subjects.

5. ALDH2 in transgenic mice, ethanol/acetaldehyde metabolism, and cardiovascular disease

Several independent ALDH2 transgenic mice have been established and studied extensively, especially with regard to the role of ALDH2 in ethanol metabolism. In one model, ALDH2 knockout mice were produced by gene interruption at the ALDH2 locus.[80] As expected, these ALDH2-null mice lacked any detectable ALDH2 enzyme activity, accumulated a high level of acetaldehyde when exposed to ethanol, and were significantly more sensitive to alcohol and acetaldehyde toxicity and damage.[81–83] Surprisingly, in these ALDH2-null mice, both acute and chronic administration of ethanol seem to produce a smaller extent of oxidative stress in the liver as measured by the decreased levels of MDA, alanine aminotransferase, TNF-α in the serum and increased level of the anti-oxidant, glutathione, when compared with the wild-type ALDH mice.[84,85] The molecular mechanism for the reduction of these oxidative stress biomarkers is not clear, but may be associated with the metabolism of ethanol itself through the microsomal CYP2E1 pathway in the liver. It appears unlikely, though, that such ethanol-induced protective effect exists in hearts of the ALDH2*2 carriers. In fact, using the same ALDH2-null mice, Wenzel *et al.*[86] demonstrated that the loss of ALDH2 enzyme activity led to increased mitochondrial oxidative stress in aortic endothelia by three pro-oxidant stimuli, nitroglycerin, doxorubicin, and acetaldehyde (*Figure 1*).

Overexpression of ALDH2 wild-type enzymes appeared to confer multiple beneficial effects to the heart tissue and cardiac functions in another transgenic mice model. Ma *et al*.[87] explored the effect of ALDH2 overexpression on acute ethanol-induced myocardium damage. Acute ethanol challenge (3 g/kg) severely impaired myocardial and myocyte functions in the wild-type FVB mice as a result of acetaldehyde toxicity. This is evidenced by the reduction in maximal velocity of pressure development and decline ($\pm dP/dt$), left ventricular developed pressure, cell shortening, prolonged relengthening duration, and an increase in cardiac protein carbonyl level within 24 h after ethanol administration. These negative outcomes were much reduced in mice expressing four-fold higher levels of ALDH2. In this study, overexpression of ALDH2 was associated with a suppression of phosphatase activity which leads to enhanced Akt and AMP-activated protein kinase activities and regulation of their downstream targets Foxo3 transcription factor and caspase-3.[87,88] Thus, ALDH2 appears critical in protecting the heart from aldehyde toxicity and is able to elicit signalling events that can protect against myocardial damages caused by acute ethanol toxicity.

Acetaldehyde toxicity also results in an exacerbated cardiac hypertrophy and contractile defect.[89,90] In models of chronic alcohol ingestion, mimicking human alcoholism, overexpression of the ALDH2 enzyme dramatically reduced the complications of the cardiovascular system. Control mice placed on a 4% alcohol liquid diet for 14-week developed cardiac hypertrophy and contractile defects, whereas mice overexpressing ALDH2 exhibited a significant attenuation of cardiac hypertrophy and contractile dysfunction.[34] These improvements were attributed to better calcium handling, reduction in myocardial fibrosis, reduction in protein carbonyl formation, and reduction in apoptosis, likely through a molecular mechanism associated with phosphorylation of apoptosis-stimulated kinase, glycogen synthase kinase-3β, GATA4, and cAMP-response element-binding protein. Related to

alcohol-induced cardiomyopathy, overexpression of ALDH2 can also overcome insulin resistance in the heart of chronically alcohol-fed mice via an improvement of insulin signalling.[91] This ALDH2-mediated protection mechanism also extends to the brain as apoptosis and cerebral damages were ablated in ALDH2 overexpressing mice after 12 weeks of continuous ethanol ingestion.[92]

Another approach to studying the potential consequences of E487K mutation represented in the ALDH2*2 carrying East Asians is the use of transgenic mice overexpressing ALDH2*2. Endo *et al*.[93] created these loss-of-function ALDH2 transgenic mice by overexpressing the E487K human form under a strong promoter. In these mice, expression of the dominant-negative ALDH2*2 resulted in impaired ALDH activity when using a variety of aldehydic substrates. These mice also showed increased mitochondrial oxidative stress demonstrated by an elevated level of 4-HNE–protein adducts, a decline in mitochondria respiratory function, smaller body size, reduced muscle mass, diminished fat content, osteopenia, kyphosis, and smaller heart morphology.[93] Surprisingly, despite the presence of substantial mitochondrial oxidative damage, the hearts of these ALDH2*2 transgenic mice demonstrated a greater tolerance to oxidative stress. This metabolic remodelling was attributed to increased glutathione biosynthesis to compensate for the diminished ALDH activity and the elevated oxidative stress. It should be noted that since ALDHs are tetramers, it is possible that overexpression of ALDH2*2 monomer inactivates not only the wild-type ALDH2, but may also form heterotetramers and inactive other ALDH isozymes (e.g. heterotetramer formation between ALDH2 and another highly homologous ALDH1B1).[81,94] Since ALDH2*2 levels were eight-fold higher than that of the endogenous ALDH2 wild-type protein in the mitochondria, ALDH2*2 may therefore have pleiotropic effects. This may explain why the ALDH2-null mice and mice that express ALDH2*2 only one- to three-fold over the endogenous ALDH2 protein do not show these major pathologies.[95] Whether metabolic remodelling and cardiac adaptation exist in ALDH2*2 Asians subjects therefore remains to be determined. A better transgenic animal model for the study of human ALDH2*2 deficiency would be the introduction, by gene targeting, of a single true genomic E487K point mutation to replace the wild-type ALDH2*1 allele on mouse chromosome 5 via homologous recombination.

6. Conclusion

Aldehydes are commonly formed inside cells as metabolic products (e.g. products of lipid peroxidation) or can gain access to the body from the environment (e.g. industry pollutants and food additives). Because aldehydes are chemically reactive moieties, forming adducts on a variety of macromolecules, which result in impairment or inactivation of these macromolecules, aldehydes catabolism is critical for cytoprotection, in general, and for cardioprotection, in particular. A natural cardioprotective mechanism involves a family of ALDHs and in particular the intra-mitochondrial enzyme, ALDH2. Although ALDH2 is constitutively active, it can be further activated by ischaemic preconditioning (due to εPKC-mediated phosphorylation) as well as by small molecule activators of this enzyme, Aldas. In addition for its role in removal of toxic aldehydes, the role of ALDH2 in nitroglycerin conversion to the bioactive NO highlights the importance of this enzyme in a variety of cardiovascular diseases. Epidemiological studies in humans carrying an inactivating mutation in ALDH2, combined with genetic and pharmacological studies in animal models, suggest ALDH2 as an important target for generating new treatments for heart diseases.

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Mitochondria-targeted Antioxidants as Therapies

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Abstract: Mitochondria are central to oxidative phosphorylation and much of metabolism, and are also involved in many aspects of cell death. Consequently, mitochondrial dysfunction contributes to a wide range of human pathologies. In many of these, excessive oxidative damage is a major factor because the mitochondrial respiratory chain is a significant source of the damaging reactive oxygen species superoxide and hydrogen peroxide. However, despite the clinical importance of mitochondrial oxidative damage, antioxidants have been of limited therapeutic success. This may be because the antioxidants are not selectively taken up by mitochondria, but instead are dispersed throughout the body. To address this unmet need, a series of mitochondria-targeted antioxidants have been developed over the past few years that are selectively concentrated within mitochondria *in vivo*. The accumulation of an antioxidant at the site where it is needed most has been shown to improve the outcome in a large number of animal models of diseases that involve mitochondrial oxidative damage. Mitochondria-targeted antioxidants have also been developed as pharmaceuticals and have been shown to be safe and effective in human clinical trial phase IIa studies. Therefore the mitochondria-targeted antioxidants are a new class of pharmaceuticals that can be used in a wide range of human pathologies for which current therapies are of limited efficacy. Here we survey the work that has been done to date using mitochondria-targeted antioxidants and suggest future applications.

Introduction

Mitochondria are central to energy metabolism and also play crucial roles in intermediary metabolism and in many other vital functions such as iron-sulfur center assembly, thermogenesis, and heme biosynthesis (Murphy and Smith, 2000; Saraste, 1999; Szewczyk and Wojtczak, 2002; Wallace, 1999). Mitochondria are also critically involved in cell death by both the apoptotic and necrotic pathways (Kroemer *et al.*, 1997; Lemasters *et al.*, 1998). The wide range of ways in which mitochondria contribute to the life and death of a cell makes it unsurprising that mitochondrial dysfunction plays a role in a number of human pathologies (Balaban *et al.*, 2005; Murphy, 2009b; Murphy and Smith, 2000). These include neurodegenerative diseases, cardiac dysfunction, inflammation, ischemia-reperfusion injury in heart attack and stroke, sepsis, types I and II diabetes, metabolic syndrome, and the success of organ transplantation (Ames *et al.*, 1993; Beckman and Ames, 1998; Balaban *et al.*, 2005; Finkel, 2005; Green *et al.*, 2004). Mitochondrial function can be disrupted due to genetic defects in either mitochondrial or nuclear genomes (Wallace, 1992), as a consequence of cumulative damage over the lifetime of a subject (Beckman and Ames, 1998), or in response to acute trauma (Halestrap, 2005). Furthermore, even if the primary cause of a pathology is unrelated to mitochondria, the tendency of mitochondria to initiate apoptosis or necrosis and the requirement for ATP for a cell to recover from an insult means that mitochondrial dysfunction is a significant secondary factor in determining clinical outcome. In most cases of pathological mitochondrial dysfunction, elevated oxidative damage is thought to play a role (Ames *et al.*, 1993; Beckman and Ames, 1998; Finkel, 2005; Green *et al.*, 2004). This is because the mitochondrion is a major source of the reactive oxygen species superoxide within the cell that leads to oxidative damage (Balaban *et al.*, 2005; Murphy, 2009a). As mitochondria are particularly susceptible to oxidative damage this contributes to mitochondrial dysfunction and cell death in a range of diseases (Balaban *et al.*, 2005; Murphy, 2009a).

The importance of mitochondrial oxidative damage in clinically important situations makes preventing it a compelling therapeutic target (Murphy and Smith, 2000). Furthermore, as mitochondrial oxidative damage occurs in many disorders such a therapy would be widely applicable, making this approach attractive to the pharmaceutical industry. The obvious way to decrease oxidative damage is through the use of antioxidants, thereby reducing the levels of reactive oxygen species such as superoxide and blocking the reactions that underlie mitochondrial oxidative damage. However, the many extensive clinical trials of conventional antioxidants such as Vitamin E or Vitamin C for diseases that involve mitochondrial oxidative damage have yielded disappointing results (Bjelakovic *et al.*, 2008; Cocheme and Murphy, 2010). One possible explanation for this may be that the antioxidants distribute widely in the body, with only a small fraction being taken up by mitochondria (Murphy and Smith, 2007). Consequently, the protective agents may not be locating where they are needed most in sufficient amounts to impact on the oxidative damage. To address this unmet need a number of mitochondria-targeted antioxidants have been developed (Murphy and Smith, 2007; Murphy and Smith, 2000; Skulachev *et al.*, 2009; Smith *et al.*, 1999; Szeto, 2008). These antioxidants are modified so that they are selectively concentrated within mitochondria *in vivo*. The accumulation of the antioxidant should then preferentially reduce mitochondrial oxidative damage and improve the clinical outcome. Ideally, mitochondria-targeted antioxidants should be pharmaceutically tractable and stable small molecules with acceptable oral bioavailability that are selectively taken up by mitochondria within organs where they block oxidative damage and then can be recycled back to the active antioxidant form (Murphy and Smith, 2007). A number of approaches have been used to develop mitochondria-targeted antioxidants and here we survey these approaches, report on results obtained so far, and discuss future applications.

Targeting Antioxidants to Mitochondria

Two general strategies have so far proved most useful in targeting small molecule antioxidants to mitochondria *in vivo*: conjugation to lipophilic cations (Murphy and Smith, 2007) or incorporation into mitochondria-targeted peptides (Horton *et al.*, 2008; Szeto, 2006). These approaches are outlined below.

Conjugation of antioxidants to lipophilic cations

Lipophilic cations can pass easily through the phospholipid bilayers of the plasma membrane and the mitochondrial inner membrane because the charge of the cation is effectively distributed over a large and hydrophobic surface area, thereby lowering the activation energy for their movement across the membrane (Liberman and Skulachev, 1970; Ross *et al.*, 2005). The ability of these cations to move through phospholipid bilayers enables their accumulation into the mitochondrial matrix simply in response to the large, negative-inside mitochondrial membrane potential (Liberman and Skulachev, 1970; Ross *et al.*, 2005) and does not require any specific import mechanism. The Nernst equation indicates that the uptake of lipophilic cations into the mitochondrial matrix driven only by the membrane potential increases 10-fold for every 61.5 mV. As the mitochondrial membrane potential *in vivo* is usually ~140-160 mV, this leads to ~200-400 fold accumulation of lipophilic cations into the mitochondrial matrix (Ross *et al.*, 2005). Uptake into cells is also driven by the plasma membrane potential (30 - 60 mV, negative inside) (Ross *et al.*, 2005), therefore the accumulation of these compounds into mitochondria *in vivo* relative to the extracellular environment can be up to several thousand-fold.

The best characterized and most widely used lipophilic cation for delivery of antioxidants to mitochondria is the triphenylphosphonium (TPP) cation, which was originally used to assess the mitochondrial membrane potential (Azzone *et al.*, 1984; Liberman and Skulachev, 1970; Liberman *et al.*, 1969). Since then the TPP cation has been conjugated to a range of antioxidants in order to target them to mitochondria (Murphy and Smith, 2007). There are a number of advantages of using the TPP cation approach as its uptake into mitochondria is well established

and it is also relatively straightforward to introduce the functionality into a compound late in the chemical synthesis scheme, typically by displacing a leaving group with triphenylphosphine (Smith *et al.*, 2004). A wide range of antioxidants have been targeted to mitochondria by conjugation to the TPP lipophilic cation, including Vitamin E (Smith *et al.*, 1999), ebselen (Filipovska *et al.*, 2005), lipoic acid (Brown *et al.*, 2007), plastoquinone (Skulachev *et al.*, 2009), nitroxides (Trnka *et al.*, 2008; Dhanasekaran *et al.*, 2005), and nitrones (Murphy *et al.*, 2003). The best characterized antioxidant targeted to mitochondria by conjugation to the TPP cation is MitoQ, which we will describe in detail as the uptake of other TPP-conjugated antioxidants is broadly similar.

MitoQ consists of a ubiquinone moiety linked to a TPP moiety by a ten-carbon alkyl chain (Kelso *et al.*, 2001; Murphy and Smith, 2007; Smith *et al.*, 2003). The TPP moiety on MitoQ leads to its rapid uptake across the plasma membrane, driven by the plasma membrane potential, followed by its accumulation into mitochondria within the cells that is satisfactorily described by the Nernst equation (Kelso *et al.*, 2001; Ross *et al.*, 2008). Within mitochondria MitoQ is continually recycled to the active ubiquinol antioxidant by respiratory complex II (Asin-Cayuela *et al.*, 2004; James *et al.*, 2005; James *et al.*, 2007; Kelso *et al.*, 2001). As MitoQ is largely found adsorbed to the mitochondrial inner membrane and its linker chain enables the ubiquinol component to penetrate deeply into the membrane core, it is an effective antioxidant against lipid peroxidation (Asin-Cayuela *et al.*, 2004; Kelso *et al.*, 2001). MitoQ has also been shown to protect against peroxynitrite (James *et al.*, 2007), and the ubiquinone form may also react directly with superoxide (Maroz *et al.*, 2009). In acting as an antioxidant the ubiquinol form of MitoQ is oxidized to the ubiquinone form, which is then rapidly re-reduced by complex II, restoring its antioxidant efficacy (James *et al.*, 2007). MitoQ has been shown to be protective in a large number of cell models of mitochondrial oxidative stress (reviewed in Murphy and Smith, 2007).

Mitochondria-targeted peptides that incorporate antioxidants

An alternative approach to targeting antioxidants to mitochondria is through the use of small, positively charged peptides called Szeto-Schiller (SS)-peptides (Zhao *et al.*, 2004). SS-peptides comprise four alternating aromatic/basic amino acids with a D-amino acid in the first or second position along with amidation of the C-terminus to make them more resistant to degradation (Szeto, 2006). The SS-peptides have three positive charges at physiological pH, and studies with isolated cells showed their rapid uptake through the plasma membrane and accumulation by mitochondria, where they bind to the inner membrane (Zhao *et al.*, 2003). The uptake through the cell membrane is concentration-dependent and non-saturable, suggesting that it is due to passage directly through the membrane (Zhao *et al.*, 2003). Peptides with a similar structure of alternating basic and aromatic amino acids are also taken up by mitochondria within cells due to the influence of the mitochondrial membrane potential on the positively charged peptide (Horton *et al.*, 2008). However, despite their positive charge, the uptake of SS-peptides into mitochondria does not seem to occur in response to the membrane potential and the mechanism that underlies their selective uptake by mitochondria is currently unclear (Szeto, 2006; Zhao *et al.*, 2004). Some of the SS-peptides have intrinsic antioxidant activity; for example in the SS-31 peptide, the most effective tested to date, the antioxidant activity is due to a dimethyltyrosine residue which is thought to act through its phenolic moiety (Szeto, 2006; Zhao *et al.*, 2004) while similar peptides without this residue were not protective. These SS-peptides are protective against oxidative stress in isolated mitochondria and in cell models of disease (Zhao *et al.*, 2004; Manczak *et al.*, 2010; Whiteman *et al.*, 2008).

The Uptake of Mitochondria-targeted Antioxidants by Mitochondria *In Vivo*

The *in vitro* experiments with two classes of mitochondria-targeted antioxidants, exemplified by MitoQ and SS-31, indicate that they are selectively taken up by mitochondria within cells where they decrease oxidative damage. To develop effective therapies *in vivo* it is necessary to determine whether these compounds can be delivered

safely long-term to mitochondria within living organisms. Here we assess what is known about the modes of administration, uptake, toxicity, metabolism, and distribution of these compounds *in vivo*.

Lipophilic cations in vivo

The intravenous (i.v.) toxicity of MitoQ in mice is relatively low with no toxicity at ~20 mg MitoQ/kg but toxicity is evident at ~27 mg MitoQ/kg (Smith *et al.*, 2003). To measure long-term oral toxicity young C57BL/6 mice were administered 500 μM MitoQ in their drinking water for up to 28 weeks with no evident toxicity, corresponding to a dose of ~55-80 mg MitoQ/day/kg (Rodriguez-Cuenca et al., 2010). In these experiments MitoQ did not change physical activity, O_2 consumption, food consumption, lean mass, glucose or insulin levels, insulin tolerance, or bone mineral density of treated mice, but there was a decrease in the percentage of body fat and liver and plasma triglyceride content (Rodriguez-Cuenca et al., 2010). The effects of MitoQ on gene expression in the heart and liver tissue, determined using the Affymetrix GeneChip MouseGene array of 28,853 genes, was not markedly affected by MitoQ expression and the few changes seen were minor and unrelated to any particular cellular process (Rodriguez-Cuenca *et al.*, 2010). MitoQ did not affect mitochondrial oxidative damage to the phospholipid cardiolipin (Paradies *et al.*, 2009), the accumulation of protein carbonyls (Davies *et al.*, 2001; Levine *et al.*, 1994), the activity of mitochondrial respiratory complexes, mtDNA copy number, or damage to mtDNA (Santos *et al.*, 2006). It should be noted that the demonstration that MitoQ did not decrease the levels of accumulation of oxidative damage markers in these young animals may be because the inherent oxidative damage levels were low and were not significantly different from the background levels that arise during sample processing. Nonetheless together these data suggest that the long-term oral administration of MitoQ is safe and that its effects *in vivo* are due to its antioxidant properties and not to other factors.

Studies of the uptake of [^{3}H]MitoQ into tissues following i.v. or intraperitoneal (i.p.) injection into mice showed that MitoQ was very rapidly cleared from the plasma and that substantial amounts of the compound were rapidly accumulated in the heart, brain, skeletal muscle, liver, and kidney (Smith *et al.*, 2003; Porteous *et al.*, 2010). To determine how much orally administered MitoQ was taken up into tissues, a liquid chromatography tandem mass spectrometry assay was developed to assess MitoQ content relative to a deuterated internal standard, d_3-MitoQ, by multiple reaction monitoring (Rodriguez-Cuenca *et al.*, 2010). For mice fed 500 μM MitoQ in their drinking water for 4-6 months this led to a steady-state accumulation of MitoQ that was ~113 pmol MitoQ/g in the heart, ~20 pmol MitoQ/g in the liver, and ~2 pmol MitoQ/g in the brain. Therefore, either acute i.v. or long-term oral administration of MitoQ leads to the substantial uptake of MitoQ within critical tissues such as the liver and heart.

The extensive studies undertaken in cells and isolated mitochondria strongly suggest that any MitoQ in tissues is essentially contained within mitochondria. However, to confirm that the MitoQ taken up into the tissues is predominantly mitochondrial due to the membrane potential is technically demanding. This is because isolating mitochondria from tissues requires homogenization which leads to the loss of the membrane potential and the consequent rapid efflux of MitoQ. Even so the mitochondrial localization of tissue MitoQ can be inferred from less direct methods. Administration of the mitochondrial uncoupler dinitrophenol, which decreases the mitochondrial membrane potential decreases the uptake of related TPP compounds within tissues *in vivo* (Porteous *et al.*, 2010). Another approach is to use a surrogate TPP compound containing a reactive moiety that binds covalently to protein thiols and thus labels those proteins it encounters *in vivo* which can then be assessed by immunostaining using specific antibodies (Lin, 2002). These experiments show that *in vivo* TPP compounds are only found in mitochondria to any significant extent (Porteous *et al.*, 2010; Smith *et al.*, 2003).

A final consideration is the metabolism and excretion of TPP compounds after their administration. MitoQ is excreted in the urine and bile as unmodified MitoQ and also with sulfation and glucuronidation of the ubiquinol ring (Li *et al.*, 2007; Ross *et al.*, 2008). Both the uptake and efflux of TPP compound such as MitoQ is dominated

by their Nernstian distribution indicating that the TPP compound is in facile equilibrium between the extracellular fluid, cytosol and mitochondria (Ross *et al.*, 2005). Once the level of the TPP compound in the blood decreases due to its excretion through the kidney or biliary pathways, then this equilibration process between the mitochondria and the cytosol with the extracellular fluid leads to transfer of compound to the blood and its subsequent excretion. Consequently the uptake of TPP compounds *in vivo* is effectively self limiting due to its rapid reversibility and equilibration with the plasma and mitochondrial membrane potentials.

Substantial amounts of MitoQ can be delivered to mitochondria within tissues such as the heart and liver following administration by either the i.v. or oral routes without toxicity. This opens up two major routes of administration that can be used in clinical situations for patients. Of particular note is that oral administration of MitoQ can continue long term (Rodriguez-Cuenca *et al.*, 2010), and that the i.v. administration of MitoQ leads to the very rapid (<5 minutes) uptake of the compounds into tissues, opening up the possibility of acute administration of these compounds (Porteous *et al.*, 2010).

The uptake of mitochondria-targeted peptides in vivo

SS-peptides have been administered i.v., i.p., and subcutaneously to rodents, and rats have been given daily doses of 1.5 mg/kg of SS-31 i.p. long-term (Anderson *et al.*, 2009), but there are no reports of these compounds being administered orally (Szeto, 2006). The SS-peptides are rapidly taken up by the perfused heart (Zhao *et al.*, 2004) and, following i.v. or i.p. injection, are taken up into tissues, including the skeletal muscle (Anderson *et al.*, 2009). They can also pass rapidly through the blood brain barrier following i.v. injection (Szeto, 2006). The plasma half life in rats and sheep for the SS-02 peptide is relatively long (Szeto *et al.*, 2001). However, the uptake of the SS-peptides into tissues has not been quantitated in detail and their metabolism has not yet been reported (Anderson *et al.*, 2009; Szeto, 2008; Zhao *et al.*, 2003; Yang *et al.*, 2009). Therefore the SS-peptides can be delivered to tissues *in vivo* following i.v., i.p. or subcutaneous injection although their pharmacokinetics and metabolism have not been described in detail to date.

Protective Effects of Mitochondria-targeted Antioxidants in Animal Models of Human Diseases

The studies discussed above indicate that both long-term and acute administration of both TPP- and peptide-based mitochondria-targeted antioxidants to rodents is safe. The next step is to determine whether the accumulation of these compounds in mitochondria *in vivo* is protective in animal models of diseases involving mitochondrial oxidative damage.

In the first study of its protective effects, MitoQ was administered to rats in their drinking water and the hearts were then isolated and exposed to ischemia-reperfusion (i/r) injury (Adlam *et al.*, 2005). MitoQ gave protection against heart dysfunction, tissue damage, and mitochondrial dysfunction (Adlam *et al.*, 2005). A similar study also showed that MitoQ was protective against cardiac i/r injury (Neuzil *et al.*, 2007). MitoQ was protective against the damage to endothelial cells *in vivo* associated with chronic exposure to nitroglycerin, due to protecting against oxidative damage to nitroglycerin-metabolizing enzymes within mitochondria (Esplugues *et al.*, 2006). MitoQ was protective against an increase in blood pressure in a spontaneously hypertensive rat model in which the increase in blood pressure is thought to arise from elevated mitochondrial oxidative damage in endothelial cells (Graham *et al.*, 2009). Administering MitoQ to rats or mice prior to induction of sepsis by endotoxin led to extensive protection against cardiac damage (Supinski *et al.*, 2009). In the lipopolysaccharide model of sepsis, infusion of MitoQ at the same time as induction of sepsis led to significant protection against liver damage (Lowes *et al.*, 2008). MitoQ administered by intraperitoneal injection was protective against heart damage associated with the anti-cancer compound adriamycin (Chandran *et al.*, 2009). In a rodent model of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) toxicity, MitoQ protected against *substantia nigra* damage, preserved locomotor

activity and dopamine content as well as decreased mitochondrial markers of oxidative damage (Ghosh et al., 2010). MitoQ has also shown protection against kidney damage in a model of type I diabetes (Chacko *et al.*, 2010) and against cocaine induced cardiac dysfunction (Vergeade *et al.*, 2010). In addition, MitoQ has proven useful in preserving function of the isolated organ in a model of kidney preservation for transplantation (Mitchell *et al*., 2011). Finally, a mitochondria-targeted plastoquinone is also protective against a range of disorders *in vivo* (Skulachev *et al.*, 2009), and a mitochondria-targeted nitroxide is protective *in vivo* against hypertension (Dikalova *et al.*, 2010). Together these findings show that MitoQ is protective against pathological changes in a number of animal models of mitochondrial oxidative damage that are relevant to human diseases. In addition, it suggests that many other antioxidant moieties can also be targeted to mitochondria by conjugation to the TPP moiety.

A number of studies have been carried out in animal models of disease with the mitochondria-targeted SS-peptides, most often with SS-31 which has a dimethyl tyrosine as its antioxidant moiety (Szeto, 2006). SS-31 was taken up into the heart in an *ex vivo* reperfusion system and was protective against i/r injury (Zhao *et al.*, 2004). The peptides SS-02 and SS-31 were also protective against cardiac i/r injury when added on reperfusion (Szeto, 2008). Intraperitoneal injection of the SS-31peptide leads to uptake into the brain and protection against damage to the *substantia nigra* caused by MPTP, which induces brain damage that mimics the symptoms of Parkinson's disease (Yang *et al.*, 2009). However, the SS-20 peptide, which does not have antioxidant ability *in vitro*, was also protective, which suggests that the protection in this case may not be due to its antioxidant ability (Yang *et al.*, 2009). Intraperitoneal injection of SS-31 has protective effects against insulin resistance in the skeletal muscle in a high fat fed mouse model (Anderson *et al.*, 2009). This is consistent with SS-31 decreasing oxidative damage protective against insulin resistance *in vivo* (Anderson *et al.*, 2009). Therefore the SS-peptides can be delivered *in vivo* by i.p. or i.v. administration and are then protective against mitochondrial damage in a wide range of animal models.

Human Studies with Mitochondria-targeted Antioxidants

To date, only one mitochondria-targeted antioxidant, MitoQ, has been used in humans. This work has been driven by Antipodean Pharmaceuticals Inc. (http://www.antipodeanpharma.com/). To make a stable active pharmaceutical of MitoQ it was found beneficial to make MitoQ with the methanesulfonate counter-anion and to complex this with β-cyclodextrin. This material was readily formulated into tablets that passed through conventional animal toxicity. The oral bioavailability was determined to be about 10% in rats and the major metabolites in urine were glucuronides and sulfates of the reduced hydroquinone form, along with demethylated compounds. In human phase I trials MitoQ showed good pharmacokinetic behavior with oral dosing at 80 mg (1 mg/kg) resulting in a plasma maximal concentration of 33.15 ng/ml after ~1 hour. MitoQ was first assessed to see if it could slow the progression of pathology in Parkinson's disease (Snow *et al.*, 2010). This was the PROTECT study (registered on www.clinicaltrials.gov as NCT00329056). In this 13-center study in New Zealand and Australia 128 newly diagnosed untreated patients with Parkinson's disease were enrolled in a double-blind study of two doses of MitoQ (40 and 80 mg per day) compared with placebo to see whether, over 12 months, MitoQ would slow the progression of Parkinson's disease as measured by the Unified Parkinson's Disease Rating Scale. This study showed no difference between MitoQ and placebo on any measure of Parkinson's disease progression (Snow *et al.*, 2010). The most probable explanation for the lack of effect is that by the time Parkinsonism is clinically evident it is too late to rescue the remaining dopaminergic neurons. While there was no therapeutic efficacy, this study demonstrated that MitoQ can be safely administered as a daily oral tablet to patients for a year. The second human trial was the CLEAR trial on chronic hepatitis C virus (HCV) patients (Gane *et al.*, 2010) (registered on www.clinicaltrials.gov as NCT00433108). HCV patients who were unresponsive to the conventional HCV virus treatments were chosen because there is evidence for increased oxidative stress and mitochondrial damage in liver dysfunction in these cases. The effect of oral MitoQ on serum aminotransferases in HCV infected

patients was assessed in a double-blind trial of 40 mg or 80 mg MitoQ, or matching placebo, for 28 days. Both treatment groups showed significant decreases in serum alanine transaminase. These data suggest that MitoQ reduces liver damage during chronic inflammation. More generally, this study is the first report of a potential clinical benefit from the use of mitochondria-targeted antioxidants in humans. Coupled with the one year's safety data for MitoQ from the Parkinson's disease study, this strongly suggested that MitoQ should be investigated in chronic liver diseases that involve mitochondrial oxidative damage. Consequently, a multicenter Phase IIb human trial has been initiated in the U.K. to assess the efficacy of MitoQ in non-alcoholic fatty liver disease. This is the MARVEL trial study (registered on www.clinicaltrials.gov as NCT01167088).

Conclusions and Future Challenges

Despite their central role in clinically important pathologies, mitochondria have been a neglected drug-target (Murphy, 2009b). This is in part due to the difficulty of selectively targeting molecules to the cellular organelle *in vivo*. The development of strategies to direct therapeutic antioxidants to mitochondria *in vivo* and the demonstration that this decreases pathology in a range of disorders following oral, i.v., or i.p. delivery strongly supports this approach. Furthermore, one mitochondria-targeted antioxidant, MitoQ, has been shown to be well tolerated, orally active, and safe in humans and is undergoing further phase IIb trials. The field of targeting therapeutic molecules to mitochondria is just beginning, but as mitochondrial damage contributes to so many diseases it is likely that further therapeutic compounds will be developed and applied to important human pathologies. The results to date suggest that disorders such as diabetes, metabolic syndrome, ischemia-reperfusion injury, hypertension, sepsis, and the preservation of organs for transplantation are important candidates for treatment by this approach. However it is likely that mitochondria-targeted antioxidants will also be tested in much other pathologies over the next few years.

Disclosure

M.P.M. and R.A.J.S. hold intellectual property in the area of the TPP class of mitochondria-targeted antioxidants and hold stock in, and act as consultants for, Antipodean Pharmaceuticals Inc.

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Hydroxytyrosol promotes mitochondrial biogenesis and mitochondrial function in 3T3-L1

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Abstract

Hydroxytyrosol (HT) in extra-virgin olive oil is considered one of the most important polyphenolic compounds responsible for the health benefits of the Mediterranean diet for lowering incidence of cardiovascular disease, the most common and most serious complication of diabetes. We propose that HT may prevent these diseases by a stimulation of mitochondrial biogenesis that leads to enhancement of mitochondrial function and cellular defense systems. In the present study, we investigated effects of HT that stimulate mitochondrial biogenesis and promote mitochondrial function in 3T3-L1 adipocytes. HT over the concentration range of 0.1–10 μ mol/L stimulated the promoter transcriptional activation and protein expression of peroxisome proliferator-activated receptor (PPAR) coactivator 1 alpha (PPARGC1 α , the central factor for mitochondrial biogenesis) and its downstream targets; these included nuclear respiration factors 1 and 2 and mitochondrial transcription factor A, which leads to an increase in mitochondrial DNA (mtDNA) and in the number of mitochondria. Knockdown of Ppargc1 α by siRNA blocked HT's stimulating effect on Complex I expression and mtDNA copy number. The HT treatment resulted in an enhancement of mitochondrial function, including an increase in activity and protein expression of Mitochondrial Complexes I, II, III and V; increased oxygen consumption; and a decrease in free fatty acid contents in the adipocytes. The mechanistic study of the PPARGC1 α activation signaling pathway demonstrated that HT is an activator of 5'AMP-activated protein kinase and also up-regulates gene expression of PPAR α , CPT-1 and PPAR γ . These data suggest that HT is able to promote mitochondrial function by stimulating mitochondrial biogenesis.

Keywords: 5'AMP-activated protein kinase (AMPK); Fatty acid oxidation; Mitochondrial transcription factor A (Tfam); Mitochondrial DNA (mtDNA); Nuclear respiration factors 1 and 2 (Nrf1 and Nrf2); Peroxisome proliferator-activated receptor coactivator 1 alpha (PPARGC1 α)

1. Introduction

Mitochondrial dysfunction plays a central role in a wide range of age-associated disorders and various forms of cancer [1], as well as type 2 diabetes [2]. Increasing evidence shows that mitochondrial metabolism and ATP synthesis decline in concert with a reduction of key factors regulating mitochondrial biogenesis in patients with insulin resistance, type 2 diabetes and obesity [3–6]. Key factors regulating this process include peroxisome proliferator-activated receptor (PPAR) coactivator 1 alpha (PPARGC1 α) and the nuclear respiratory factors (Nrfs). It also has been shown that a reduction of mitochondrial DNA (mtDNA) copy number in adipose tissue from diabetic volunteers and treatment with thiazolidinedione (TZD), an insulin-sensitizing drug currently used in treating type 2 diabetes, restored diminished mtDNA content and expression of genes involved in mitochondrial biogenesis and fatty acid oxidation [6,7]. Therefore, with the emerging evidence that mitochondrial dysfunction is associated with various diseases, it has been suggested that promoting mitochondrial biogenesis, just as

improving adipocyte metabolism, could be a strategy for preventing and reversing various diseases, including cardiovascular disease, cancer, insulin resistance, obesity and diabetes [2,8–10].

The Mediterranean diet has been associated with a lower incidence of certain cancers and of cardiovascular disease, which is the most common and serious complication of diabetes [11–13]. Olive oil is the principal source of fats in the Mediterranean diet, and hydroxytyrosol (HT), a polyphenolic constituent of extra-virgin olive oil, is considered to be one of the most potent determinants of its efficacy [14–17]. Studies on the mechanism of HT's action have focused on its antioxidant properties so far [14,18,19]. We have recently shown that HT protects retinal pigment epithelial cells from acrolein-induced oxidative damage and mitochondrial dysfunction by inducing detoxifying Phase II enzymes [20]. Based on our hypothesis and recent results [21,22] that mitochondrial nutrients can improve mitochondrial function through stimulating mitochondrial biogenesis, we hypothesize that the Mediterranean diet or supplementation with HT could stimulate mitochondrial function and prevent diabetes and obesity-related mitochondrial dysfunction, thus reducing the risk of cardiovascular disease. Therefore, in the present study, we determined whether treatment of 3T3-L1 adipocytes with HT could improve mitochondrial function by stimulating mitochondrial biogenesis. It has been observed that the expression of regulatory factors for mitochondrial biogenesis was reduced in adipose tissues of diabetic and obese subjects [3,6]. Therefore, we used adipocytes as a model to study the effects of HT. In the present study, we first examined the effect of HT on the protein expression of PPARGC1 α , the key regulator of mitochondrial biogenesis, and of its downstream targets, nuclear respiration factors 1 and 2 (Nrf1 and Nrf2) and mitochondrial transcription factor A (Tfam). Second, we examined mtDNA; protein expression of Mitochondrial Complexes I, II, III, IV and V; and mitochondrial mass/numbers. Third, we monitored the expression of proteins or genes related to fatty acid oxidation, adipogenesis and mitochondrial function, including activities of Mitochondrial Complexes I, II, III, IV and V; oxygen consumption; and free fatty acid (FFA) content. Fourth, we investigated the effects of HT on signalling pathways involving phosphorylation of 5'AMP-activated protein kinase (AMPK) and acetyl-CoA carboxylase (ACC).

2. Materials and methods

2.1. Materials

The AMPK activator, 5-amino-imidazole-4-carboxamide-riboside (AICAR), was from Sigma (St. Louis, MO); anti-phospho-(Thr 172)-AMPK (pAMPK), total AMPK (tAMPK), anti-phospho-ACC (Ser79) (pACC) and total ACC (tACC) were from Cell Signaling Technology, Inc. (Beverly, MA); anti-OxPhos Complexes I, II, III, IV and V were from Invitrogen (Carlsbad, CA); anti- α -tubulin and anti- β -actin were from Sigma; anti- PPARGC1 α (Santa Cruz, Heidelberg, Germany); the Reverse Transcription System Kit was from Promega (Mannheim, Germany); HotStarTaq was from Takara (Otsu, Shiga, Japan); primers were synthesized by Bioasia Biotech (Shanghai, China); and TRIzol and other reagents for cell culture were from Invitrogen. HT was from DSM Nutritional Products Ltd., Switzerland, and used in all experiments.

2.2. Cell culture and differentiation

Cell culture and differentiation of 3T3-L1 cells have been extensively used as a model of adipogenic differentiation and insulin action. 3T3-L1 cells undergo growth arrest and initiate a program of differentiation manifested by large lipid droplet accumulation upon hormonal stimulation. In parallel, these cells become sensitive to insulin, express Glut4 and display insulin-induced activation of glucose uptake

comparable to that seen in primary adipose cells [23]. In the present study, murine 3T3-L1 pre-adipocytes (American Type Culture Collection, Manassas, VA) were cultured in DMEM supplemented with 10% (v/v) fetal bovine serum and allowed to reach confluence. Differentiation of pre-adipocytes was initiated with 1.0 μ mol/L

insulin, 0.25 μ mol/L dexamethasone and 0.5 mmol/L 3-isobutyl-1-methylxanthine in DMEM supplemented with 10% (v/v) fetal bovine serum. After 48 h, the culture medium was replaced with DMEM supplemented with 10% fetal bovine serum and 1.0 μ mol/L insulin. The culture medium was changed every other day with DMEM containing 10% (v/v) fetal bovine serum. Cells were used at 9 to 10 days following induction of differentiation and when 90% exhibited the adipocyte phenotype.

2.3. Transient transfection and promoter activity assay

A 2-kb Ppargc1 α promoter in pGL3-basic luciferase reporter construct was a gift from Dr. X. Ge (Shanghai Institutes of Biological Sciences, Chinese Academy of Sciences, Shanghai, China). Cells were fully differentiated as described above and then seeded in 24-well plates at about 80% confluence and grown overnight. The cells were transiently transfected with pGL3-Ppargc1 α or pGL3-basic plasmid using the Cell Line Nucleofector Kit from Amaxa (Gaithersburg, MD) following the manufacturer's instructions. The Renilla vector was used to monitor the transfection efficiency. The transfected cells were cultured for 18‑20 h and then incubated with HT (1 μ mol/L) for 24 h. Cells were lysed, and the reporter activity was measured by a luciferase assay kit (KenReal, Shanghai, China) with a luminometer (Berthold Technologies, Bad Wildbad, Germany). The relative Luc activity was calculated as the ratio of firefly Luc activity to Renilla luc activity. Transfections were performed in duplicate and repeated at least three times.

2.4. Western blot analysis

After treatment with HT, cells were washed twice with ice-cold phosphatebuffered saline (PBS), lysed in sample buffer (62.5 mmol/L Tris‑Cl, pH 6.8, 2% SDS and 5 mmol/L dithiothreitol) at room temperature and vortexed. Cell lysates were then boiled for 5 min and cleared by centrifugation (13,000 rpm, 10 min at 4°C). Protein concentrations were determined using the Bio-Rad DC protein assay. The soluble lysates (10 μ g per lane) were subjected to 10% SDS-PAGE; proteins were then transferred to nitrocellulose membranes and blocked with 5% nonfat milk/TBST for 1 h at room temperature. Membranes were incubated with primary antibodies directed against anti- α -tubulin (1:5000), PPARGC1 α (1:1000), phospho-(Thr 172)-AMPK (1:1000), tAMPK (1:1000), phospho-ACC (Ser79) (1:1000), tACC (1:1000), anti-OxPhos Complex I (NADH ubiquinone oxidoreductase 39-kDa subunit, 1:2000), anti-OxPhos Complex II (succinate‑ubiquinone oxidoreductase 70-kDa subunit, 1:2000), anti-OxPhos Complex III (ubiquinol‑cytochrome c oxidoreductase core II 50 kDa, 1:2000) or anti-OxPhos Complex V (ATP synthase, 53 kDa) in 5% milk/TBST at 4°C overnight. After washing membranes with TBST three times, membranes were incubated with horseradish-peroxidase-conjugated secondary antibody for 1 h at room temperature. Western blots were developed using ECL (Roche, Mannheim, Germany) and quantified by scanning densitometry [24].

2.5. RNA isolation and reverse transcription polymerase chain reaction

After incubation, cells werewashed twice with ice-cold PBS. Total RNAwas isolated using the single-step TRI reagent, and 1 μ g RNA was reverse transcribed into cDNA. In brief, the isolated RNA was dissolved in sterile water and 2.5 mmol/L Mg2+, 1 mmol/L dNTPs, 0.5 μ g oligodT15, 25 U AMV reverse transcriptase and 10× RT buffer to give a final volume of 20 μ l. The sample was incubated at 25°C (10 min), 42°C (60 min) and 99°C (5 min). cDNA was diluted in DNase-free water (1:25) before quantification by real-time PCR. The primers for quantification of mRNA by real-time quantitative PCR for Nrf1, Nrf2, Tfam, Cpt1a, Ppara, Pparg and 18S rRNA mRNAs were the same as those published previously [22]. Quantitative PCR was performed using Mx3000P (see above). Each quantitative PCR was performed in triplicate. The mouse 18S rRNA gene served as the endogenous reference gene. The evaluation of relative differences of PCR product among the treatment groups was carried

out using the $\Delta\Delta$CT method. The reciprocal of 2CT (using CT as a base 2 exponent) for each target genewas normalized to that for 18S rRNA, followed by comparison with the relative value in control cells. Final results are presented as percentage of control.

2.6. RT-PCR for mtDNA

Total DNA and mtDNA were extracted using a kit (QIAamp DNA Mini Kit; Qiagen, Hilden, Germany), and quantitative PCR was done using 18S rRNA primers for a nuclear target sequence and primers for the mitochondrial D-loop as an mtDNA target [22]. Quantitative PCR was performed using a real-time PCR system (Mx3000P; Stratagene, Amsterdam, the Netherlands). Reactions were performed with 12.5 μl SYBR Green Master Mix (ABI, Warrington, UK), 0.5 μl of each primer (10 μmol/L) and 100 ng template (DNA) or no template (NTC), with RNase-free water being added to a final volume of 25 μl. The cycling conditions were as follows: 50°C for 2 min, initial denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 30 s, 55°C for 1 min and 72°C for 30 s. Each quantitative PCR was performed in triplicate. The following primers were used: mitochondrial D-loop: forward, 5′-AATCTACCATCCTCCGTG-3′; reverse, 5′-GACTAATGATTCTTCACCGT; 18S rRNA: forward, 5′-CATTCGAACGTCTGCCCTATC- 3′; reverse, 5′-CCTGCTGCCTTCCTTGGA-3′. The mouse 18S rRNA gene served as an endogenous reference gene. Melting curves were performed to ensure specific amplification. The standard curve method was used for relative quantification. The ratio of mitochondrial D-loop to 18S rRNA was then calculated. Final results are presented as percentage of control.

2.7. Cell respiration test

Oxygen consumption by intact cells was measured as an indication of mitochondrial respiration activity [22]. The BD Oxygen Biosensor System utilizes an oxygensensitive fluorescent compound [tris 1,7-diphenyl-1,10 phenanthroline ruthenium(II) chloride] embedded in a gas-permeable and hydrophobic matrix permanently attached to the bottom of a multiwell plate. The concentration of oxygen in the vicinity of the dye is in equilibrium with that in the liquid media. Oxygen quenches the dye in a concentration-dependent manner. The fluorescence correlates directly to oxygen consumption in the well. This unique technology allows homogenous instantaneous detection of oxygen levels. After treatment, adipocytes were washed in Krebs‐Ringer solution buffered with HEPES (KRH) buffer plus 0.1% bovine serum albumin (BSA). Cells from each condition were divided into aliquots in a BD Oxygen Biosensor System plate (BD Biosciences) in triplicate. The number of cells contained in equal volumes did not differ statistically among samples treated with various nutrients and nutrient concentrations. Plates were sealed and “read” on a fluorescence spectrometer (Molecular Probes, Sunnyvale, CA) at 1-min intervals for 60 min at an excitation of 485 nmand emission of 630 nm [25]. Results are expressed as the slope of time-varying fluorescence intensity.

2.8. Mitochondrial mass analysis

The fluorescent probe MitoTracker Green FM (Molecular Probes, Eugene, OR) was used to determine the mitochondrial mass of adipocytes [22]. In brief, adipocytes treated with HT for 48 h were trypsinized and centrifuged at 3000×g at 4°C for 5 min, resuspended in KRH buffer and 0.1% BSA (w/v) and then incubated with 0.1 μmol/L MitoTracker Green FM in KRH buffer for 30 min at 37°C. Cells were centrifuged at 3000×g at 4°C for 5 min and resuspended in 400 μl of fresh KRH buffer. Fluorescence was analyzed by flow cytometry (FACS Calibur, Becton Dickinson, Mountain View, CA).

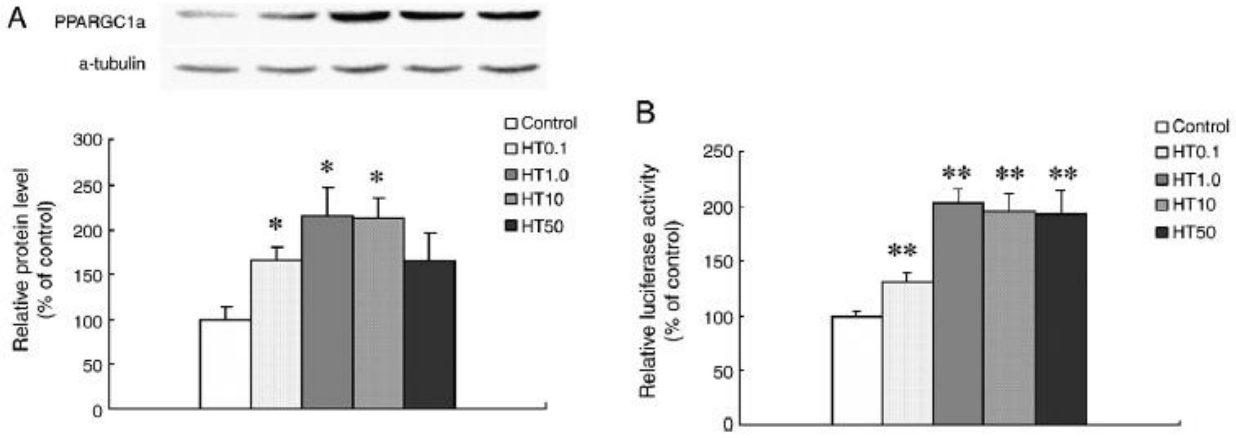


*Fig.1. Effect of HT treatments on protein expression and transcriptional activation of PPARGC1 α. 3T3-L1 adipocytes were treated for 48 h with HT at concentrations of 0.1, 1, 10 and 50 μmol/L. Cells were subsequently solubilized into SDS sample buffer and analyzed by Western blotting with antibodies against α-tubulin and PPARGC1 α. (A) Upper panel: Immunoblots for representative samples of steadystate levels of proteins; lower panel: Quantitative values (in percentage) were tabulated for PPARGC1 α : α-tubulin ratios determined by densitometry. (B) Relative luciferase reporter activity. Values are mean±S.E. of the results from four independent experiments. *Pb.05 versus control (without HT treatment).*

2.9. Electron microscopic observation

3T3-L1 adipocytes on Day 8 of differentiation were seeded on glass coverslips. On Day 9, cells were treated with HT (1.0 μ mol/L) for 48 h. On Day 10, adipocytes were fixed overnight with 2.5% (v/v) glutaraldehyde in 0.1 mol/L sodium phosphate buffer (pH 7.3).

They were postfixed with 2% (w/v) OsO4 in the same buffer, followed by block staining with 1% (w/v) uranyl acetate. After dehydration with a graded ethanol series, they were washed with propylene oxide and embedded in Spurr's low viscosity resin. Silver to gold sections were cut and examined using a Philips CM 10 (Eindhoven, the Netherlands) transmission electron microscope at a 60-kV accelerating voltage [26].

Measurements were made on six individual adipocytes treated with or without HT. For each individual adipocyte in each image, the number of mitochondria and the total mitochondrial sectional area were determined. All electron microscopic photographs were analyzed by observers blind with respect to treatments [22].

2.10. Mitochondrial isolation

Following addition of trypsin, the cells were pelleted by centrifugation at 300×g for 5 min at 4°C. All of the subsequent steps were performed on ice. The resulting pellet was then resuspended in 0.5 ml of mitochondrial isolation buffer (215 mmol/L mannitol, 75 mmol/L sucrose, 0.1% BSA, 1 mmol/L EGTA and 20 mmol/L HEPES, pH 7.2) and homogenized on ice with a 2-ml glass homogenizer (Dounce, Fisher Scientific, Pittsburgh, PA). The mitochondria were then purified by differential centrifugation at 1300×g for 5 min to pellet unbroken cells and the nuclei. The supernatant was then centrifuged at 13,000×g for 10 min to pellet the mitochondria. The pellet was resuspended in EGTA-free isolation buffer [22].

2.11. Assays for activities of Mitochondrial Complexes I, II, III, IV and V

Adipocytes were cultured in 100-mm plates, washed in PBS, resuspended in an appropriate isotonic buffer (0.25 M sucrose, 5 mM Tris‑HCl, pH 7.5, and 0.1 mM phenylmethylsulfonyl fluoride) and homogenized. Mitochondria

were isolated by differential centrifugation of the cell homogenates. NADH‑CoQ oxidoreductase (Complex I) activity was tested by monitoring the reduction of 2,6-dichlorophenol indophenol at 600 nm upon addition of assay buffer (finally, 0.05 M Tris‑HCl, pH 8.1, 0.1% BSA (w/v), 1 mM antimycin A, 0.2 mM NaN3 and 0.05 mM coenzyme Q1) [27].

Assays of succinate‑CoQ oxidoreductase (Complex II), CoQ‑cytochrome c reductase (Complex III) and cytochrome c oxidase (Complex IV) were performed spectrometrically using conventional assays [27] with minor modifications. Complex V (ATP synthase) activity was measured as oligomycin-sensitive, Mg2+-ATPase activity [27].

2.12. Determination of FFA and glycerol content in the cell culture supernatant

Adipocytes cultured in six-well plates were stimulated with 1.0 μM HT for 72 h; then, the FFA content of the supernatant was estimated by a commercially available FFA kit (Jiancheng Biochemical Inc.). The test is based on the reaction of FFAs with Cu2+ to form copper salt, which is detected photometrically by absorbance at 440 nm. The glycerol content of the supernatant was determined by a free glycerol reagent kit from Sigma (Cat #F6428). In brief, 10-μl samples were added to wells of 96-well plates, 200 μl of glycerol reagent was pipetted into each well and all samples were incubated for 5 min at 37°C. The absorbance of each sample was spectrophotometrically measured at 540 nm, with water as reference, with three samples per condition.

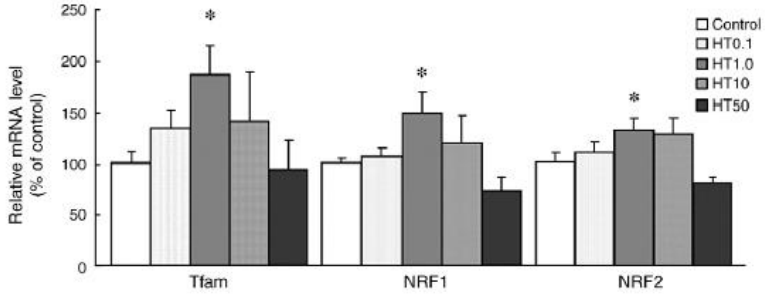


*Fig. 2. Effect of HT treatments on mRNA levels of Tfam, Nrf1 and Nrf2 in adipocytes. 3T3-L1 adipocytes were treated for 48 h with HT at 0.1, 1.0, 10 and 50 μmol/L. mRNA levels of Tfam, Nrf1 and Nrf2 were analyzed by quantitative RT-PCR with gene-specific oligonucleotide probes in adipocytes. The cycle number at which the various transcripts were detectable was compared with that of 18S rRNA as an internal control. Results were expressed as percentage of untreated control cells. Values are mean±S.E. of the results from at least four independent experiments. *Pb.05 versus control cells without HT treatment.*

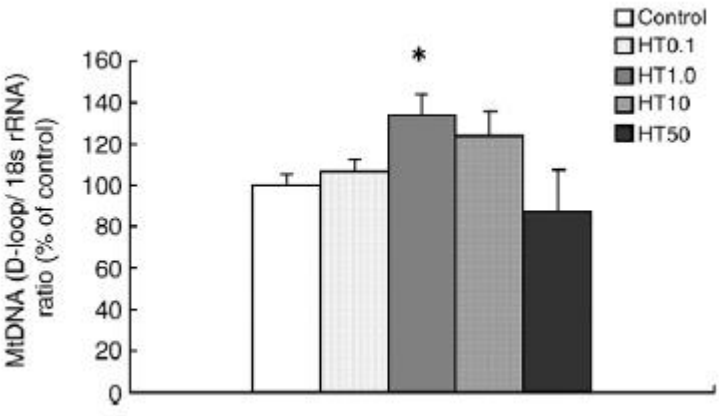


*Fig. 3. Effect of HT treatments on expression of mtDNA. 3T3-L1 adipocytes were treated for 48 h with HT at 0.1, 1.0, 10 and 50 μmol/L. DNA was isolated and PCR products were quantified using SYBR Green fluorescence. Quantitative values (in percentage) were tabulated for D-loop:18S rRNA ratios. Values are mean±S.E. of the results from seven independent experiments. *Pb.05 versus control without HT taken as 100%.*

2.13. Determination of triglycerides using Oil Red O staining

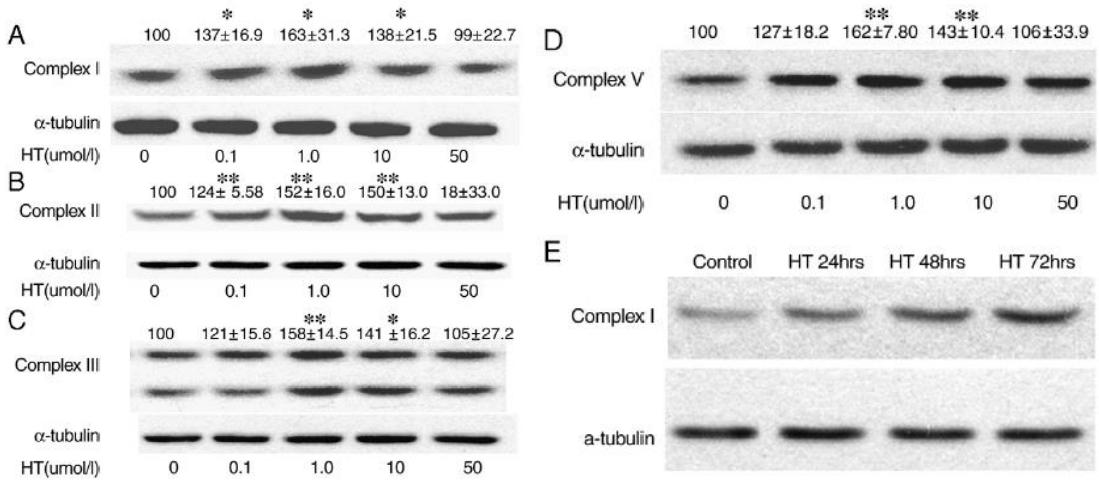
After the induction of differentiation, adipocytes cultured in 24-well plates were stimulated with HT (1.0 μM) for 72 h. Cells were washed twice with PBS and fixed with 10% formalin in PBS for 1 h. After being washed three times with PBS, cells were stained with Oil Red O (six parts of 0.6% Oil Red O dye in isopropanol and four parts of water) for 1 h, and the excess of the stain was removed by washing with water. Then, stained cells were put in a fume cabinet until they are dried, and the stained oil droplets were dissolved in isopropanol containing 4% Nonidet P-40 overnight. The triglycerides in adipocytes were quantified by measuring the absorbance at 520 nm.

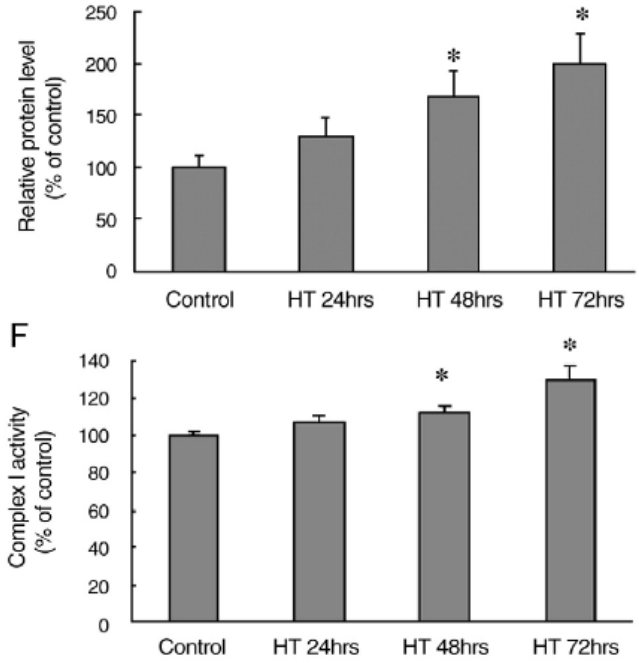
2.14. Construction of RNAi adenovirus and transient transfection

The siRNA targeting of Ppargc1α has been described previously [28]. The selected sequence was screened using a BLAST search to ensure that only the Ppargc1α genewas targeted. The double-strand oligonucleotides used in the present study were as follows: sense, 5′-GATCCGGTGGATTGAAGTGGTGTAGttcaagagaCTACACCACTTCAATCCACCTTTTTTCTGCAGG-3′; antisense, 5′-AATTCCTGCAGAAAAAAGGTGGATTGAAGTGGTGTAGtctcttgaaCTACACCACTTCAATCCACCG-3′. 3T3-L1 adipocytes were seeded into sixwell plates at about 80% confluence the day before virus infection. Cells were then incubated with recombinant virus (Ad-siRNA-Ppargc1α or Ad-siRNA-control) at a concentration of 2×104 virus particles per cell. After incubation for 2 h, fresh growth mediumwas added and cells were further cultured for 4 h and then stimulated with HT μmol/L) for 48 h.

2.15. Statistical analysis

All data are representative of at least three independent experiments. Data are presented as means±S.E.M. Statistical significance was calculated by SPSS 10.0 software using one-way ANOVA, with P values b.05 considered significant.





3. Results

3.1. HT stimulated transcriptional activity and protein expression of PPARGC1 α

PPARGC1 α is a key factor that drives mitochondrial biogenesis, which also plays an important stimulatory role in thermogenesis and fatty acid oxidation in muscle and adipose tissues [29–31]. Treatment of adipocytes with HT at 0.1–50 μ mol/L resulted in a dose-dependent curve of stimulation of expression of PPARGC1 α by Western blot and its promoter transcription by luciferase reporter assay. Both assays showed that the most significant stimulation occurred at 0.1 and 10 μ mol/L (Fig. 1A and B).

3.2. HT up-regulated genes involved in mitochondrial biogenesis and fatty acid oxidation

Ppargc1 α autoregulates its gene expression, along with the expression of Nrf1 and Nrf2, which are mitochondrial transcription factors encoded by nuclear genes. Nrf1 also induces the expression of Tfam [29–31]. Therefore, we examined the effects of HT on the mRNA expression of Nrf1, Nrf2 and Tfam. Treatment of HT at 0.1–50 μ mol/L resulted in bell-shaped response curves of mRNA expression of Nrf1, Nrf2 and Tfam, similar to that of PPARGC1 α expression. However, the increase was significant only at 1 μ mol/L of HT for all three factors (Fig. 2).

3.3. HT treatment increased mtDNA

mtDNA content decreases age-dependently and may be one of the causal factors in age-related type 2 diabetes [32]. Tfam is involved in regulating expression of nuclear genes encoding some major mitochondrial proteins that regulate mtDNA transcription and replication. Its level is proportional to that of mtDNA [29–31]. Because HT stimulated the mRNA expression of Tfam, it is expected that mtDNA copy number should be increased. mtDNA expression was quantified by real-time PCR measuring the ratio of D-loop to 18s rRNA levels. The D-loop region is known as the major site of transcription initiation on both the heavy and light strands of mtDNA. As shown in Fig. 3, the HT treatment at 1 μ mol/L resulted in a significant increase in the ratio of mitochondrial D-loop/18s rRNA.

3.4. HT promoted the protein expression of OxPhos Complexes I, II, III and V

Tfam, along with other nuclear-encoded mitochondrial proteins, is imported into mitochondria by the protein import machinery and regulates the expression of the 13 mtDNA-encoded proteins, which are components of Respiratory Chain Complexes I (ND1–6 and 4L) and III (Cyt b – cytochrome b) and ATP synthase (A8 and A6). The nuclear DNA-encoded mitochondrial proteins and the mtDNA-encoded proteins are assembled to form multisubunit enzyme complexes required for oxygen consumption and ATP synthesis [29–31]. As shown in Fig. 4, treatment of HT (48 h) significantly increased the expression of Complex I (Fig. 4A) and Complex II (Fig. 4B) at 0.1,1 and 10 μ mol/L and Complex III (Fig. 4C) and Complex V (Fig. 4D) at 1 and 10 μ mol/L, respectively. The increases in protein expressionwere about 1.5- to 1.7- fold compared with control samples. The rationale for choosing the 48-h treatment for all experiments was based on the time-dependent effects of HT treatment: as shown in Fig. 4E and F, HT treatment effected a dose-dependent increase of Complex I expression and activity after periods varying from 24 to 72 h, with significant stimulation beginning at 48 h.

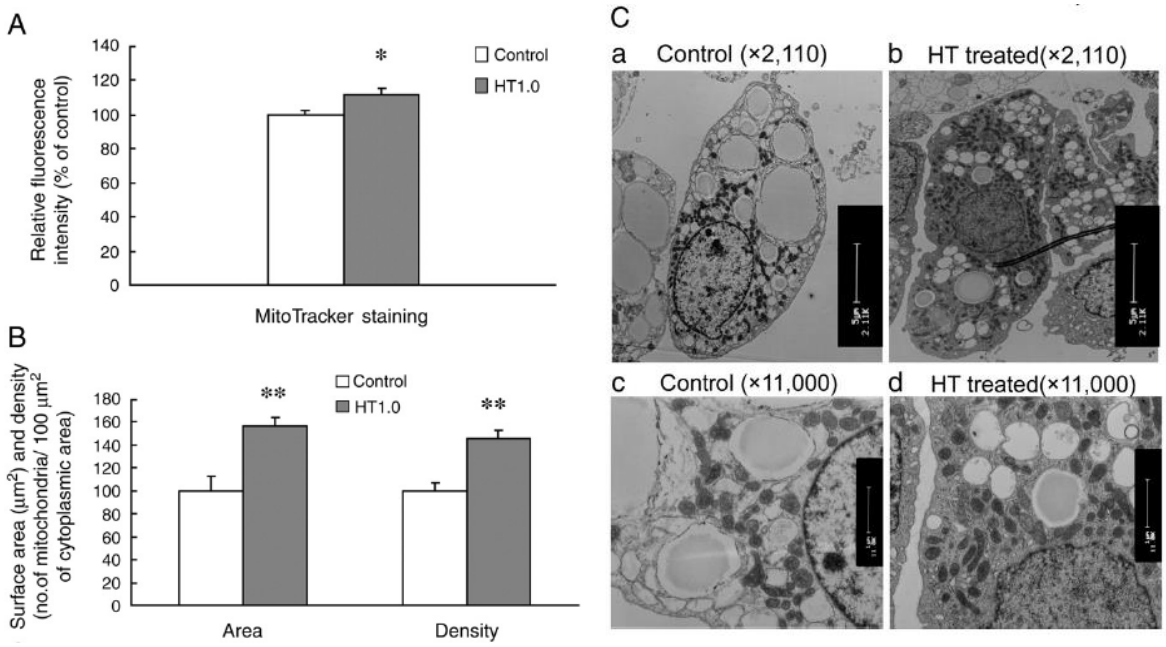


*Fig. 5. Effect of HT treatments on mitochondrial mass and ultrastructural changes under the electron microscope. Adipocytes were stimulated with HT at 1.0 μM for 48 h. (A) Mitochondrial mass was estimated by MitoTracker (100 nmol/L) staining with flow cytometry. Results were expressed as fold increase of the fluorescence intensity over untreated control cells. Values are mean±S.E. of the results from four independent experiments. *Pb.05 versus control cells without HT treatment. (B) Morphometric analysis of surface area and density of mitochondria under the electron microscope. Values are means±S.E. of data from six cells. **Pb.01 versus control cells without HT treatment. (C) Representative illustrations of mitochondrial profiles under the electron microscope (magnification, ×2110 and ×11,000).*

3.5. HT increased adipocyte mitochondrial mass

Mitochondrial formation is dependent on the assembly of large hetero-oligomeric complexes, and this assembly requires coordination between the nuclear and mitochondrial genomes [29–31]. We examined whether HT-induced activation of PPARGC1α and its downstream signaling leads to an increase in mitochondrial numbers. First, we used a specific dye, MitoTracker Green FM, which accumulates inside mitochondria, to label and quantify mitochondria in cells. As shown in Fig. 5A, treatment of HT at 1.0 μ mol/L resulted in a significant increase in fluorescence intensity, suggesting an increase in mitochondrial mass. Mitochondrial morphology was also examined under the electron microscope. As shown in Fig. 5B, quantitative analysis (six cells were analyzed)

demonstrated that the treatment with HT at 1.0 μ mol/L for 48 h significantly increased mitochondrial section area and density. A representative control adipocyte and a 1.0-μ mol/L HT-treated adipocyte are shown in Fig. 5C.

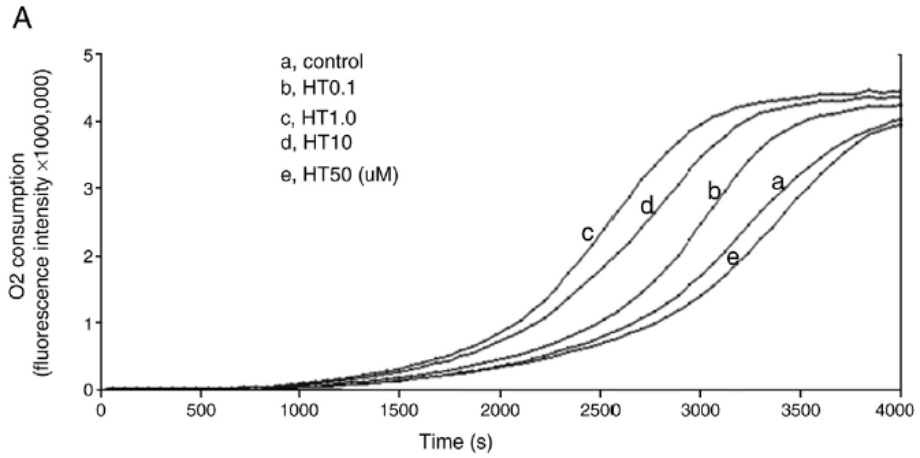
3.6. HT augmented oxygen consumption and activities of Mitochondrial Complexes I, II, III, IV and V

An increase in mitochondrial formation should be accompanied by an increase in mitochondrial function [29–31]. We examined oxygen consumption and activities of Mitochondrial Complexes I, II, III, IV and V. As shown in Fig. 6A and B, the basal rate of oxygen consumption was significantly increased in adipocytes by treatment with HT at concentrations between 1 and 10 μ mol/L; the optimal increase was found to be at 1 μ mol/L. As shown in Fig. 7, treatment with HT (48 h) significantly increased the activities of Complex I (Fig. 7A) at 1 μ mol/L HT; Complex II (Fig. 7A) at 0.1, 1 and 10 μ mol/L; Complex III (Fig. 7B) at 1 and 10 μ mol/L; and Complex IV (Fig. 7B) and Complex V (Fig. 7B) at 1 and 10 μ mol/L.

3.7. HT up-regulated fatty-acid-oxidation-related expressions of Ppara, Cpt1 and Pparg genes

PPAR α is an important regulator of mitochondrial biogenesis and β-oxidation. CPT-1 is the gatekeeper of mitochondrial fatty acid oxidation because it regulates long-chain fatty acid transport across the mitochondrial membrane by converting acyl-CoA into acylcarnitine.

PPAR γ plays an important role not only in adipogenesis but also in regulating lipid metabolism in mature adipocytes. To study the effects and mechanism of HT on mitochondrial biogenesis and fatty acid oxidation, the effects of HT on Ppara, Cpt1 and Pparg were studied by quantitative RT-PCR. As shown in Fig. 8, HT showed dosedependent increases in mRNA expression of Cpt1, Ppara and Pparg with significant increases at 1.0 μ mol/L HT for Cpt1 and Ppara and at 1.0 and 10 μ mol/L HT for Pparg. However, HT did not increase PPAR induction of the reporter gene in PPAR α or PPAR γ transactivation assays (data not shown).



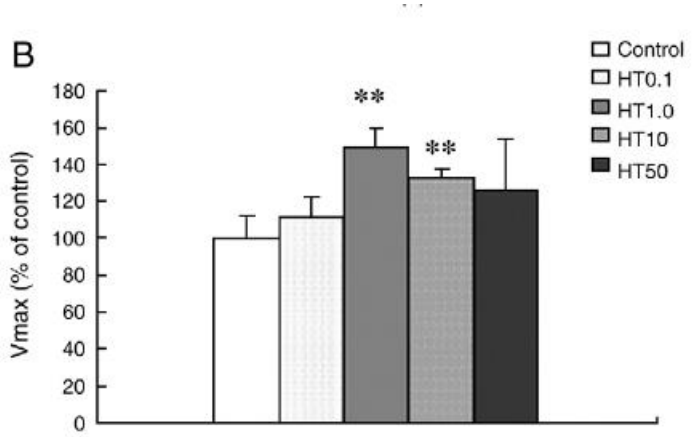


*Fig. 6. Effect of HT treatments on oxygen consumption in 3T3-L1 adipocytes. 3T3-L1 adipocytes were treated for 48 h with various concentrations of HT as indicated. (A) Representative oxygen consumption curves. Cells were trypsinized and equal volumes of cells were separated into aliquots in wells of a 96-well BD Oxygen Biosensor plate. Plates were covered and fluorescence in each well was recorded over time with a fluorescence microplate spectrophotometer. (B) Quantitative changes in the respiratory rate of adipocytes under each condition were calculated by determining the kinetic parameters. Vmax=maximum oxygen consumption rate. Final results are presented as percentage of control. Values are mean±S.E. of the results from three independent experiments. **Pb.01 versus control cells without HT treatment taken as 100%.*

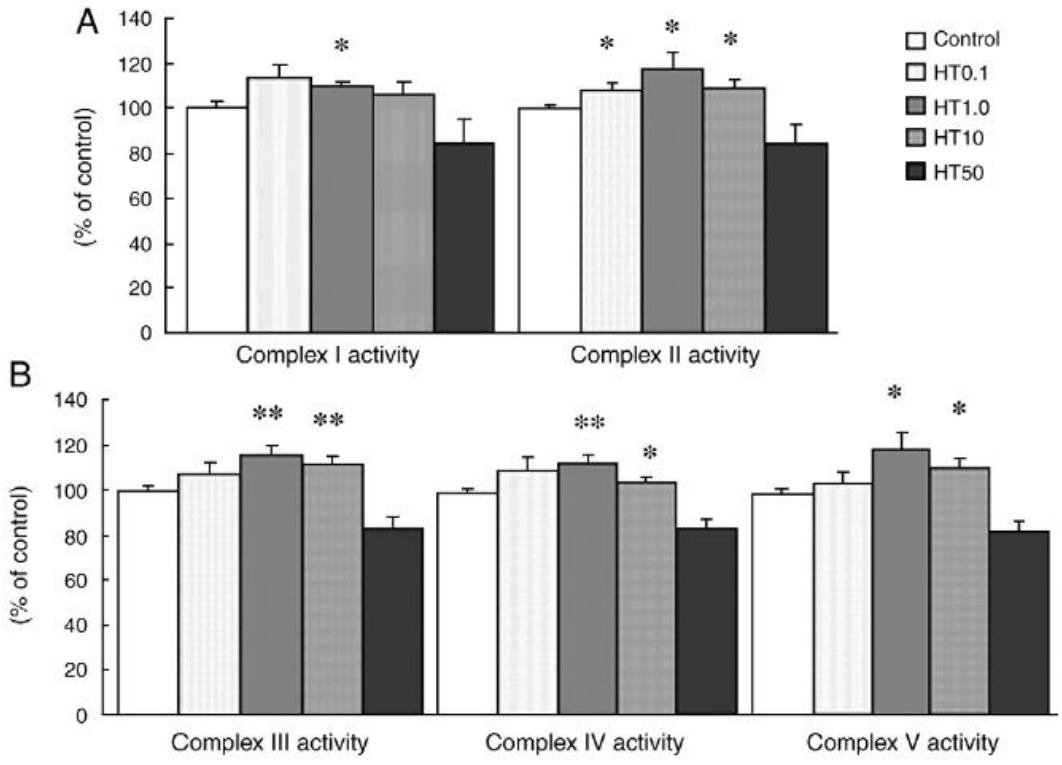


*Fig. 7. Effect of HT treatments on activities of mitochondrial complexes in adipocytes. 3T3-L1 adipocytes were treated for 48 h with HT at 0.1, 1.0, 10 and 50 μmol/L. Cells were then washed; mitochondria were isolated, and the complex activities were assayed. (A) Complexes I and II; (B) Complexes III, IV and V. Final results are presented as percentage of control. Values are mean±S.E. of the results from at least four independent experiments. *Pb.05 and **Pb.01 versus control cells without HT treatment.*

3.8. HT decreased the FFA content in the supernatant of treated adipocytes

A chronic, high concentration of plasma FFAs is one of the factors that contributes to the underlying pathophysiology of type 2 diabetes, including development of insulin resistance. FFA treatment impaired insulin-receptor-mediated signal transduction and decreased insulinstimulated GLUT4 translocation and glucose transport. In one study, FFAs activated the stress/inflammatory kinases JNK, IKKb and SOCS-3; increased secretion of the inflammatory cytokine TNF- α ; and decreased secretion of adiponectin into the medium [33]. Pharmacological agents that effectively lower FFA (such as TZD) are likely to have a significant effect in reducing fasting plasma glucose. Therefore, reducing FFA might be a target for treating obesity and type 2 diabetes. Therefore, we examined whether HTcan target FFA levels. As shown in Fig. 9, treatment of adipocytes with HT (1.0 μ mol/L) for 72 h significantly reduced the FFA content of the supernatant (Pb.01), although the glycerol and triglyceride content did not show distinct change compared with nontreated controls.

3.9. HT-activated phosphorylation of AMPK and ACC

One of the important pathways for activating PPARGC1 α involves AMPK [34]. AMPK phosphorylates a number of targets, resulting in increases in glucose transport, fatty acid oxidation and gene transcription. One example of these targets is ACC. ACC is a biotindependent enzyme that catalyses the irreversible carboxylation of acetyl-CoA to produce malonyl-CoA. The carboxylation inhibits β -oxidation, but when ACC is phosphorylated by AMPK, its activity is decreased [35]. AICAR, a pharmaceutical AMPK activator, has been shown to cause a time- and concentration-dependent increase in phosphorylation of AMPK and ACC [36]. As shown in Fig. 10C and D, HT affected ACC phosphorylation and AMPK phosphorylation similarly. We studied the effects of HTon the phosphorylation of AMPK and ACC, using AICAR as positive control. HT treatment (1.0 μ mol/L) of 3T3-L1 adipocytes caused a time-dependent increase in the phosphorylation of AMPK (Fig. 10A and B) over the time range of 5, 15, 30 and 60 min, with the maximum increase at 30 min. Both HT (1.0 μ mol/ L) and AICAR (0.5 mmol/L) significantly affected AMPK phosphorylation (Fig. 10C and D). That HT, at about 1/500th the concentration, induced a similar degree of AMPK and ACC phosphorylation as AICAR suggests that HT is the more potent AMPK activator.

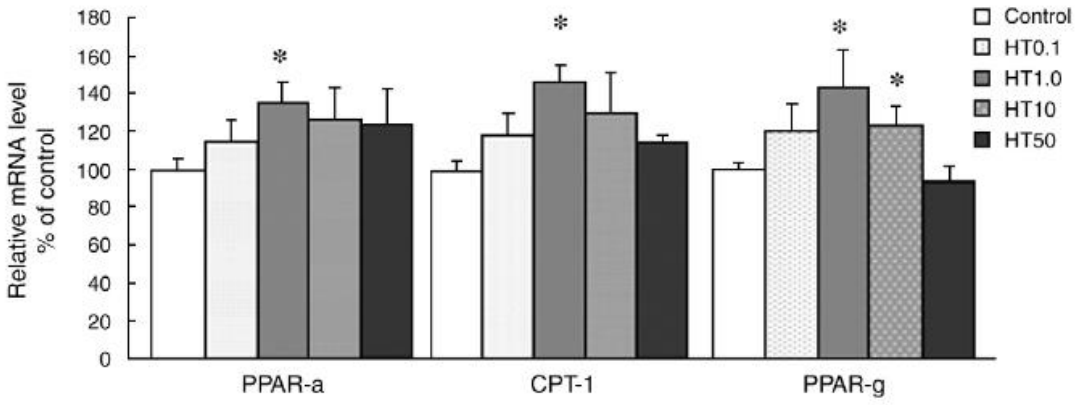


*Fig. 8. Effect of HT treatments on expression of Cpt1a and Ppara mRNA. 3T3-L1 adipocytes were treated for 48 h with HT at 0.1, 1.0, 10 and 50 μmol/L, and total RNA was isolated. PCR fluorescence products were quantified using SYBR Green. The cycle number at which the various transcripts were detectablewas compared with that of 18S rRNA as an internal control. Results are expressed as percentage of control. Values are mean±S.E. of the results from at least four independent experiments. *Pb.05 versus control without HT treatment.*

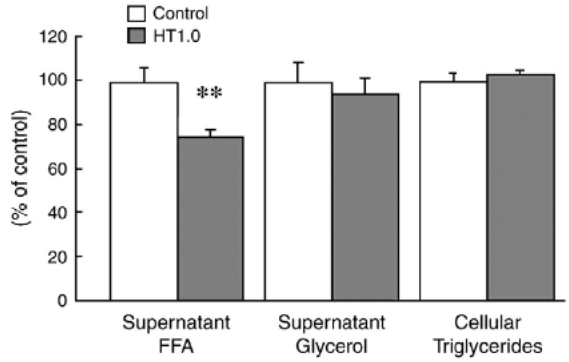


*Fig. 9. Effect of HT treatments on levels of supernatant FFAs, glycerol and triglycerides in 3T3-L1 adipocytes. Adipocytes were stimulated with HT at 1.0 μmol/L for 72 h. FFA and glycerol contents in the supernatants of cells were detected. Results are expressed as a percentage fold increase of the metabolite concentration over untreated control cells. Values are mean±S.E. of the results from six independent experiments. **Pb.01 versus control without HT treatment.*

3.10. Knockdown of Ppargc1α blocked the effects of HT

To better determine how important Ppargc1α is in producing the stimulatory effects of HT on mitochondrial function and biogenesis in 3T3-L1 adipocytes, we knocked down Ppargc1α by siRNA. As shown in Fig. 11, introducing Ad-siRNA-Ppargc1α to the cells decreased PPARGC1α protein levels at the 24-h and 48-h time points. The AdsiRNA-control did not affect PPARGC1α expression (Fig. 11A). Next, we determined the effects of Ppargc1α on the mtDNA quantity and Complex I expression. Knockdown of Ppargc1α suppressed them significantly (Fig. 11B), indicating the important role of Ppargc1α.

These results also suggest that Ppargc1α is an important target in the process by which HT is able to stimulate mitochondrial function and biogenesis in adipocytes. No big difference was observed between

control and Ad-siRNA-control groups, and HT stimulated both mtDNA and Complex I in Ad-siRNA-control cells, but for Ad-siRNA-Ppargc1α cells, mtDNA and Complex I were dramatically inhibited and HT treatment could not reverse this inhibition. These data suggest a crucial role of Ppargc1α in the effects of HT. Both mtDNA and Complex I levels tended to be higher in the Ad-siRNA-Ppargc1α cells treated with HT compared to untreated cells, but the increases were not significantly different.

4. Discussion

Identifying the mitochondrial dysfunction mechanisms and developing mitochondrial targeting drugs/nutrients have formed a new discipline of mitochondrial medicine and opened up avenues for manipulating mitochondrial function and health [21,37–39]. It is well reported that mitochondrial biogenesis could, in part, underlie the central role of adipose tissue in the control of whole-body metabolism and the actions of some insulin sensitizers [25] and that mitochondrial dysfunction might be an important contributing factor in type 2 diabetes [9]. Mitochondrial loss in adipose tissue is correlated with the development of type 2 diabetes [2]. Hence, it is possible that stimulating mitochondrial biogenesis may reduce the symptoms of metabolic syndrome. In the present study, we showed that HT over the concentration range of 0.1–10 μmol/L stimulated the protein expression of PPARGC1α – the central factor for mitochondrial biogenesis – and the mRNA of its downstream targets, Nrf1, Nrf2 and Tfam. HT increased the quantity of mtDNA and the protein expression of Mitochondrial Complexes I, II, III and V; consequently, mitochondrial numbers increased. This increase in mitochondrial biogenesis was

accompanied by an enhancement of mitochondrial function, including an increase in the activity and protein expression of Mitochondrial Complexes I, II, III and V and oxygen consumption, as well as a decrease in FFAs. Using siRNA, we further demonstrated that a key factor for HT to target is PPARGC1 α . These data suggest that HT, as a mitochondrial targeting nutrient, is able to promote mitochondrial function by stimulating mitochondrial biogenesis.

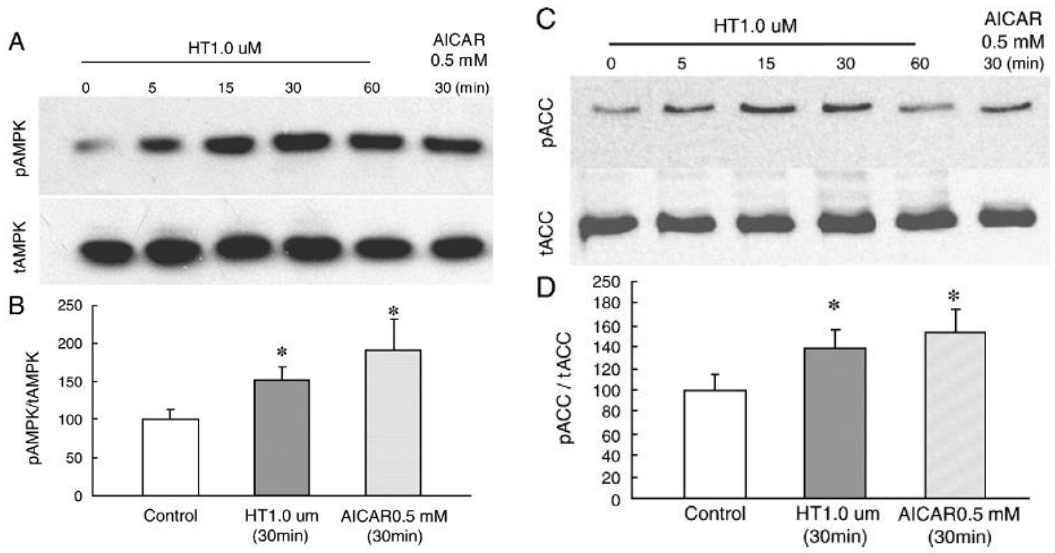


*Fig. 10. Effect of HT treatments on AMPK and ACC phosphorylation in 3T3-L1 adipocytes. AMPK phosphorylation and ACC phosphorylation were determined by Western blotting using lysates of 3T3-L1 adipocytes that had been cultured for 5, 15, 30 or 60 min with 1.0 μM HT and for 30 min with AMPK activator AICAR at 0.5 mM. Proteins were prepared, applied (20 μg/lane) and separated. After transfer to a nitrocellulose membrane, polyclonal sera specific for phospho-AMPK (pAMPK), tAMPK, phospho-ACC (pACC) and tACC were applied overnight, and detection was carried out as described in Materials and Methods. (A) A representative image of AMPK phosphorylation. (B) Quantitative values (in percentage) are tabulated for pAMPK:tAMPK ratios. (C) A representative image of ACC phosphorylation. (D) Quantitative values (in percentage) are tabulated for pACC:tACC ratios. Values are mean±S.E. of the results from four independent experiments. *Pb.05 versus control cells without HT treatment.*

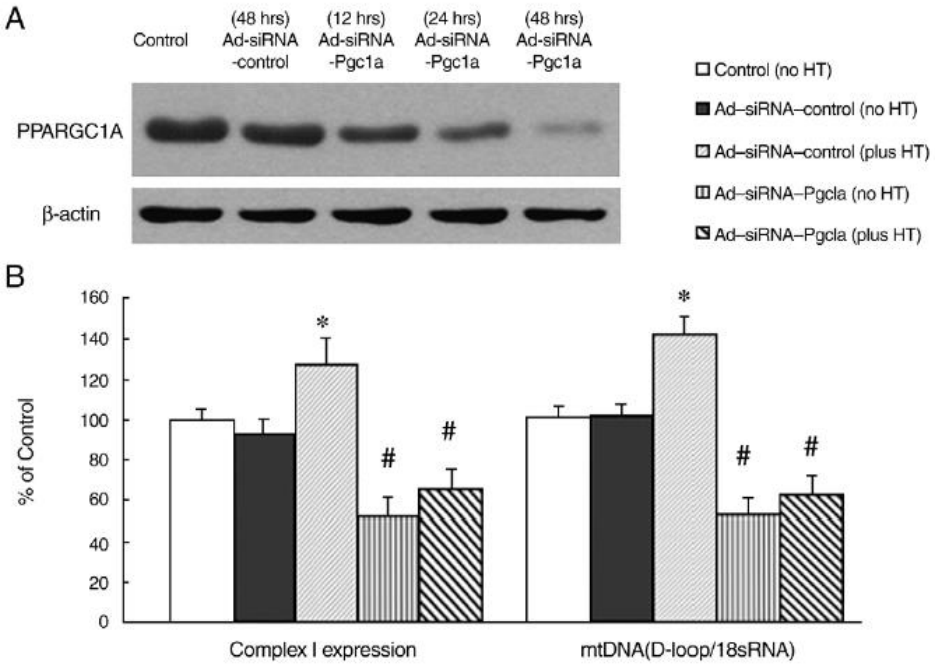


*Fig. 11. Effect of PPARGC1a (Pgc1a) silencing on HT-stimulated mtDNA and Complex I expression. Cells were infected with Ad-siRNA-Ppargc1 α or Ad-siRNA-control as described. DNA and protein were isolated and detected by RT-PCR and Western immunoblotting. (A) PPARGC1 α protein expression after 12, 24 and 48 h of adenovirus infection. (B) Complex I expression in adipocytes treated with or without HT (1.0 μ mol/L) and mtDNA quantity in adipocytes treated with or without HT (1.0 μ mol/L). Values are mean±S.E. of the results from three independent experiments. *Pb.05 versus Ad-siRNA-control without HT treatment.*

Mitochondrial fatty acid oxidation is one of the key processes in ATP production. Mitochondrial biogenesis and remodeling in white adipocyte tissue enhance fatty acid uptake and oxidation, indicated by increased oxygen consumption. The increase in oxygen consumption is accompanied by an increase in expression of Cpt1 and Ppara, suggesting that HT stimulates mitochondrial biogenesis, leading to increased fatty acid oxidation. We hypothesized that the protective effects of mitochondrial antioxidants and nutrients on mitochondria may include (a) protecting mitochondria from oxidative damage and thus slowing down the loss of mitochondria, (b) stimulating repair of damaged mitochondria, (c) stimulating degradation of damaged mitochondria (by lysosomes) and (d) stimulating de novo mitochondrial biogenesis [21]. α -Lipoic acid and acetyl-L-carnitine are two examples of mitochondrial nutrients. In our previous experiments, we [22] have demonstrated that treatments using a combination of R- α -lipoic acid and acetyl-L-carnitine at concentrations of 0.1, 1 and 10 μ M for 24 h significantly increased mitochondrial mass, expression of mtDNA, mitochondrial complexes, oxygen consumption and fatty acid oxidation in 3T3-L1 adipocytes. These changes were accompanied by an increase in the mRNA expression of Cpt1a and expression of several transcription factors involved in mitochondrial biogenesis, including Ppargc1 α , Tfam, Nrf1 and Nrf2. We concluded that the combination of R- α -lipoic acid and acetyl-L-carnitine may act as Pparg and Ppara dual ligands to complementarily promote mitochondrial synthesis and adipocyte metabolism.

HT has long been considered as a potent antioxidant polyphenol [14,15,17]. However, its effect on mitochondrial biogenesis has never been studied. Therefore, this is the first study to show that HT is able to act as a mitochondrial targeting nutrient and provides a new mechanism of the efficacy of the Mediterranean diet on lowering the risk of various diseases, including cardiovascular disease, cancer, diabetes and obesity. As we know, cardiovascular disease is the most common and most serious complication of diabetes and obesity. Because mitochondrial respiration plays a critical role in glucose metabolism, mitochondrial dysfunction has been shown

to be associated with diabetes and obesity. The Mediterranean diet, including a high intake of HT, may stimulate mitochondrial biogenesis and function (enhancement of fatty acid oxidation) and, thus, reduce the risk of obesity and diabetes, leading to a lowered risk of cardiovascular disease. It seems that mitochondrial biogenesis and the phase II antioxidant system are closely related or coupled because the transcriptional coactivator PPARGC1 α was shown to suppress ROS and neurodegeneration [40]. Therefore, it is possible that HT, a potent antioxidant and Phase II enzyme inducer, may enhance mitochondrial biogenesis and improve mitochondrial function by suppressing ROS and stimulating the Phase II antioxidant system to strengthen the cell's antioxidant defenses, in addition to its direct effect on mitochondrial assembly as demonstrated here.

Mitochondrial biogenesis is a complicated process. The activation of PPARGC1 α is associated with a number of signaling pathways involving the activation of AMPK [34], intracellular calcium and the subsequent activation of calcium-sensitive signaling of calcium/ calmodulin-dependent protein kinase [41] and nitric oxide [42] and cAMP-responsive element binding protein [29‑31]. We investigated the possible involvement of the AMPK signaling pathway by detecting the phosphorylation of AMPK and ACC (Fig. 10). Whether HT also affects other signaling pathways of PPARGC1 α activation needs to be studied further.

In addition to stimulating mitochondrial biogenesis, AMPK was also shown to increase muscle fatty acid oxidation and insulin sensitivity. The antidiabetic drug metformin activates AMPK [43]. gACRP30 or globular adiponectin, the globular subunit of ADIPOQ, improves insulin sensitivity and increases fatty acid oxidation. The mechanism by which gACRP30 exerts these effects is possibly due to activation of AMPK and inactivation of ACC [44]. The potent effect of HT on phosphorylation of AMPK and ACC is consistent with the decrease in FFA and the increases in Cpt1 and Ppara as indexes of increased fatty acid oxidation. The potent effect of HT on phosphorylation of AMPK and ACC in our 3T3-L1 adipocytes suggests that HT might be potentially effective in increasing fatty acid oxidation and improving insulin sensitivity in diabetes and obesity.

In addition to the effects on increasing the mRNA expression of Ppara and Cpt1a for stimulation of fatty acid oxidation, HT also induced gene mRNA expression, but not transactivation, of Pparg. Oil Red O staining indicates smaller oil droplets in adipocytes. At the same time, the cellular triglyceride level did not increase (Fig. 9), and the FFA levels in the cell culture supernatantwere reduced, suggesting that HT promoted fat burning. This reasoning is in accordance with Wilson-Fritch et al. [23], who describe that PPAR γ plays an important role not only in adipogenesis but also in regulating lipid metabolism in mature adipocytes. PPAR γ activity can be modulated by direct binding of low-molecular-weight ligands. For example, the clinically effective antidiabetic drugs such as TZD are high-affinity agonist ligands for PPAR γ , leading to a net flux of fatty acids from the circulation and other tissues into adipocytes [8]. Interestingly, HT did not increase the levels of cellular triglycerides and FFAs but rather led to decreased level of FFAs in the cell culture supernatant and to smaller lipid droplets in adipocytes. Since HT only up-regulates PPAR α /PPAR γ expression but has no PPAR agonistic activity, these findings could be interpreted by the presence of sufficient PPAR agonistic molecules (e.g., fatty acids) in the cellular system, to favour adipocyte differentiation. Our findings indicate that HT in adipocytes may support adipocyte differentiation but does not result in increased fat storage. This is in accordance with observations that a 2-week supplementation of HT did not cause any change in body weight in normal rats [Albino: HanWistar (SPF), doses up to 450 mg/kg]. Also, HT did not influence body weight or body composition in mice [C57BL/6NCrl: (SPF), doses up to 300 mg/kg]. The net effect of HT on adipogenesis can be compared with the action of the novel non-TZD selective PPAR γ modulator (nTZDpa). Berger et al. [45] found that, in cell-based assays for transcriptional activation, nTZDpa served as a selective, potent PPAR γ partial agonist andwas able to antagonize the activity of PPARg full agonists; nTZDpa also displayed partial agonist effects when its ability to promote adipogenesis in 3T3-

L1 cells was evaluated. Interestingly, nTZDpa, unlike the TZD, caused reductions in weight gain and adipose depot size when it was administered to fat-fed C57BL/6J mice.

In conclusion, we showed that HT is a nutrient that effectively stimulates mitochondrial biogenesis and function. This mitochondrial targeting property may provide a possible mechanism for the efficacy of the Mediterranean diet for lowering the risk of cardiovascular disease and also suggests that HT may be used as a therapeutic intervention for preventing and treating type 2 diabetes and obesity.

Acknowledgments

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Hydroxytyrosol protects against oxidative damage by simultaneous activation of mitochondrial biogenesis and phase II detoxifying enzyme systems in retinal pigment epithelial cells

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Abstract

Studies in this laboratory have previously shown that hydroxytyrosol, the major antioxidant polyphenol in olives, protects ARPE-19 human retinal pigment epithelial cells from oxidative damage induced by acrolein, an environmental toxin and endogenous end product of lipid oxidation, that occurs at increased levels in age-related macular degeneration lesions. A proposed mechanism for this is that protection by hydroxytyrosol against oxidative stress is conferred by the simultaneous activation of two critically important pathways, viz., induction of phase II detoxifying enzymes and stimulation of mitochondrial biogenesis.

Cultured ARPE-19 cells were pretreated with hydroxytyrosol and challenged with acrolein. The protective effects of hydroxytyrosol on key factors of mitochondrial biogenesis and phase II detoxifying enzyme systems were examined. Hydroxytyrosol treatment simultaneously protected against acroleininduced inhibition of nuclear factor-E2-related factor 2 (Nrf2) and peroxisome proliferator-activated receptor coactivator 1 alpha (PPARGC1α) in ARPE-19 cells. The activation of Nrf2 led to activation of phase II detoxifying enzymes, including γ-glutamyl-cysteinyl-ligase, NADPH (nicotinamide adenine dinucleotide phosphate)-quinone-oxidoreductase 1, heme-oxygenase-1, superoxide dismutase, peroxiredoxin and thioredoxin as well as other antioxidant enzymes, while the activation of PPARGC1α led to increased protein expression of mitochondrial transcription factor A, uncoupling protein 2 and mitochondrial complexes.

These results suggest that hydroxytyrosol is a potent inducer of phase II detoxifying enzymes and an enhancer of mitochondrial biogenesis. Dietary supplementation of hydroxytyrosol may contribute to eye health by preventing the degeneration of retinal pigment epithelial cells induced by oxidative stress.

Keywords: Hydroxytyrosol; RPE cells; Acrolein; AMD; Mitochondrial biogenesis; Phase II enzymes

1. Introduction

Age-related macular degeneration (AMD) is the leading cause of vision loss in the Western world among people over 65 y of age [1], and worldwide, it is the third most common cause of blindness [2]. AMD is characterized by an age-related degeneration of retinal pigment epithelium (RPE) and the photoreceptors in the macular area of the retina. The underlying cause of the disease is unknown, but oxidative stress is involved [3], suggesting that consumption of diets rich in antioxidants may be of benefit.

The Mediterranean diet has been associated with a lower incidence of not only certain cancers, but also cardiovascular disease, which is the most common and serious complication of diabetes [4–6], all conditions associated with oxidative stress. Olives and olive oil are considered an important part of the Mediterranean diet.

Evidence has accumulated recently that in addition to olive lipids, which are rich in monounsaturated fatty acids, antioxidant polyphenols such as hydroxytyrosol also contribute to the health effects of olives [7–10].

Work in our laboratory has led to the proposal that oxidative damage to mitochondria in RPE cells may contribute to the retinal degeneration observed in AMD and the compounds that protect mitochondrial function may prevent or alleviate this damage. Our work also showed that acrolein, a lipid oxidation end product and mitochondrial toxin [11], causes oxidative mitochondrial damage in RPE cells; moreover, hydroxytyrosol protects RPE cells against this acrolein-induced oxidative stress [12]. To investigate the underlying mechanisms, the same acrolein model is now used to study the effects of hydroxytyrosol on the induction of phase II detoxifying enzymes and stimulation of mitochondrial biogenesis, two of the most important pathways for cells to fight against oxidative stress.

Table 1
Primers and annealing temperatures

Gene	Annealing temp. (°C)	Forward primer	Reverse primer
18SrRNA	55	CATTCGAACGTCTGCCCTATC	CCTGCTGCCTTCCTTGGA
Cu/ZnSOD	55	CGGAGGCTTTGAAGGTGTGG	CTCCAACATGCCTCTCTTCATCC
MnSOD	55	AGGTTAGATTTAGCCTTATTCCAC	TTACTTTTTGCAAGCCATGTATCTTTC
UCP-2	55	TACAAAGCCGGATTCCGGCAGC	CTCCTTGGATCTGTAACCGGAC
PRDX3	60	CCTTTGGATTTCACCTTTGTGTG	CAAACCACCATTCTTTCTTGGTG
PRDX5	60	CCAATCAAGGTGGGAGATGCC	GCAGGTGTGTCTTGGAACATC
TRX2	60	GTCCACACCACTGTGCGTGG	TTGCAGGGAGATGGCTCAGCG

When cells are subjected to a variety of oxidative environmental stresses, they typically respond by inducing a coordinated expression of genes encoding the set of phase II detoxifying enzymes (Fig. 13) , principally mediated by activation of the transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) [15,16]. Nrf2 controls the orchestrated expression of phase II enzymes and genes involved in oxidative defense, although normally Nrf2 protein is kept inactive in the cytoplasm by complexing with its cytosolic inhibitor keap-1. Upon activation and release from keap-1, Nrf2 protein translocates to the nucleus, where it binds to promoters containing antioxidant response elements, resulting in the transactivation of the respective genes for phase II detoxifying enzymes.

Key phase II detoxifying enzymes include glutathione (GSH) Stransferase (GST), heme oxygenase-1 (HO-1), NAD(P)H quinone oxido-reductase-1 and γ -glutamyl cysteine ligase (GCL), enhanced expression of which leads to an increase in levels of endogenous antioxidants such as the major thiol antioxidant GSH and reduced quinones [13,15,17].

Phase II enzymes perform a variety of vital cellular functions important for protecting against oxidative damage. GCL controls the production of GSH, the major endogenous antioxidant thiol. GSH reductase (GR) catalyses the NADP-dependent reduction of GSSG (oxidized GSH) to GSH to maintain a high cytoplasmic GSH:GSSG ratio. GSH peroxidase (GPx), an enzyme widely present in many tissues, is thought to be an important cellular H2O2 detoxifier in neurons [18] and mice lacking GPx develop cataracts at a young age [19]. NAD(P)H:quinone oxidoreductase (NQO1) reduces quinones via a two-electron reduction and converts the dopamine quinones into less toxic hydroquinones that may be further detoxified via conjugation to sulfate or glucuronic acid [20].

Therefore, NQO1 is likely to play a crucial role in the protection of cells against oxidative damage. HO-1 produces the antioxidant bilirubin; it is typically associated with an increased production of ferritin, which results in reduced amounts of free iron [21], the main catalyst of the Fenton reaction. HO-1 expression is ubiquitous and its activity

is increased by many types of agents, particularly those involved in oxidative stress such as heme, metalloporphyrins and transition metals.

The cytoplasmic antioxidant system [including NQO1, GST, GCL and Cu/Zn superoxide dismutase (SOD)] is mainly controlled by Nrf2. In contrast, the mitochondrial antioxidant system [thioredoxin-2, peroxiredoxin (Prdx)3, Prdx5 and Mn SOD] is modulated through the transcription factor FOXO3a.

Inducing phase II enzymes and stimulating mitochondrial biogenesis may also enhance other antioxidant defense systems, such as the antioxidant enzyme catalase. The functions of catalase include catalyzing the decomposition of hydrogen peroxide to water and oxygen to remove free radicals and protect cells from oxidative damage. The role of mitochondrial dysfunction in the aging process [22] and in the development of chronic degenerative diseases, such as Type 2 diabetes [23] and neurodegenerative diseases [24], is being increasingly acknowledged. One underlying mechanism of mitochondrial dysfunction is the loss of mitochondria. For example, mitochondrial loss in adipose tissue is correlated with the development of Type 2 diabetes [25]. Thus, an effective strategy for preventing and treating mitochondrial dysfunction-related disease should be the effective stimulation of mitochondrial biogenesis. This may be achieved by activation of the key factor promoting mitochondrial biogenesis, peroxisome proliferator-activated receptor coactivator 1 alpha (PPARGC1 α) [26]. PPARGC1 α possesses dual activities – stimulation of mitochondrial electron transport while enforcing suppression of reactive oxygen species (ROS) – and may serve as an adaptive set-point regulator, capable of providing an accurate balance between metabolic requirements and cytotoxic protection [27]. Therefore, its dual activities of inducing mitochondrial biogenesis and suppressing ROS make PPARGC1 α an almost ideal target protein for the control or limiting of damage associated with mitochondrial dysfunction.

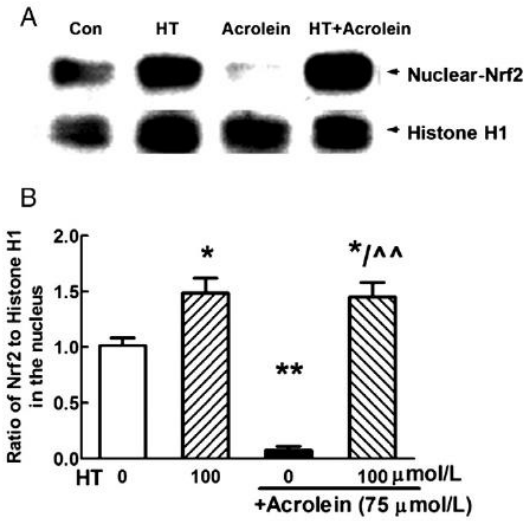

*Fig. 1. Hydroxytyrosol increased nuclear Nrf2 levels both in untreated and in acroleinchallenged ARPE-19 cells. Hydroxytyrosol pre-treatment (100 μ mol/L, 48 h) and acrolein exposure (75 μ mol/L, 24 h): (A) Western blot image and (B) Quantification of Western blots from four separate experiments. *Pb.05, **Pb.01 vs. control cells (no acrolein, no hydroxytyrosol) and ^^Pb.01 vs. acrolein-treated cells.*

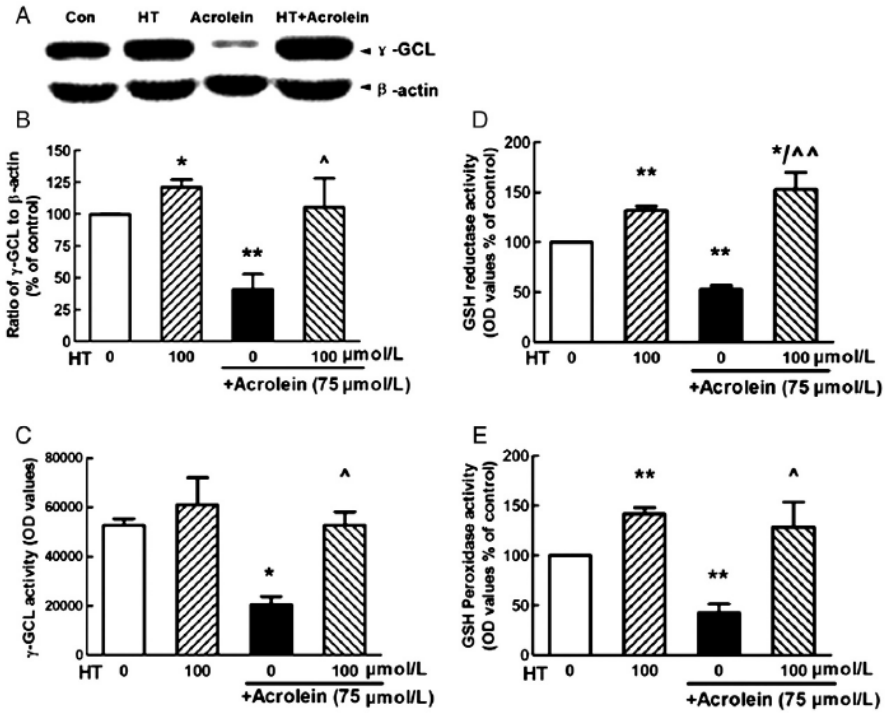

*Fig. 2. The induction of γ-GCL by hydroxytyrosol (100 μmol/L, 48 h) in ARPE-19 cells without and with exposure to acrolein challenge (75 μmol/L, 24 h). (A) Representative Western blot image and (B) quantification of Western blot of GCL protein expression. Values are means±S.E. of four separate experiments. (C) GCL activity assay. Values are means±S.E. of one representative experiment chosen from three separate experimental repeats, all of which had the same trend. (D) GR activity and (E) GPx activity. Values are means±S.E of data from three separate experiments, each experiment performed in triplicate. *Pb.05, **Pb.01 vs. control (hydroxytyrosol 0 μmol/L) and ^Pb.05, ^^Pb.01 vs. acrolein-treated.*

Signalling molecules upstream of PPARGC1, such as adenosine monophosphate kinase (AMPK) [28], nitric oxide [29], and calcium [30], can also promote mitochondrial biogenesis. Of these, AMPK also regulates other metabolic pathways, including the cellular uptake of glucose, the β-oxidation of fatty acids and the biogenesis of glucose transporters [31]. The enzyme nitric oxide synthase (NOS) produces NO, and one isoform, endothelial cell NOS (eNOS), is an upstream regulating factor for mitochondrial biogenesis [32]. Finally, uncoupling protein 2 (UCP2), a mitochondrial factor controlled by PPARGC1 α, is involved in maintaining acceptable ROS levels and is neuroprotective during ischemia/reperfusion [33]; it therefore may play a role in preventing and correcting mitochondrial dysfunction. Polyphenols have been shown to protect RPE from oxidativestress-induced death [34] and to induce phase II detoxifying enzymes [35]. Hydroxytyrosol is the major antioxidant polyphenol in olives and has been shown to have beneficial effects on human health.

Results from our previous experiments have shown that hydroxytyrosol exhibits protective effects against acrolein-induced toxicity in the human retinal pigment epithelial cell line ARPE-19 [12]. Pretreatment with hydroxytyrosol dose-and time-dependently protected the ARPE-19 cells from acrolein-induced oxidative damage and mitochondrial dysfunction. A short-term pretreatment (48 h) with over 75 μmol/L hydroxytyrosol was required for protection while a long-term pretreatment (7 days) showed protective effects with as little as 5 μmol/L or more, suggesting that lower long-term doses of hydroxytyrosol treatment can achieve similar protective effects as the higher short-term doses. These results suggest that hydroxytyrosol may be a mitochondrial protecting nutrient even at a relatively low concentration when given for an extended period of time. Our

hypothesis is that the mechanism behind hydroxytyrosol's protective effects against acrolein-induced RPE damage may be related to its capability to activate simultaneously both mitochondrial biogenesis and phase II detoxifying enzyme systems. In the present study, these pathways are investigated using acrolein-challenged ARPE-19 cells.

2. Materials and methods

2.1. Reagents

Acrolein was purchased from Sinopharm Chemical Reagent (Shanghai, China). Pure (N99%), synthetic hydroxytyrosol was a gift from DSM Nutritional Products, Kaiseraugst, Switzerland. The reverse transcription system kit was purchased from Promega (Mannheim, Germany) and HotStarTaq from Takara (Otsu, Shiga, Japan). Primers were synthesized by Bioasia Biotech (Shanghai, China). TRIzol and reagents for cell culture were from Invitrogen. Anti-oxphos complexes I, II, III and V were from Invitrogen (Carlsbad, CA, USA); nuclear-encoded subunits of the mitochondrial oxphos complexes detected were: for complex I, the 39-kDa protein (alpha subunit 9); for complex II, the 30 kDa subunit B protein (iron-sulfur protein); for complex III, the core I protein; and for complex V, the alpha-subunit (F1 complex). All other reagents were purchased from Sigma–Aldrich (St. Louis, MO, USA).

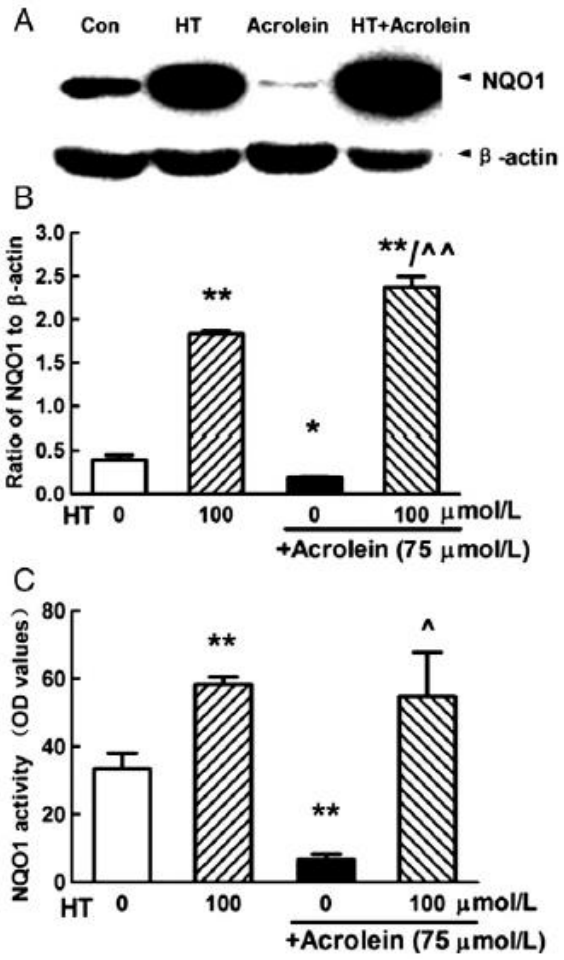

Fig. 3. The induction of NQO1 by hydroxytyrosol (100 μmol/L, 48 h) in ARPE-19 cells without and with exposure to acrolein (75 μmol/L, 24 h). (A) Representative Western blot image and (B) quantification of Western blot. Values are means±S.E. of three separate experiments. (C) NQO1 activity. Values are means±S.E of data from three separate experiments; each

*experiment performed in triplicate. *Pb.05 and **Pb.01 vs. control cells (hydroxytyrosol 0 μmol/L); ^Pb.05 and ^^Pb.01 vs. acrolein-treated.*

2.2. Cell culture

The human ARPE-19 cell line was obtained from Dr. Nancy J. Philp and was cultured according to her methods [36]. The ARPE-19 cells were maintained in Dulbecco's modified Eagle's medium (DMEM)-F12 medium supplemented with 10% fetal bovine serum, 0.348% sodium bicarbonate, 2 mmol/L L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Cell cultures were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO2. The medium was changed every 3–4 days. ARPE-19 cells were used within 10 generations.

2.3. Acrolein exposure and hydroxytyrosol supplementation

All experiments were performed with an 80% confluent monolayer. Hydroxytyrosol was dissolved in dimethylsulfoxide (DMSO) (final DMSO concentration ≤0.025%). Acrolein was dissolved in DMEM-F12 medium immediately before each experiment and was incubated with cells for 24 h as an acute toxicity model [12,37]. The protective effects of hydroxytyrosol were studied with the acute toxicity model by pre-treating cells with hydroxytyrosol for 48 h followed by 24 h acrolein treatment in the absence of hydroxytyrosol.

2.4. Western blot analysis

After pre-treatment with hydroxytyrosol and following acrolein treatment, cellswere washed twice with ice-cold phosphate-buffered saline, lysed in sample buffer [62.5 mmol/L Tris-HCl pH 6.8, 2% (w/v) sodium dodecyl sulfate (SDS), 5mmol/L dithiothreitol] at room temperature and vortexed. Cell lysates were then boiled for 5min and cleared by centrifugation (13,000×g, 10min at 4°C). Protein concentrationswere determined using a protein assay kit (Bio-Rad DC; Hercules, CA, USA). The soluble lysates (10 μg per lane) were subjected to 10% (w/v) SDS-polyacrylamide gel electrophoresis; proteinswere then transferred to nitrocellulose membranes and blocked with 5% (w/v) nonfat milk/Trisbuffered saline Tween 20 (TBST) solution for 1 h at room temperature.Membranes were incubated overnight at 4°C with primary antibodies directed against anti- β -actin (1:5,000), anti- γ -GCL (1:1,000), anti-NQO1 (1, 1:2000), anti-HO-1 (1:2,000), anticatalase (1:1000), anti-PPARGC1 α (1:1000), anti-total AMPK, anti-phosphor AMPK (1:1000), anti-eNOS (1:1000), anti-Tfam (1:1000), anti-Complex I (1:2000), anti- Complex II (1:2000), anti-Complex III (1:2000) and anti-Complex V (1:2000) in 5% (w/ v) milk/TBST. After washing the membranes with TBST three times, membranes were incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Western blots were developed using electrochemiluminescence (Roche, Mannheim, Germany) and quantified by scanning densitometry [38].

2.5. Nuclear Nrf2 analysis

Total protein and nuclear protein were isolated for Western blot analysis of Nrf2 levels. Nrf2 was probed with anti-Nrf2 antibodies (Santa Cruz) at 1:1000 in the nuclear protein fraction. Histone H1 was used as loading control for nuclear proteins. Antihistone H1 antibody (Sigma) was used at 1:2000. Chemiluminescence detection was done with an ECL Western blotting detection kit from Amersham Pharmacia [39].

2.6. Enzyme activity measurements

NQO1 activity was measured as the dicoumarol-inhibitable fraction of 2,6-dichlorophenolindophenol (DCPIP) reduction in the cell cytosol in the presence or absence of activators of NQO1. DCPIP was used as the electron acceptor; reduction was measured [40]. GCL activity was assayed with the NDA method described previously [41]. Cellular GR activity was measured by the nicotinamide adenine dinucleotide phosphate (NADPH) method described previously [42]. Cellular GPx activity was measured by the NADPH method described previously [43]. Catalase activity was assayed by the method of Aebi [42].

2.7. Quantitative reverse transcriptase-polymerase chain reaction

Real-time PCR was used to measure gene expression levels of Cu/Zn superoxide dismutase (Cu/Zn SOD, SOD1), Mn SOD (SOD2), uncoupling protein 2 (UCP2), peroxiredoxin 3 (PRDX3), peroxiredoxin 5 (PRDX5) and thioredoxin-2 (TRX2). Total RNA (1 μ g), isolated by TRIZOL Reagent (Invitrogen, Carlsbad, CA, USA) from cells cultured in 6-well plates, was reverse transcribed using Revertra Ace (Toyobo, Japan) following the supplier's instructions. Primers were designed using Premier Primer 5 software (Palo Alto, CA, USA). Triplicate PCR reactions were carried out with real-time PCR Master Mix (Toyobo, Japan). PCR was performed on a Multiplex Quantitative PCR System Mx3000P (Stratagene, Cedar Creek, TX, USA) as follows: after an initial step of 10 min at 95°C, the samples were subjected to 40 cycles of 30s denaturizing at 95°C, 1 min annealing at 55–60°C and 30 s extension at 72°C. Melting curves were assessed over the range 55°C-99°C to ensure specific DNA amplification. The cycle number at which the various transcripts were detected (Ct) was compared with that of 18S-RNA, referred to as ΔCt. The relative gene level is expressed as $2(-\Delta\Delta Ct)$, in which $\Delta\Delta$Ct equals ΔCt of the detected gene minus ΔCt of 18SrRNA ([29]). The nucleotide sequences and annealing temperatures of primers used for real-time PCR or cDNA probe construction (5′ to 3′) are shown in Table 1.

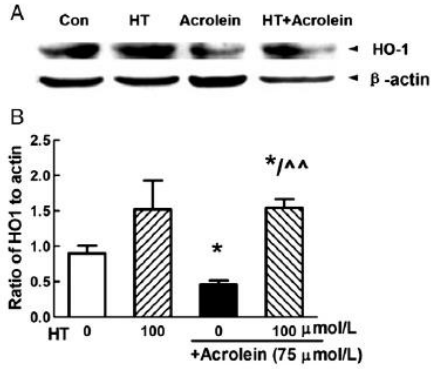

*Fig. 4. The induction of heme oxygenase-1 (HO-1) protein by hydroxytyrosol (100 μ mol/L, 48 h) in ARPE-19 cells without and with exposure to acrolein (75 μ mol/L, 24 h). (A) Representative Western blot image and (B) quantification of Western blot of HO-1 from three separate experiments. *Pb.05 vs. control cells (hydroxytyrosol 0 μ mol/L) and ^^Pb.01 vs. acrolein-treated.*

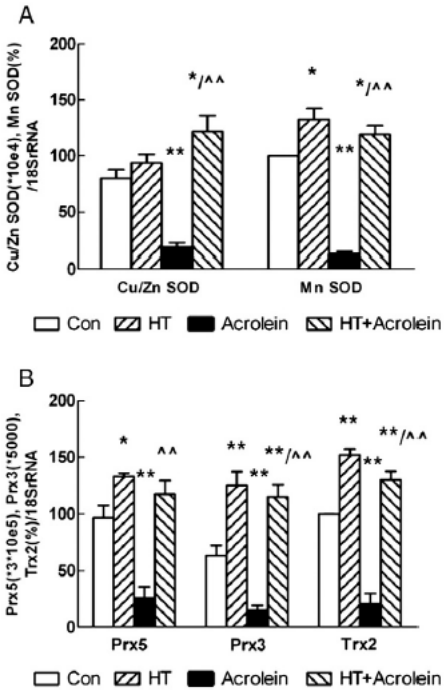

*Fig. 5. Hydroxytyrosol increased the transcription levels of cytoplasmic and mitochondrial antioxidant defense system genes both in untreated and in acroleinchallenged ARPE-19 cells pretreated with hydroxytyrosol (100 μmol/L; 48 h) before acrolein exposure (75 μmol/L; 24 h). RNA was isolated, reverse-transcribed to cDNA and analyzed by real time PCR to measure expression levels of the target genes as ratios to 18SrRNA. mRNA levels of (A) SOD1 (Cu/Zn SOD) and SOD2 (Mn SOD); and (B) PRDX5 (Prx5), PRDX3 (Prx3), and TRX2. Values are means±S.E.M. from four separate experiments for Cu/Zn SOD, PRDX5, TRX2, and five separate experiments for Mn SOD and PRDX3. *Pb.05, and **Pb.01, vs. control cells; ^Pb.05 and ^^Pb.01 vs. acrolein group.*

2.8. Statistical analysis

Results are presented as means±S.E. Group comparisons were made by one-way analysis of variance, followed by determination of significant differences using post hoc comparisons with a Tukey HSD test. Pb.05 was considered significant.

3. Results

3.1. Hydroxytyrosol increases nuclear Nrf2

The central factor controlling phase II detoxifying enzyme activation is the activation of Nrf2. Acrolein treatment (75 μmol/L, 24 h) significantly suppressed nuclear Nrf2 levels to about 15% of control cell levels. Hydroxytyrosol pretreatment (100 μmol/L for 48 h for all experiments, same as in our previous study [12]) abolished the acrolein effect and further enhanced nuclear Nrf2 protein levels (Fig. 1). In addition, hydroxytyrosol significantly increased (to 34% above control cell values) nuclear translocation of Nrf2 in cells not challenged with acrolein (Fig. 1).

3.2. Hydroxytyrosol boosts phase II detoxifying enzymes

GCL expression and activity were significantly decreased by acrolein to approximately 50% of control levels, and hydroxytyrosol pretreatment effectively protected cells against the effects of acrolein (Fig. 2A-C). Hydroxytyrosol also significantly enhanced GCL protein expression in unchallenged cells (Fig. 2A and B). GSH reductase (GR) activity was reduced by approximately 55% by acrolein, and again, hydroxytyrosol pretreatment abolished the acrolein effect (Fig. 2D). Hydroxytyrosol alone also significantly stimulated GR activity in unchallenged cells (Fig.

2D). GSH peroxidase (GPx) activity dropped by 45% after acrolein treatment (Fig. 2E). Hydroxytyrosol pretreatment completely protected against the acrolein-induced GPx activity decrease. Hydroxytyrosol treatment alone significantly increased GPx activity (Fig. 2E). Acrolein induced a significant decrease in both expression (Fig. 3A and B) and activity (Fig. 3C) of NQO1, and this decrease was prevented by hydroxytyrosol pretreatment. Moreover, hydroxytyrosol pretreatment significantly induced NQO1 protein expression (about 4 fold) and activity (about 2 fold) in unchallenged cells. Acrolein also significantly decreased HO-1 protein expression. Hydroxytyrosol pretreatment protected cells against this decrease and also enhanced HO-1 expression in cells not challenged with acrolein (Fig. 4).

3.3. Hydroxytyrosol increases antioxidant gene transcription

Acrolein treatment significantly repressed the gene expression of Cu/Zn SOD, Mn SOD, PRDX5, PRDX3 and TRX2 in ARPE-19 cells by 75–88% (Fig. 5). Hydroxytyrosol pretreatment restored all these transcripts to above their control levels, by significantly increasing their expression 4.5-fold to 10-fold over the acrolein-depressed levels (Fig. 5).Moreover, hydroxytyrosol significantly up-regulated transcript levels of all but Cu/Zn SOD in cells not challenged with acrolein (by 30–98%; Fig. 5).

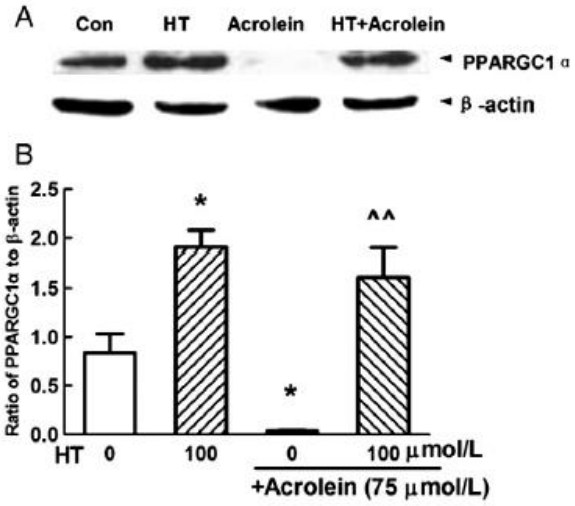

*Fig. 6. Hydroxytyrosol increased PPARGC1α protein expression both in untreated and in ARPE-19 cells pretreatedwith hydroxytyrosol (100 μmol/L; 48 h) before acrolein exposure (75 μmol/L; 24 h). (A) Representative Western blot image and (B) quantification of PPARGC1α Western blots.Values aremeans±S.E. of three separate experiments. *Pb.05 vs. control cells (hydroxytyrosol 0 μmol/L), and ^^Pb.01 vs. acrolein-treated.*

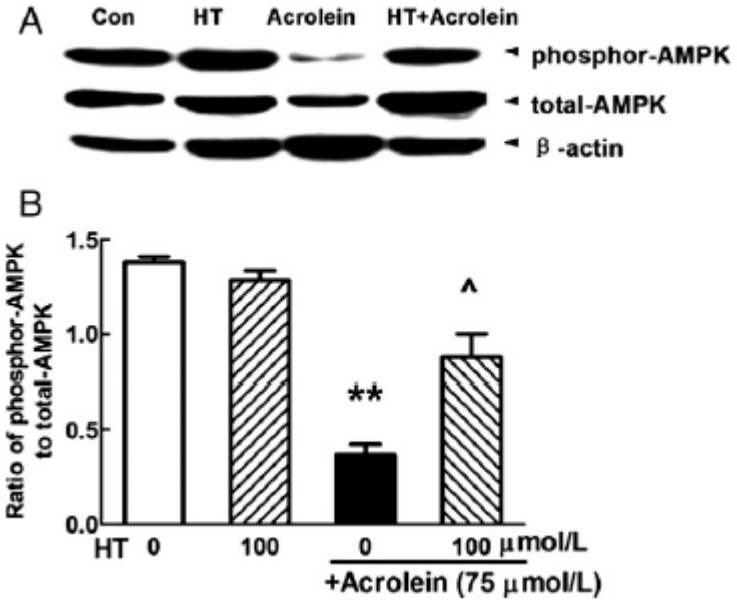

*Fig. 7. Hydroxytyrosol prevented acrolein-induced suppression of the PPARGC1 α - activating signal AMPK. Stimulation of AMPK phosphorylation by hydroxytyrosol (100 μ mol/L, 48 h) in ARPE-19 cells without and with exposure to acrolein (75 μ mol/L, 24 h). (A) Representative Western blot images and (B) quantification of AMPK phosphorylation Western blots. Values are means±S.E. of three separate experiments. **Pb.01 vs. control (hydroxytyrosol 0 μ mol/L) and ^Pb.05 vs. acrolein-treated.*

3.4. Hydroxytyrosol increases PPARGC1 α expression

PPARGC1 α protein expression was strongly suppressed in ARPE-19 cells after acrolein challenge (Fig. 6), while being markedly stimulated by hydroxytyrosol. Hydroxytyrosol pretreatment not only prevented the acrolein-induced suppression of PPARGC1 α but nearly doubled its control cell levels (Fig. 6).

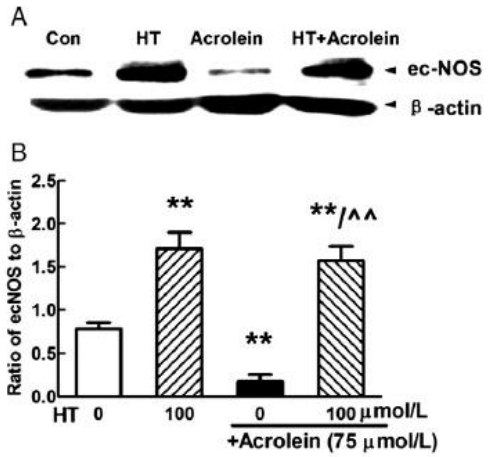

*Fig. 8. Hydroxytyrosol prevented acrolein-induced suppression of the PPARGC1 α - activating signal eNOS (ecNOS). Stimulation of eNOS signaling by hydroxytyrosol (100 μ mol/L, 48 h) in ARPE-19 cells without and with exposure to acrolein (75 μ mol/L, 24 h). (A) Representative Western blot images and (B) quantification of eNOS Western blots. Values are means±S.E. of four separate experiments. **Pb.01 vs. control (hydroxytyrosol 0 μ mol/L) and ^^Pb.01 vs. acrolein-treated.*

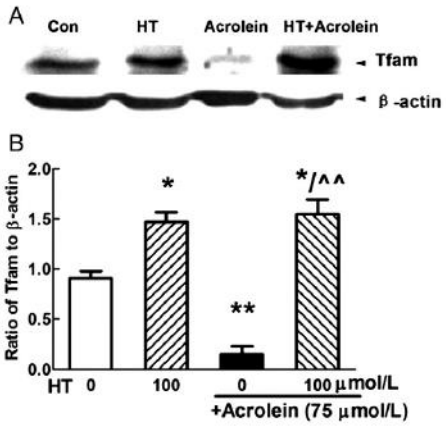

*Fig. 9. Stimulation of the PPARGC1 α-regulated mitochondrial marker Tfam by hydroxytyrosol (100 μmol/L, 48 h) in ARPE-19 cells without and with exposure to acrolein challenge (75 μmol/L, 24 h). (A) Representative Western blot image and (B) quantification of Tfam Western blots. Values are means±S.E. of three separate experiments. *Pb.05, **Pb.01 vs. control (hydroxytyrosol 0 μmol/L) and ^^Pb.01 vs. acrolein-treated.*

3.5. Hydroxytyrosol prevents acrolein-induced suppression of AMPK and eNOS

Acrolein treatment caused a significant inhibition of the PPARGC1 α -activating signal AMPK phosphorylation and hydroxytyrosol pretreatment prevented this decrease (Fig. 7). However, hydroxytyrosol did not influence AMPK phosphorylation in nonacrolein- challenged cells. Just as with AMPK phosphorylation, acrolein treatment severely decreased eNOS protein expression, and the decrease of this PPARGC1 α -activating signal was prevented by hydroxytyrosol pretreatment (Fig. 8). Moreover, hydroxytyrosol also increased eNOS protein expression in non-acrolein-challenged cells.

3.6. Hydroxytyrosol increases PPARGC1 α -regulated mitochondrial markers

Protein expression levels of Tfam (Fig. 9) and the mitochondrial electron transport complexes I, II, III and V (Fig. 10) were significantly decreased by acrolein treatment and rescued by hydroxytyrosol pretreatment. Hydroxytyrosol significantly up-regulated Tfam and the mitochondrial complex proteins in cells not challenged by acrolein (Figs. 9,10). UCP2 gene expression was significantly decreased by acrolein and the acrolein effect was abolished by hydroxytyrosol (Fig. 11). Hydroxytyrosol also induced basal UCP2 expression.

3.7. Hydroxytyrosol increases catalase

Catalase protein expression and activity were significantly suppressed by acrolein (Fig. 12A-C). Hydroxytyrosol pretreatment not only abolished the acrolein-induced decreases in both these measures, but raised catalase expression above basal level as well. In addition, hydroxytyrosol significantly increased basal catalase protein expression and activity in unchallenged cells (Fig. 12B and C).

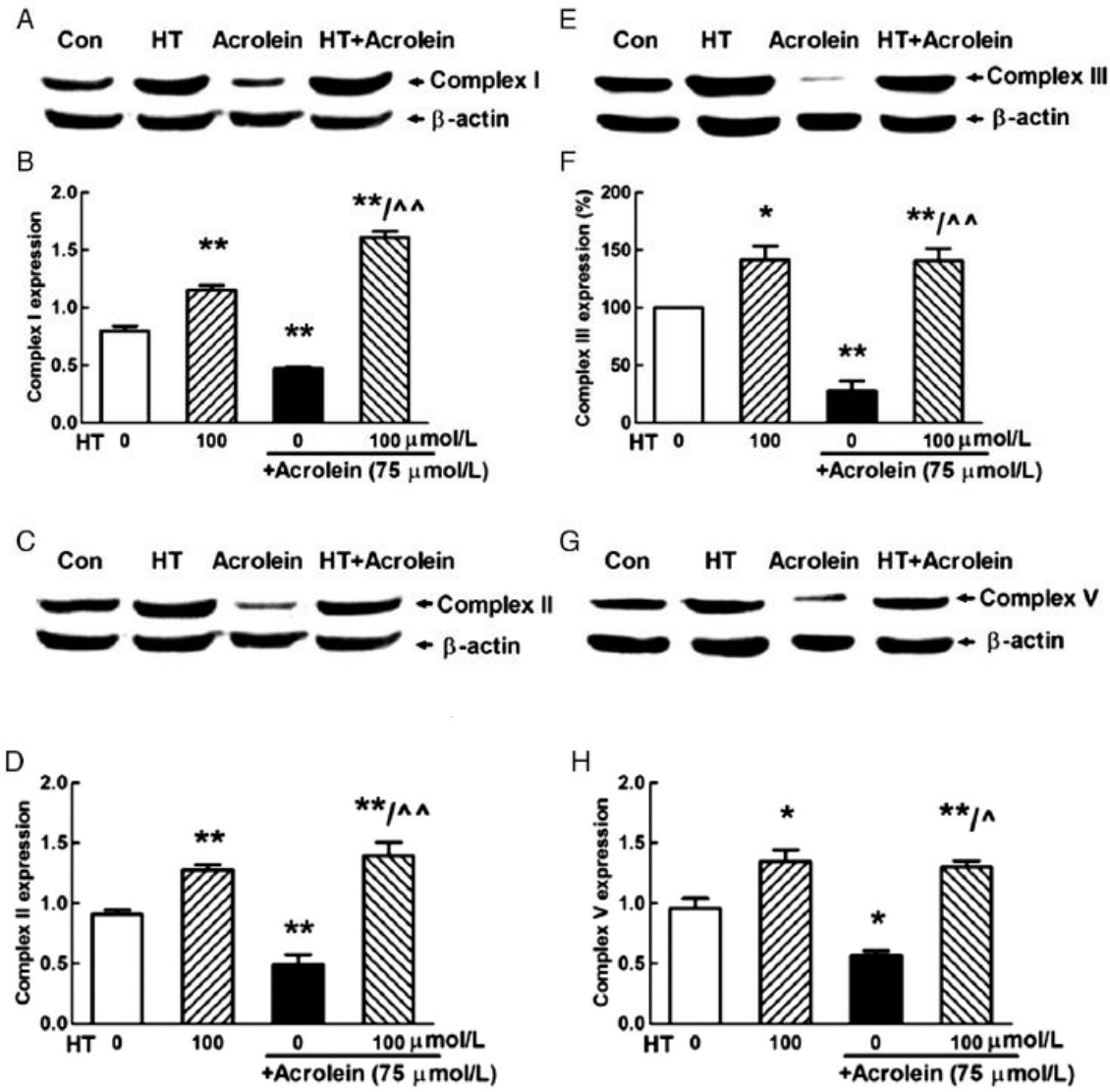

*Fig. 10. Hydroxytyrosol protected ARPE-19 cells from acrolein challenge and increased protein expression of electron transfer complexes I, II, III, and V in both basal and challenged cells. Following pretreatment of cells with hydroxytyrosol (100 μ mol/L, 48 h) and treatment or not with acrolein (75 μ mol/L, 24 h), protein expression of the electron transfer complexes was detected by Western blotting. (A, C, E and G) show representative images and (B, D, F and H) display protein expression ratios relative to beta actin for complexes I, II, III and V, respectively. Values are means±S.E. of three to five experiments. *Pb.05 and **Pb.01 vs. control (no acrolein, no hydroxytyrosol); ^Pb.05 and ^^Pb.01 vs. acrolein-treated.*

4. Discussion

Mechanistic studies of hydroxytyrosol action so far have focused on its antioxidant activity [7,44,45]. Our previous study demonstrated that hydroxytyrosol protects RPE cells from acrolein-induced oxidative damage and mitochondrial dysfunction [12]. The present study further explores the underlying mechanisms of hydroxytyrosol's protective effects in RPE cells. Our study has focused mainly on the following two pathways:

1. Stimulation of endogenous antioxidant systems, including assessment of phase II detoxifying enzymes, mitochondrial antioxidants and catalase and

2. Stimulation of mitochondrial biogenesis.

Our results may be summarized in a mechanistic model of how hydroxytyrosol protects RPE damage by simultaneously activating mitochondrial biogenesis and phase II detoxifying enzyme systems (Fig. 13).

4.1. Phase II enzyme induction by hydroxytyrosol

The role of the activation of the transcription factor Nrf2 and its repression by Keap1 in the control of phase II gene expression (Fig. 13, right hand side) has been well established during the last decade.

Recently, there has been great interest in finding natural phase II detoxifying enzyme inducers to enhance antioxidant response systems for health maintenance. Sulforaphane [46], lipoic acid [20,47,48] and lipoamide [49] are some examples. The present study investigated whether hydroxytyrosol may activate Nrf2 expression to promote the expression of phase II detoxifying enzymes [15]. As clearly shown, hydroxytyrosol indeed activated Nrf2 and increased the protein expression and activities of GCL, NQO1 and HO-1. These results strongly suggest that hydroxytyrosol confers additional antioxidant protection in addition to its direct antioxidant activities, such as free radical scavenging and iron chelation. Our previous work has demonstrated that hydroxytyrosol protects against the acroleininduced decrease in Nrf2 expression on the total protein level [12,17]. Because Nrf2 causes transcriptional activation of antioxidant response elements in the nucleus, the increase in nuclear Nrf2 level should be the key index for phase II detoxifying enzyme activation, resulting in enhancement of the antioxidant defense system. Phase II detoxifying enzymes are part of an elaborate system for protection against the toxicity of xenobiotics and reactive oxygen and nitrogen species that are constant dangers to the integrity of mammalian DNA [46] and lipids. Induction of phase II detoxifying enzymes is also an effective means for achieving protection against a variety of carcinogens and other oxidative damage in animals and humans [50].

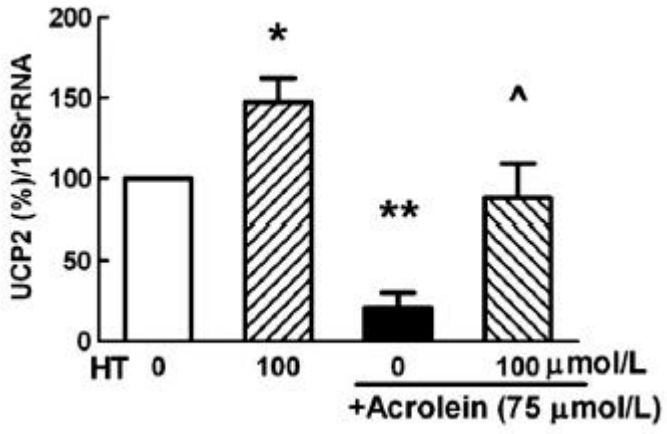

*Fig. 11. Hydroxytyrosol treatment increased the gene expression of UCP2 and protected this gene from decrease by acrolein challenge (75 μ mol/L, 24 h). ARPE-19 cells were treated with 100 μ mol/L hydroxytyrosol for 48 hours; then RNA was isolated, reversetranscribed to cDNA, and UCP2 gene expression relative to 18SrRNA was determined by real time PCR. Data are means±S.E. from five separate experiments. *Pb.05, and **Pb.01vs. control; ^Pb.05 vs. acrolein-treated.*

4.2. Stimulation of mitochondrial biogenesis by hydroxytyrosol

Mitochondrial function is related to mitochondrial content, which can be affected by exercise and environmental factors [28] (Fig. 13, left). The key regulators of mitochondrial biogenesis include PPARGC1 α and its upstream signals, including AMPK, calcium/calmodulin-dependent protein kinase IV and nitric oxide [26,28–31]. Stimulation of PPARGC1 α can suppress neurodegeneration [27], while repression of PPARGC1 α can lead to mitochondrial dysfunction and neurodegeneration [51]. Work in our laboratory with two mitochondrial nutrients, lipoic acid

and/or acetyl-L-carnitine in cellular and animal models of diabetes and Parkinson's disease has shown that lipoic acid and/or acetyl-L-carnitine are able to stimulate mitochondrial biogenesis in neurons [52], adipocytes [53] and muscle [54]. The present study was carried out to determine whether stimulation of mitochondrial biogenesis is a mechanism by which hydroxytyrosol can improve mitochondrial function, and thus reduce oxidative damage in acrolein-induced RPE toxicity. The present study demonstrates that hydroxytyrosol stimulated the expression of PPARGC1 α , the key factor for mitochondrial biogenesis and the protein expression of Tfam, a key transcription factor involved in mitochondrial biogenesis and target gene of PPARGC1 α . In line with this, hydroxytyrosol also increased the protein levels of mitochondrial complexes I, II, III and V. Moreover, hydroxytyrosol up-regulated gene expression of UCP2, also a PPARGC1 α target gene involved in mitochondrial function. These results suggest that hydroxytyrosol stimulates mitochondrial biogenesis and function.

The dual activities of mitochondrial biogenesis and antioxidant defense mediated by PPARGC1 α are closely coupled. However, based on our results on the activation of the upstream signals for regulating PPARGC1 α , viz., phosphorylation of AMPK and expression of eNOS, the induction of phase II detoxifying enzymes by hydroxytyrosol seems to constitute a pathway independent of the dual activities of PPARGC1 α . The detailed molecular mechanisms for this warrant further study.

Aldehydes, including acrolein, are important oxidative stress biomarkers of lipid peroxidation, and have been shown to increase during aging and in diseases [55]. Intravitreous injection of paraquat provides a new model of oxidative damage-induced retinal degeneration. Intraocular injection of paraquat caused condensation of chromatin and thinning of the inner and outer nuclear layers indicating cell death, and terminal deoxynucleotidyl transferasemediated dUTP-biotinide end-labeling demonstrated that one mechanism of cell death was apoptosis. The cell death and apoptosis were accompanied by increases in acrolein, superoxide radicals and carbonyl adducts in the retina and retinal pigmented epithelium [56]. Acrolein toxicity was also demonstrated in retinal ganglion cell (E1A-NR.3) cultures [57]. Although the level of acrolein in retina is not known yet, in our estimation, it may be similar to 4-hydroxynonenal levels because both compounds have been used as indices of oxidative damage in cone cell death in retinitis pigmentosa [58]. Therefore, use of acrolein and hydroxytyrosol in model systems may have practical impact for elucidating the mechanisms of retinal degeneration and finding strategies for its prevention and treatment. It should be pointed out that primary human fetal RPE cells generally are considered a better model than the ARPE-19 cell line for use in RPE studies. However, work in our laboratory [37] has previously demonstrated that primary human fetal RPE cells are comparable to the ARPE-19 cells in sensitivity to acrolein toxicity and lipoic acid protection. Thus, all our experiments have been performed with the ARPE cell line. Of course, further studies with human fetal RPE and animal models are warranted.

In conclusion, hydroxytyrosol has been shown to be a potent inducer of phase II detoxifying enzymes, and a stimulator of mitochondrial biogenesis in retinal pigment epithelial cells. These results provide new mechanistic insights into how hydroxytyrosol may contribute to eye health.

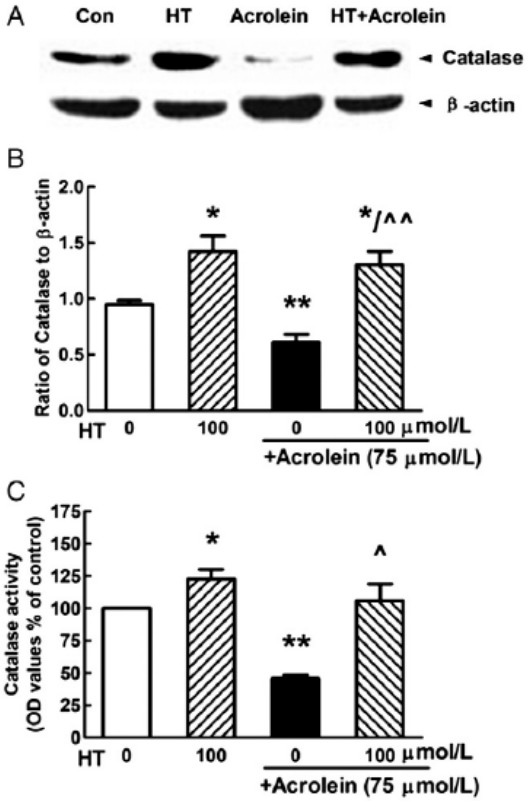

*Fig. 12. Hydroxytyrosol increased catalase both in untreated and in acrolein-challenged ARPE-19 cells. Hydroxytyrosol (100 μ mol/L, 48 h) up-regulated catalase protein and activity levels in untreated ARPE-19 cells, and prevented their suppression by acrolein (75 μ mol/L, 24 h). Catalase protein expression is shown as (A) a representative Western blot image and (B) as quantification of Western blots. Values represent the arithmetic means±S.E. of data from three separate experiments. (C) Catalase activity. Values are means±S.E. of data from three separate experiments, each experiment performed in triplicate. *Pb.05 and **Pb.01 vs. control (hydroxytyrosol 0 μ mol/L); ^Pb.05 and ^^Pb.01 vs. acrolein-treated.*

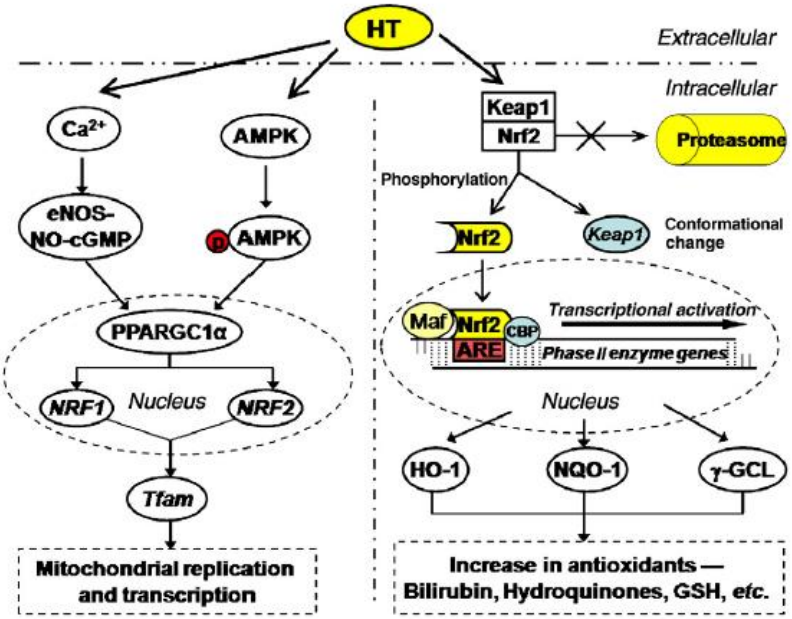

Fig. 13. Schematic illustration of the possible mechanisms of hydroxytyrosol protection against acrolein-induced oxidative damage and mitochondrial dysfunction. There are two pathways related to hydroxytyrosol protection of oxidative injury. The first one is the stimulation of mitochondrial biogenesis by up-regulating AMPK, eNOS and the PPARGC1 α signaling pathway; the second is the induction of phase II detoxifying enzymes by up-regulating the Keap1/Nrf2 pathway. The interactions shown are based on information derived from our past and recent studies [13,14]. Lines terminating in arrows represent positive regulation. This scheme is adapted from Toxicology 246 2008 24–33 and Neurochemical research 33 2008194–203.

Reference [5] The American Society for Nutritional Sciences 2001 **131**, n9 2515S-2522S

Why Is l-Glutamine Metabolism Important to Cells of the Immune System in Health, Postinjury, Surgery or Infection?

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Abstract

Glutamine is normally considered to be a nonessential amino acid. However, recent studies have provided evidence that glutamine may become "conditionally essential" during inflammatory conditions such as infection and injury. It is now well documented that under appropriate conditions, glutamine is essential for cell proliferation, that it can act as a respiratory fuel and that it can enhance the function of stimulated immune cells. Studies thus far have determined the effect of extracellular glutamine concentration on lymphocyte proliferation and cytokine production, macrophage phagocytic plus secretory activities and neutrophil bacterial killing. Other cells of the immune system remain to be studied. The high rate of glutamine utilization and its importance to the function of lymphocytes, macrophages and neutrophils have raised the question "why glutamine?" because these cells have access to a variety of metabolic fuels both in vivo and in vitro. I have attempted to answer this question in this article. Additionally, knowledge of the rate of utilization and the pathway of metabolism of glutamine by cells of the immune system raises some intriguing questions concerning therapeutic manipulation of utilization of this amino acid such that the proliferative, phagocytic and secretory capacities of cells of the defense system may be beneficially altered. Evidence to support the hypothesis that glutamine is beneficially immunomodulatory in animal models of infection and trauma, as well as trauma in humans, is provided.

Lymphocytes, macrophages and neutrophils play important roles in the immune and inflammatory response. Mature lymphocytes recirculate via blood and lymph through lymphoid tissues in a relatively quiescent state until stimulated to proliferate during, for example, a bacterial or viral infection. T-Lymphocytes are required to stimulate macrophage and B-lymphocyte activities mainly via production of regulatory cytokines. B-Lymphocytes produce and secrete antibodies in response to antigenic stimuli. By contrast, macrophages are terminally differentiated end-cells in which the ability to proliferate is gradually lost. They originate, as do all cells of the immune system, in the bone marrow and enter the blood as immature macrophages, termed monocytes. Monocytes enter the tissues and serous cavities of the body where they mature into macrophages and subsequently phagocytose foreign material and apoptosing host cells, they present antigen at the cell surface in association with major histocompatibility complex II (MHC II),[3] and they secrete inflammatory cytokines and free radicals when stimulated to do so. Neutrophils constitute 60% of the circulating leukocytes. They act as first-line-of-defense cells in the blood and at sites of infection. Their function is to remove and destroy foreign material by phagocytosis either alone or in cooperation with antigen-specific defenses. They digest and dismantle the phagocytosed material by production of superoxide and exposure to hydrolytic enzymes in specialized "phagolysosomes." They differ from macrophages in that they die via apoptosis after digesting foreign antigen and they cannot present antigen.

In recent years, the molecular biology of these cells and the process of chemical communication among them has attracted considerable interest, and much progress has been made in our understanding of some regulatory aspects of the immune system. This system is of fundamental importance not only in preventing or limiting

infection, but also in the overall process of repair and recovery from injury. It is therefore of importance in clinical conditions of trauma, sepsis, burns and recovery from surgery.

Despite the undoubted importance of the cells of the immune system, it was surprising that until recently, relatively little was known about their metabolism, the fuels they require to carry out their functions, the rates of utilization and fates of these fuels and any implications for the overall metabolic homeostasis of the animal. Indeed, it was not until the pioneering work of Eric Newsholme's laboratory in the early/mid 1980s that it was established that immune cells such as lymphocytes and macrophages could utilize glutamine at high rates in addition to glucose (Ardawi and Newsholme 1983, Curi et al. 1986, Newsholme, P. et al. 1986 and 1987). It was generally thought at that time that glutamine was only a quantitatively important fuel for cells of the intestine, liver and some tumors. The importance of glutamine metabolism for immune cell function has recently become apparent and is discussed in detail in this review.

Glutamine metabolism in isolated cells

The importance of glutamine to cell survival and proliferation in vitro was first reported by Ehrensvard et al. (1949) but was more fully described by Eagle et al. (1956). Glutamine had to be present at 10- to 100-fold in excess of any other amino acid in culture and could not be replaced by glutamic acid or glucose. This work led to the development of the first tissue culture medium that contained essential growth factors, glucose, 19 essential and nonessential amino acids at approximately physiologic concentrations and a high concentration of glutamine (2 mmol).

It is now clear that glutamine is utilized at high rates by isolated cells of the immune system such as lymphocytes, macrophages and neutrophils [Table 1; also see Newsholme et al. (1999), Calder and Yaqoob (1999) and Wilmore and Shabert (1998) for reviews]. Although the activity of the first enzyme responsible for the metabolism of glutamine, glutaminase, is high in these cells, the rate of oxidation is low. Much of the glutamine is converted to glutamate, aspartate [via tricarboxylic acid (TCA) cycle activity], lactate and under appropriate conditions CO_2 (Table 1).

Addition to incubation medium	Glucose	Glutamine	Lactate	Glutamate	Aspartate	$^{14}CO_2$ production
	nmol/(h · mg protein)					
Mouse macrophages						
Glucose	−355[2]	—	632	—	—	11
Glutamine	—	−186	33	137	25	9
Rat lymphocytes						
Glucose	−42	—	91	—	—	1.5
Glutamine	—	−223	9	132	59	6.1
Rat neutrophils						
Glucose	−460	—	550	—	—	2.4
Glutamine	—	−770	320	250	68	6.5

TABLE 1 - *Rates of utilization of glucose or glutamine and of production of lactate, glutamate, aspartate and $^{14}CO_2$ by isolated incubated mouse macrophages, rat lymphocytes or rat neutrophils*[1]

[1] Data from Ardawi and Newsholme (1983), Newsholme, P. et al. (1987) and Pithon-Curi et al. (1997).

[2] Negative sign indicates utilization.

The importance of glutamine to the function of immune cells in vitro.

T-Lymphocytes.

The major functions of T-lymphocytes in vivo are to proliferate in response to antigenic stimuli, to produce cytokines essential to the propagation of the immune response and to up-regulate specific cytokine receptors on the T-cell surface, which will further enhance rates of proliferation. The concentration of extracellular glutamine appears to regulate T-lymphocyte proliferation (Fig. 1), the rate of interleukin (IL)-2 production and IL-2 receptor expression (Table 2).

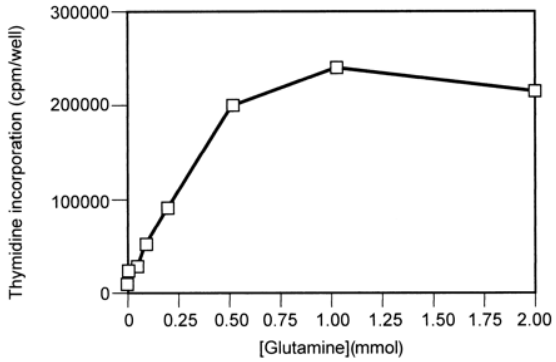

FIGURE 1 - Lymphocyte proliferation is dependent on extracellular glutamine concentration. Lymphocytes obtained from rat spleens were incubated in vitro in RPMI medium containing antibiotics. They were exposed to the T-cell mitogen concanavalin A at the start of the incubation in medium containing various extracellular glutamine concentrations. Proliferation is expressed as increase in radioactively labeled thymidine incorporation into DNA during the last 18 h of a 66-h incubation. [Adapted from Yaqoob and Calder (1997).]

Source of lymphocytes	Authors	Effect of increasing extracellular glutamine concentration
Rats	Ardawi and Newsholme (1983)	Increase in rate of proliferation in response to a mitogenic stimulus
	Szondy and Newsholme (1989)	
Mice	Griffiths and Keast (1990)	
	Yaqoob and Calder (1997)	
Humans	Parry-Billings et al. (1990)	
	Chuang et al. (1990)	
Rats	Calder and Newsholme (1992)	Increase in interleukin (IL)-2 production in response to mitogenic stimulus
Mice	Yaqoob and Calder (1997)	
Humans	Rhode et al. (1996)	
	Yaqoob and Calder (1998)	
Rats	Yaqoob and Calder (1997)	Increase in IL-2 receptor expression

TABLE 2 - The effect of glutamine on T-lymphocyte function

B-Lymphocytes.

The differentiation of B-lymphocytes into antibody synthesizing and secreting cells is glutamine dependent and increases significantly over a range of physiologic glutamine concentrations (Crawford and Cohen 1995). This effect could not be mimicked by a variety of other amino acids.

Lymphokine-activated killer cells (LAK cells).

Glutamine has been reported to support the potential of LAK cells to kill target cells (Juretic et al. 1994). These authors concluded that glutamine deficiency limited the number of activated cells generated in response to a stimulus.

Macrophages.

In contrast to lymphocytes, which have the potential for rapid division, macrophages are terminally differentiated cells that have lost their ability to divide. However, they are metabolically active cells that have high rates of phagocytosis, pinocytosis, protein secretion, free radical secretion (superoxide, nitric oxide), eicosanoid production and membrane recycling and synthesis. All of these processes are linked to the overall function of the macrophage, which is to destroy foreign material via exposure to free radicals and hydrolytic enzymes, antigen presentation to T-lymphocytes (in association with MHC II molecules) and activation of lymphocyte subpopulations via cytokine secretion. It has been demonstrated that when the extracellular glutamine concentration was reduced from 2 to 0.2 mmol, MHC class II expression was reduced by 40% and a decreased level of tetanus toxoid–induced antigen presentation was observed (Spittler et al. 1995). In addition phagocytosis of complement opsinized *Escherichia coli* was decreased in the low glutamine environment (Spittler et al. 1995). Glutamine availability has also been reported to influence the phagocytic uptake of unopsinized yeast cell walls (Parry-Billings et al. 1990a) and of opsinized sheep red blood cells (Wallace and Keast 1992) by mouse macrophages.

Macrophages can be found in various states of activation in vivo. However, macrophages obtained from experimental animals or matured from human moncytes may be specifically stimulated to produce a fairly homogenous population. For example, intraperitoneal injection of bacillus Calmette-Guerin (BCG)-vaccine into mice will lead 4–7 d later to a peritoneal population of *activated* macrophages. These cells are characterized by their enhanced free radical and cytokine secretory activity, ruffling of their membranes, phagocytic capacity and increased metabolism compared with the resident peritoneal cavity macrophage population, which has received no equivalent stimuli. The rate of glutamine utilization of these different macrophage populations had not been determined until relatively recently (Murphy and Newsholme 1998). It is now widely accepted that macrophage activation in vivo (via exposure to BCG) or in vitro [via exposure to lipopolysaccharide (LPS)] leads to a significant increase in glutamine utilization **(Table 3**).One possible explanation for enhanced rates of glutamine utilization is to satisfy the large demand for arginine by activated macrophages. Macrophages are unable to utilize extracellular arginine after activation because the enzyme arginase is rapidly secreted from the cells under these conditions (Murphy and Newsholme 1998), which subsequently depletes the extracellular arginine concentration. Arginine is the immediate precursor of nitric oxide synthesis catalyzed by the enzyme inducible nitric oxide synthase (iNOS) in the macrophage. The secretion of arginase, coupled with enhanced rates of intracellular arginine synthesis from glutamine (Murphy and Newsholme 1998, Newsholme et al. 1999), provides the conditions in which nitric oxide synthesis can occur in cells that normally have a large capacity to produce urea. The same metabolic pathway required for glutamine conversion to arginine in the mouse macrophage also exists in human monocytes (Murphy and Newsholme 1998). Nitric oxide levels determined via the stable oxidative end product, nitrite, reach significant levels after cell culture in the absence of arginine for 48 h (Table 4), coincidentally the same time required to reach maximal rates of glutamine utilization (Table 3).

Conditions	Resident macrophage		Activated macrophage	
	Time of preincubation (h)			
	24	48	24	48
	nmol glutamine consumed/(h · mg protein)			
5 mmol glucose+ Arginine				
Control	20.7 ± 0.3	37.9 ± 4.2	73.8 ± 9.4	95.5 ± 12.7
+LPS	26.7 ± 3.0†	71.0 ± 9.3†	100.4 ± 15.0	118.1 ± 23.5
5 mmol glucose− Arginine				
Control	56.1 ± 6.1	104.6 ± 10.2	126.8 ± 18.6	121.1 ± 4.0
+LPS	74.1 ± 6.4*	127.5 ± 15.9	168.7 ± 26.4	136.6 ± 5.1*

TABLE 3 - Glutamine utilization rates in resident and activated murine macrophages[1, 2, 3]

[1] Cells were isolated and purified via adherence to tissue culture plastic as previously described. After purification, cells were preincubated for 24 or 48 h at 37°C, in 95% air and 5% CO_2, in selected tissue culture media supplemented with a combination of 2 mmol glutamine with 5 mmol glucose and with or without 0.4 mmol l-arginine. After preincubation, the media were replaced and the cells were incubated for 1 h under the conditions described in the table in fresh media; glutamine utilization was determined and was linear over this period.

*[2] P values, where indicated, refer to the statistical difference between glutamine utilization in the presence of 15 µg/mL lipopolysaccharide (LPS) compared with its absence. * P < 0.05; † P < 0.01. Values are expressed as means ± sem, of three or more incubations.*

[3] Data from Murphy and Newsholme (1998).

Conditions	Resident macrophage		Activated macrophage	
	24 h	48 h	24 h	48 h
	nmol nitrate/(h · mg protein)			
0 mmol L–Arginine				
Control	0.0 ± 0.0	4.8 ± 0.8	0.0 ± 0.0	8.4 ± 1.2
+LPS	0.0 ± 0.0	6.6 ± 0.7†	0.0 ± 0.0	11.4 ± 1.1*
0.06 mmol L–Arginine				
Control	8.1 ± 0.8	8.8 ± 1.4	16.3 ± 1.2	16.5 ± 1.2
+LPS	11.0 ± 1.1*	10.4 ± 0.9	21.9 ± 2.9*	18.7 ± 2.8
0.4 mmol L–Arginine				
Control	14.2 ± 3.5	13.8 ± 1.2	36.5 ± 3.1	46.3 ± 2.3
+LPS	22.3 ± 1.7†	15.1 ± 0.7*	42.5 ± 0.7†	47.4 ± 0.9

TABLE 4 - Nitrite production in resident and BCG-activated murine macrophages

[1] Peritoneal murine macrophages were isolated and purified as previously described. After purification via adherence to tissue culture plastic, cells were cultured for 4, 24 or 48 h at 37°C, in 95% air and 5% CO_2 in tissue culture media supplemented with 2 mmol glutamine and 5 mmol glucose in the absence or presence of varying concentrations of l-arginine. After preincubation,

the media were replaced and the cells were incubated for 1 h under the conditions described below after which nitrite production was determined. Nitrite production was linear over this period.

[2] P-*values, where indicated, refer to the statistical difference between glutamine utilization in the presence of 15 µg/mL lipopolysaccharide LPS compared with its absence. ** P *< 0.05; †* P *< 0.01. Values are expressed as means ± sem of three or more incubations.*

[3] Data from Murphy and Newsholme (1998).

Wallace and Keast (1992) demonstrated that murine macrophages stimulated with LPS secreted increasing amounts of IL-1β as the availability of extracellular glutamine increased. More recently Yassad et al. (1997) and Murphy and Newsholme (1999) demonstrated that enhancement of IL-6 and tumor necrosis factor (TNF)-α secretion, respectively, by LPS-stimulated macrophages, was dependent upon extracellular glutamine availability. TNF-α, IL-1β and IL-6 are quantitatively the most important cytokines produced by LPS-stimulated macrophages. Murphy and Newsholme (1999) also demonstrated that in addition to murine macrophage TNF-α production, the production of the quantitatively important human monocyte-derived cytokine, IL-8, was also dependent upon the availability of extracellular glutamine **(Fig. 2**).

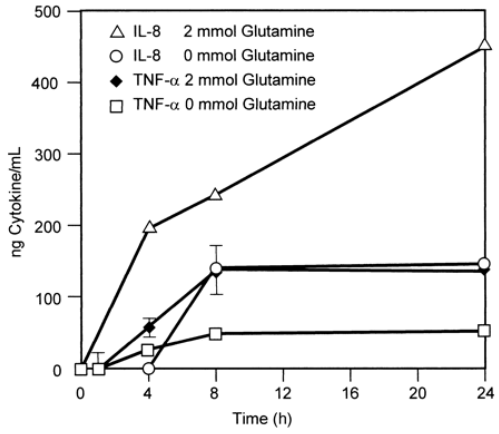

FIGURE 2 -Tumor necrosis factor (TNF)-α and interleukin (IL)-8 production from murine macrophages and human monocytes, respectively, after incubation in the presence or absence of l-glutamine and lipopolysaccharide (LPS). Bacillus Calmette-Guerin (BCG)-activated murine macrophages were isolated and purified as described in Murphy and Newsholme (1999). After purification via adherence, the cells were incubated for up to 24 h in MEM tissue culture medium in the presence or absence of 2 mmol glutamine and presence or absence of 15 µg/mL bacterial LPS. TNF-α production was determined using a DuoSet immunosorbant ELISA test kit as described by Murphy and Newsholme (1999). Cytokine concentration is expressed as ng/mL, which was adjusted to reflect the amount produced from 1 mg cell protein (after cell protein assay).

Macrophage ATP generation and O_2 consumption rates.

Macrophages are known to have a large oxidative capacity and their O_2 consumption rates [515 nmol/(h · mg protein)] are similar to those of sheep heart [696 nmol/(h · mg protein)] and rat liver [520 nmol/(h · mg protein)] in vitro as calculated by Newsholme (1987) using original data from Krebs, Johnson and Karnovsky. Additionally, Newsholme (1987) calculated ATP generation rates for isolated and incubated macrophages in vitro and cultured macrophages, taking into account oxygen utilized by the NADPH oxidase of these cells. The ATP generation rate in the presence of both glucose and glutamine was 930 nmol/(h · mg protein), based on known pathways of metabolism. Glucose contributed 62% and glutamine 38% to the energy requirement of the cell. Because the ATP concentration of the macrophage is ∼7 nmol/mg protein (Newsholme, P. et al. 1987), then the total ATP concentration of the cell must have been turned over at least 2 times/min. The macrophage, when studied over longer periods (82 h of culture), had a similar dependency on these fuels in which glucose contributed 68% and

glutamine 32% to the energy needs of the cell. The major difference in metabolism between freshly isolated cells and cultured cells is that a greater proportion of glutamine carbon is fully oxidized in culture; thus the overall rate of glutamine utilization is lower [55 nmol/(h · mg protein)].

Neutrophils and monocytes.

A recent study by Furukawa et al. (2000) demonstrated that neutrophils and monocytes obtained from patients over a 7-d period after gastrointestinal operations respond to glutamine addition to cell culture medium by increasing their phagocytic activity and rate of production of superoxide (a key free radical required for bacterial killing) in a dose-dependent manner. The degree of improved neutrophil and monocyte phagocytosis and superoxide production with glutamine addition was positively correlated with severity of inflammatory stress and more severe plasma glutamine depletion in vivo (see later). The work by Furukawa et al. (2000) complements a previous study (Saito et al. 1999) in which it was shown that bacterial killing by neutrophils from postoperative patients is enhanced by glutamine in vitro. Neutrophils were cultured in either 0.5 or 1.0 mmol glutamine. The number of viable *E. coli* decreased by 26% as the extracellular glutamine concentration was increased.

A recent study by Garcia et al. (1998) may throw some light on the mechanism by which glutamine may provide increased antimicrobial activity in neutrophils. They demonstrated that 2 mmol extracellular glutamine was able to attenuate the adrenaline-induced inhibition of superoxide production in these cells (**Fig. 3**). In addition, Costa Rosa et al. (1995) demonstrated that glucose 6-phosphate dehydrogenase is inhibited in macrophages by adrenaline, but $NADP^+$-dependent malate dehydrogenase is activated under the same conditions, resulting in a situation in which a considerable proportion of NADPH generating capacity is provided via glutamine metabolism (see below). The adrenaline concentration in "stressed" postoperative patients (whose plasma glutamine concentration will be low) is likely to be high, thus inducing an inhibitory effect on neutrophil and monocyte superoxide generating capacity. Once incubated in 2 mmol glutamine in vitro, the superoxide generating capacity of the neutrophils is thus restored.

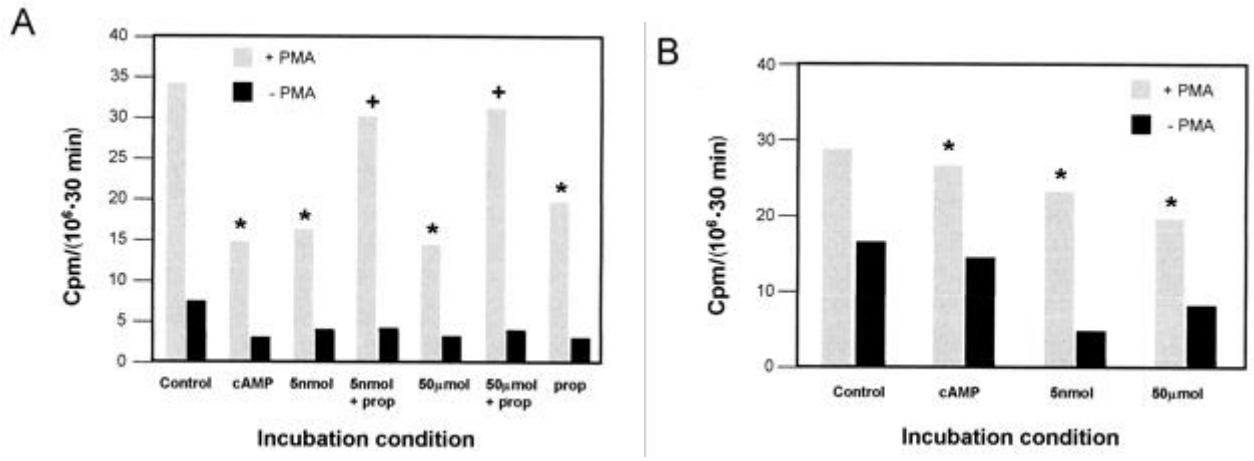

FIGURE 3 - The inhibitory action of adrenaline on rat neutrophil superoxide production is attenuated by the presence of 2 mmol extracellular glutamine. Rat peritoneal neutrophils (3 × 10^6) were incubated in Krebs-Ringer buffer, pH 7.4, in the presence of 5 mmol glucose (A) or 2 mmol glutamine (B), lucigenin at 0.25 mmol, supplemented with adrenaline at 5 nmol/L or 50 µmol/L, dibutyryl cAMP at 100 µmol/L or propranolol (prop) at 200 µmol. Phorbol 12-myristate 13-acetate (PMA; 100 ng) was added as a stimulus. Chemiluminescence was directly proportional to superoxide production and was determined using a liquid scintillation counter. [Adapted from Garcia et al. (1999).]

A rationale for high rates of glutamine utilization in lymphocytes, macrophages, and neutrophils

For many years, the question of the advantage of a high rate of glycolysis in tumor cells has been discussed (Newsholme, E. A. et al. 1987). A similar question can be raised concerning glutaminolysis, i.e., the pathway of incomplete glutamine oxidation that occurs at high rates in cells of the immune system. Glutamine carbon may be converted to glutamate and subsequently via the TCA cycle to malate. The action of $NADP^+$-dependent malate dehydrogenase [malic enzyme, which is present in lymphocytes, macrophages, monocytes and neutrophils (Newsholme et al. 1999)], converts malate and $NADP^+$ to pyruvate and NADPH, from which the "final" product of metabolism, lactate (in glutaminolysis) or CO_2, can be produced. I have outlined here that similar metabolic characteristics apply to various cells of the immune system despite the fact that their cell biology is different. Hence, any hypothesis must explain high rates of glutamine utilization in cells with widely different cell-biological characteristics. Glycolysis and glutaminolysis both provide metabolic intermediates for biosynthetic pathways [e.g., glycolysis provides glucose 6-phosphate for formation of ribose 5-phosphate and glycerol 3-phosphate for phospholipid synthesis; glutaminolysis provides glutamine (by increasing the availability of intracellular glutamine), ammonia and aspartate for purine and pyrimidine synthesis and ultimately for DNA and RNA synthesis]. Although the capacity for rapid cell division is retained by isolated lymphocytes, this does not apply to isolated neutrophils or macrophages, which are terminally differentiated cells with little capacity for cell division. However, neutrophils and macrophages have a large phagocytic capacity (requiring a high rate of lipid turnover and synthesis) and a large secretory activity, e.g., free radicals and cytokines. The mechanism by which glutamine can act to allow high rates of secretory product formation and release and sustain cell proliferation must account for the diverse nature of these secretory products and requirements for cell division and thus must contain at least one common metabolic product.

In the formation of the reactive species, nitric oxide and superoxide, NADPH is required by the enzymes responsible for free radical production, iNOS and NADPH oxidase, respectively. NADPH is also required for the formation of new proteins, DNA or RNA. Glutamine, via catabolic metabolism involving $NADP^+$-dependent malate dehydrogenase [glutamine → glutamate → 2-oxoglutarate → malate → pyruvate; (Newsholme et al. 1996)] can thus generate considerable NADPH for cell requirements. The $NADP^+$-dependent malate dehydrogenase step will result in the formation of pyruvate, which can be converted either to lactate (ending the pathway of glutaminolysis) or to acetyl-CoA and thus CO_2. Thus, depending upon the energy demands placed on the cell, glutamine may be partially oxidized in the pathway of glutaminolysis or may be fully oxidized (at least in macrophages), but the outcome of metabolism in either case is NADPH production. Glucose may also generate NADPH, via metabolism through the pentose phosphate pathway. However, during periods of active pinocytosis and phagocytosis, glucose carbon may be diverted toward lipid synthesis, and therefore the pentose-phosphate pathway may be compromised (Newsholme et al. 1996). Additionally, glutamine carbon may be used for new amino acid synthesis in periods of active synthesis and secretion.

I am therefore making a case for NADPH as the "common factor" that links the diverse effects for which glutamine is responsible in cells of the immune system. Evidence in support of my hypothesis is provided by the beneficial effect of glutamine on superoxide generation in neutrophils and monocytes (Furukawa et al. 2000, Garcia et al. 1998, Saito et al. 1999) and recent in vitro data that cell proliferation in response to growth factors is positively related to the level of superoxide produced intracellularly (Suh et al. 1999). Superoxide generation in cells requires the electron donating ability of NADPH if generated via the enzyme NADPH oxidase, which directly reduces molecular oxygen. The latter enzyme is quantitatively the most significant source of superoxide in immune cells.

The concept of "conditional deficiency"—muscle and glutamine production in stress

Under normal dietary conditions, very little of the glutamine derived from dietary protein enters the blood stream. The epithelial cells of the intestine will consume much of the dietary-derived glutamine and utilize it as a

respiratory fuel. There are a number of tissues that may serve the role of glutamine producers for other organs and tissues of the body. The lung may produce glutamine in acute situations (Souba et al. 1990a) and the brain will synthesize glutamine for conversion to the neurotransmitter glutamate when required. However, skeletal muscle is able to produce glutamine in large amounts; it contains 90% of the whole-body glutamine pool (Darmaun et al. 1986) and can release glutamine in significant quantities [in catabolic stress in humans, glutamine comprised 26% of the released amino acids, (Wilmore and Shabert 1998)]. Glutamine synthetase in skeletal muscle is sensitive to regulation by glucocorticoids (Calder and Yaqoob 1999, Max et al. 1988), and the stress response will result in an increase in activity of this enzyme and the release of glutamine into the blood stream [also sensitive to glucocorticoid regulation (Parry-Billings et al. 1990b)]. TNF-α has also been shown to induce glutamine synthetase gene expression in cultured skeletal muscle cells (Chakrabarti 1998), thus providing a possible link between increased macrophage activity and glutamine utilization at sites of inflammation and increased amino acid metabolism in muscle. Release of glutamine exceeds synthesis in skeletal muscle in conditions of stress, resulting in the lowering of intracellular glutamine concentration, leading to enhanced rates of protein breakdown. However, plasma glutamine concentrations are also decreased in stress situations such as burns (Parry-Billings et al. 1990a) trauma (Jensen et al. 1996, Long et al. 1995), premature birth (Lacey et al. 1996) and sepsis (Ardawi 1991).

As has been described elsewhere in this review, glutamine is utilized at high rates by various immunologic tissues and cells. During inflammatory states, which occur in conditions such as sepsis and injury, the glutamine consumption in immunologic tissues and cells increases. This increase in glutamine consumption, coupled with enhanced utilization by other tissues, results in a demand for glutamine that outstrips supply. As a result, blood, immunologic tissue and muscle glutamine levels fall. The low concentrations of glutamine limit the function of key tissues and cells, especially cells of the immune system. It has been estimated that when plasma glutamine levels fall in a “glutamine-deficient” state, e.g., <0.4 mmol compared with “normal” levels of plasma glutamine, e.g., >0.6 mmol, then the immune system is compromised (Wilmore and Shabert 1998). If this hypothesis is correct, then providing exogenous glutamine to infected or stressed animals or humans and thus raising plasma concentrations to normal should enhance immunologic responses and improve outcome.

Glutamine supplementation, immune function and survival in animal models of infection and trauma

There have been several well-designed animal studies that have reported that glutamine can improve survival rates after infection. These studies were reviewed in detail previously (Calder and Yaqoob 1999, Wilmore and Shabert 1998); thus, I will provide only a brief summary of the findings here. Glutamine-supplemented parenteral nutrition improved survival (75 vs. 45% in the control, standard parenteral nutrition group) in rats after cecal ligation and puncture (Ardawi 1991). Glutamine improved nitrogen balance, attenuated the loss of glutamine from the skeletal muscle intracellular pool, enhanced muscle protein synthesis and decreased protein degradation. Delivery of glutamine-supplemented parenteral nutrition improved survival (92 vs. 55% in the control group) after intraperitoneal injection of live *E. coli* into rats (Inoue et al. 1993). In a different model of infection and glutamine supplementation, mice were fed diets containing casein or casein supplemented with 20 or 40 g glutamine/kg for 10 d and then inoculated intravenously with live *Staphylococcus aureus* (Suzuki et al. 1993). Mortality was assessed over the following 20 d in which the mice consumed the same diets. During this period, 20% of the mice consuming control diets survived, whereas survival increased to 40 and 70% of the mice in the 20 and 40 g/kg groups, respectively. However, immune function was not assessed in any of these studies.

There are a limited number of studies that have addressed the question of whether dietary glutamine enhances immune cell function in infected animals. Yoo et al. (1997) reported that proliferation of blood lymphocytes from *E. coli*–infected piglets was significantly higher if the piglets consumed a diet containing 40 g glutamine/kg

compared with a diet that was not supplemented with glutamine. Infusion of the dipeptide alanyl-glutamine into septic rats increased in vitro proliferation of mitogen-stimulated blood lymphocytes (Yoshida et al. 1992). Additionally, glutamine or alanyl-glutamine, provided parenterally, maintained the lymphocyte yield from Peyer's patches and intestinal integrity in influenza virus–inoculated mice (Li et al. 1998). It is likely that glutamine, via supply of NADPH and possibly other key metabolites, is able to sustain lymphocyte proliferation and viability in these animals.

Provision of glutamine in trauma in humans

At least 16 randomized, blind, controlled clinical trials have demonstrated beneficial effects of glutamine-supplemented parenteral and/or enteral nutrition in catabolic patient subgroups, including those with inflammatory bowel disease [recently reviewed by Jonas and Ziegler (1999)] and those patients receiving abdominal radiation treatment (Souba et al. 1990b). These studies reported improved nitrogen balance, muscle mass and/or gut integrity. In contrast, a number of other studies have not demonstrated improved clinical outcomes e.g., chemotherapy-induced toxicity (van Zaanen et al. 1994), bone-marrow transplantation (Schloerb and Skikne 1999) and Crohn's disease (Den Hond et al. 1999). However, reduced hospital infection rates with glutamine-enriched nutrition have been shown in adult patients after bone-marrow transplantation (Ziegler et al. 1992), major trauma (Houdijk et al. 1998) and in premature infants requiring intensive care (Neu et al. 1997). In addition to these reports, in vitro studies and clinical results have demonstrated a potent effect of glutamine supplementation on human immune cell number and function [reviewed by Ziegler (2000)].

Role of glutamine in the pathogenesis of Type-1 diabetes

It has been proposed that the availability of extracellular glutamine, as an essential amino acid for lymphocyte function, could play a role in the pathogenesis of some autoimmune conditions such as Type-1 diabetes (Wu et al. 1991). Indeed, the administration of the antiglutamine utilization drug acivicin delayed or stopped the progression of the disease in diabetes-prone rats (Misra et al. 1996). We demonstrated recently that the addition of the glutaminase inhibitor 6-diazo 5-oxo norleucine to macrophages before exposure to clonal rat pancreatic β-cells in vitro virtually abolished the lytic capacity of the macrophage toward the target β-cells (Murphy and Newsholme 1999). We speculated that this inhibition of destructive capability is achieved via inhibition of secretion of TNF-α, a cytokine to which the β-cell is particularly sensitive (Dunger et al. 1996, Mandrup-Paulsen et al. 1986, Pukel et al. 1988). We recently obtained evidence that glutamine utilization is increased in macrophages exposed to Type-1 diabetic patient serum (unpublished work) and that the glutamine concentration in the plasma of moderately ketoacidotic patients at diagnosis is significantly elevated, thus adding further weight to the argument that this amino acid is important to the pathogenic process.

CONCLUSIONS

From the initial observations made in Eric Newsholme's laboratory in the 1980s, it has now become clear that many cells of the immune system utilize glutamine at high rates. More recently, glutamine utilization has been linked to functional activities of cells of the immune system such as proliferation, antigen presentation, cytokine production, nitric oxide production, superoxide production and phagocytosis. Many of these functional parameters appear to be directly or indirectly dependent upon the intracellular supply of NADPH. The initial pathway of glutamine metabolism, which is common to all cells of the immune system, can generate NADPH from $NADP^+$, thus providing a possible link between high rates of glutamine utilization and the beneficial effect on the many diverse functions of immune cells. Glutamine supplementation of diet or parenteral nutrition has resulted in beneficial clinical outcomes. In the future, it may be possible to manipulate glutamine metabolism in vivo. This approach may provide novel treatment for a growing list of diseases in which glutamine utilization may directly

or indirectly contribute to disease pathogenesis such as acquired immunodeficiency syndrome, sickle cell anemia, obesity and diabetes.

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Footnotes

↵[1] Presented at the International Symposium on Glutamine, October 2–3, 2000, Sonesta Beach, Bermuda. The symposium was sponsored by Ajinomoto USA, Incorporated. The proceedings are published as a supplement to *The Journal of Nutrition*. Editors for the symposium publication were Douglas W. Wilmore, the Department of Surgery, Brigham and Women's Hospital, Harvard Medical School and John L. Rombeau, the Department of Surgery, the University of Pennsylvania School of Medicine.

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↵[3] Abbreviations used: BCG, bacillus Calmette-Guerin; iNOS, inducible nitric oxide synthase; LAK cells, lymphokine-activated killer cells; LPS, lipopolysaccharide; MHC, major histocompatibility complex; TCA, tricarboxylic acid.

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Methionine residues as endogenous antioxidants in proteins

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ABSTRACT

Cysteine and methionine are the two sulfur-containing residues normally found in proteins. Cysteine residues function in the catalytic cycle of many enzymes, and they can form disulfide bonds that contribute to protein structure. In contrast, the specific functions of methionine residues are not known. We propose that methionine residues constitute an important antioxidant defense mechanism. A variety of oxidants react readily with methionine to form methionine sulfoxide, and surface exposed methionine residues create an extremely high concentration of reactant, available as an efficient oxidant scavenger. Reduction back to methionine by methionine sulfoxide reductases would allow the antioxidant system to function catalytically. The effect of hydrogen peroxide exposure upon glutamine synthetase from *Escherichia coli* was studied as an *in vitro* model system. Eight of the 16 methionine residues could be oxidized with little effect on catalytic activity of the enzyme. The oxidizable methionine residues were found to be relatively surface exposed, whereas the intact residues were generally buried within the core of the protein. Furthermore, the susceptible residues were physically arranged in an array that guarded the entrance to the active site.

Methionine and cysteine are the two sulfur-containing amino acids that occur in proteins. The structural and catalytic roles of cysteine have been defined for many proteins, but this is not the case for methionine. There are as yet no enzymes for which methionine has been shown to function in the catalytic cycle. Although there are many examples from which methionine has been postulated to play an essential structural role, as yet none have been shown to require the thiol ether, which distinguishes methionine from other residues. For example, in α-antitrypsin, oxidation of Met-358 to methionine sulfoxide destroys the antiproteinase activity, presumably by interfering with complex formation with the target proteinase (1). However, the thiol ether is not required for interaction, since replacement of the methionine with valine gives a fully active antiproteinase (2).

All amino acids are susceptible to oxidation, although their susceptibilities vary greatly (3). Organisms have evolved complex antioxidant defenses to minimize oxidative damage to proteins and other macromolecules. They also possess repair systems for reversing some oxidative modifications and disposal systems for removing modified macromolecules that are not repaired. Oxidative modification of residues within proteins may be mediated by a variety of physiologic and non-physiologic systems, including oxidases, ozone, hydrogen peroxide, superoxide, γ-irradiation, metal-catalyzed oxidation, “leakage” from the electron transport chain, and “auto-oxidation” of flavins or xenobiotics. Methionine residues are remarkable for their high susceptibility to oxidation by most of these systems, with the product generally being methionine sulfoxide. The work of Weissbach, Brot, and colleagues (4) established the widespread existence of methionine sulfoxide reductases, capable of reducing either the free or the protein-bound methionine sulfoxide back to methionine. Thus, a repair mechanism exists for dealing with the product of the relatively facile reaction of oxidants with methionine residues.

This led us to hypothesize that methionine residues could function as a “last chance” antioxidant defense system for proteins. As endogenous components of the protein, their effective concentration is very high, providing effective scavenging of oxidants before they can attack residues that are critical to structure or function. If this hypothesis were correct, one should be able to detect oxidation of solvent-exposed methionine residues before oxidation of other residues. In 1975, Shechter and colleagues reported that only the surface-exposed methionine

residues of native proteins were oxidized by chloramine-T or *N*-chlorosuccinimide (5). More recently, the oxidative inactivation of α-2-macroglobulin has been studied in detail and the results obtained are consistent with the hypothesis (6). This antiproteinase loses activity when exposed to activated neutrophils, or to a model system consisting of chloramine. Consumption of eight equivalents of chloramine caused oxidation of eight methionine residues to methionine sulfoxide. Continued exposure caused oxidation of six additional residues of methionine and of a single tryptophan residue. The fractional loss of the tryptophan residue matched the fractional inactivation of the α-2-macroglobulin. These results are consistent with the suggestion that methionine residues scavenge oxidants that could otherwise attack the tryptophan residue that is essential to function.

The locations of the susceptible methionine residues in the α-2-macroglobulin sequence were not determined, and no crystal structure is available to disclose their three-dimensional location. We therefore chose to study the oxidative modification of bacterial glutamine synthetase by hydrogen peroxide, with the purpose of establishing the susceptibility of methionine residues to oxidation, their location within the protein, and the relation of oxidation to loss of catalytic competence. We found that surface-exposed methionine residues surrounding the entrance to the active site are preferentially oxidized without loss of catalytic activity, consistent with the hypothesis that methionine residues function as an endogenous antioxidant defense system.

MATERIALS AND METHODS

Glutamine Synthetase Preparations.

Unadenylylated glutamine synthetase was purified (7) from an overproducing *Escherichia coli*, YMC 10/pgln6, and stored at 20 mg/ml in 10 mM imidazole, 100 mM KCl, 1 mM $MnCl_2$ (pH 7.0). Activity was assayed at pH 7.57 with the γ-glutamyl transferase assay (8). Treatment with H_2O_2 was carried out in 50 mM potassium phosphate (pH 7.5). Both the stock buffer and water were treated with Chelex (Bio-Rad) to minimize metal-catalyzed oxidation of glutamine synthetase (9). One milligram glutamine synthetase in 1 ml total volume was incubated for 2 hr at 37°C in a 12 × 75-mm glass test tube, capped to minimize evaporative losses. H_2O_2 was varied from 0–160 mM (Fisher, 30%). After incubation, an aliquot was taken for activity determination and the remainder dialyzed with dialysis cassettes (Pierce) to remove H_2O_2. Samples were dialyzed against five changes of 10 mM potassium phosphate, 100 mM KCl, 10 mM $MgCl_2$ (pH 7.4). Whatever method is used to accomplish this buffer exchange, care must be taken to assure removal of the H_2O_2. If H_2O_2 carries over into the formic acid used for CNBr cleavage, it will react to yield performic acid, which readily oxidizes methionine residues.

CNBr Cleavage and Amino Acid Analysis.

CNBr cleaves peptide bonds on the carboxyl side of methionine, yielding homoserine; it does not cleave at methionine sulfoxide (10). CNBr cleavage was carried out on 200 μg of protein dried by vacuum centrifugation (Savant) in 4-ml glass vials (Wheaton 224882) fitted with Teflon-lined caps. CNBr was prepared as a 10 M stock solution in acetonitrile, then diluted to 100 mM with 70% formic acid just before use. One hundred microliters was added to the vial, which was capped and incubated for 1 hr at 70°C in a hood. Five microliters was transferred to another vial for amino acid analysis, and both samples were dried by vacuum centrifugation. Hydrogen chloride hydrolysis and amino acid analysis were carried out on samples with and without CNBr treatment (6).

Proteolytic Susceptibility.

Surface hydrophobicity of the 1-anilinonaphthalene-8-sulfonic acid glutamine synthetases was assessed with the fluorescent probe (Sigma). One hundred micrograms of protein was incubated for 30 min at 37°C with 100 mM 1-anilinonaphthalene-8-sulfonic acid in 50 mM Hepes, 100 mM KCl (pH 7.8) with a total volume of 1 ml as described

(11). The 20S proteasome was purified from rat liver essentially as described (11). Proteolytic degradation of glutamine synthetase was assessed with a fluorimetric method adapted to an HPLC detector (12).

Simultaneous Sequencing of Peptide Mixtures.

We wished to quantify the fractional modification of each methionine residue in the oxidatively modified proteins. While this might have been accomplished by reverse-phase HPLC mapping of proteins cleaved by specific proteases, it is a challenging process complicated by varying recoveries and changing retention times caused by oxidation of the methionine residues. We therefore used the technique of simultaneous sequencing of a peptide collection. Simultaneous sequencing by an automated Edman sequencer generates quantitative results from which one can determine the location and extent of covalent modifications, provided that the sequence of the protein is known and the cleavage methods are selected to minimize or eliminate ambiguities. The technique has been used to establish the histidine residues modified by metal-catalyzed oxidation of glutamine synthetase and the cysteine residue modified by glutathiolation in carbonic anhydrase (13, 14).

Treatment of unmodifiied glutamine synthetase with CNBr should yield 17 peptides. Sequencing of this collection of peptides will generate multiple phenylthiohydantoin–amino acids in each cycle of Edman degradation. The peptide collection and resultant phenylthiohydantoin–amino acid patterns will change upon formation of methionine sulfoxides, because CNBr does not cleave these residues. Ambiguities may result from multiple oxidations, but whether this actually occurs can only be determined experimentally for each protein.

The CNBr peptide collection of each of the 16 glutamine synthetase preparations was loaded onto the sequencing column of a Hewlett–Packard G1005A automated sequencer equipped with a model 1040 diode array spectrophotometer. Five cycles were run on each protein, providing an overdetermination useful for confirming the status of each methionine. In addition to cleavage at each methionine by CNBr, the 70% formic acid also cleaved each of the three Asp–Pro sequences (15). These additional peptides were useful for assessing recovery of peptides from each sample. For example, cleavage at Asp-103–Pro-104 places an arginine in cycle 2, the only one that occurs in that cycle. Examination of the yield of arginine established that recovery was reproducible from sample to sample. Table 1 shows the expected effect of oxidation of each methionine in the first five cycles.

Methionine no.	Oxidized by H_2O_2	Cycle 1	Cycle 2	Cycle 3	Cycle 4	Cycle 5
8	Yes	Leu	Asn	Glu	His	Glu
48	Yes	Phe	Asp	Gly	Ser	Ser
65	Yes	Val	Leu	Met		
68	Yes	Pro	Asp	Ala	Ser	Thr
195	Yes	Cys	Leu	Val	Met	
199	No	Glu	Gln	Gln		
202	No	Gly	Leu	Val	Val	Glu
228	No	Thr	Lys	Lys	Ala	Asp
256	?	Pro	Lys	Pro	Met	
260	No	Phe	Gly	Asp	Asn	Gly
268	No	His	Cys	His	Met	
272	No	Ser	Leu	Ser	Lys	Asn
331	Yes	Leu	Ala	Tyr	Ser	Ala
376	No	Ala	Gly	Leu	Asp	Gly
392	Yes	Asp	Lys	Asn	Leu	Tyr
455	Yes	Thr	Pro	His	Pro	Val

Table 1 Susceptibility of methionine residues in glutamine synthetase to oxidation and the expected decrease in residue yields during simultaneous peptide sequencing

Oxidation of a methionine residue prevents cleavage by CNBr, thus causing a decrease in yield of residues in the following peptide. Short, hydrophilic peptides may exhibit poor recovery, and that was the case for P^{257}KPM so that the status of Met-256 could not be determined. Oxidation of the carboxyl-terminal methionine of such peptides causes an increase in recovery of the preceeding residues because the resultant peptide is longer. This phenomenon was useful in monitoring the status of Met-68, whose oxidation caused an increase in valine in cycle 1 because of improved retention of Val-66. Also, methionine sulfoxide was reduced back to methionine under conditions of Edman sequencing so that yields of methionine during sequencing increased as residues were oxidized. For example, no methionine was detected in cycle 3 of the control glutamine synthetase, but yields increased as Met-68 was oxidized. The second column refers to oxidation by exposure to hydrogen peroxide.

RESULTS

Methionine/Methionine Sulfoxide by Amino Acid Analysis.

Exposure of glutamine synthetase to increasing concentrations of hydrogen peroxide for a fixed time (2 hr) generated a series of modified proteins with increasing methionine sulfoxide content, approaching a plateau of eight residues at 160 mM peroxide (Fig.1). Extrapolation to infinite peroxide concentration suggests that a maximum of 10 of the 16 methionine residues per subunit could be oxidized (Fig.1). The γ-glutamyl transferase activity of the enzyme was almost unchanged, decreasing only 15% in the sample exposed to 160 mM peroxide. Thus, oxidation of methionine residues provides a route for scavenging hydrogen peroxide without loss of catalytic activity.

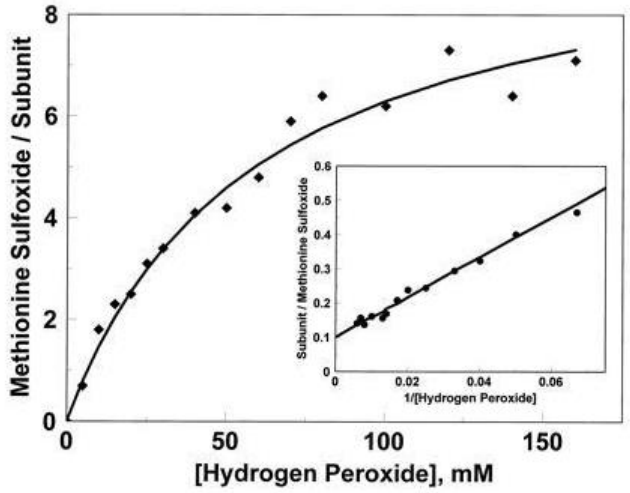

Figure 1 : Oxidation of methionine residues in glutamine synthetase by hydrogen peroxide, determined by amino acid analysis. A double reciprocal plot of the same data is shown (*Inset*), demonstrating that extrapolation to infinite peroxide concentration would cause oxidation of 10 of the 16 methionine residues in each subunit.

Effect of Oxidation on Susceptibility to Proteolysis.

Oxidation of glutamine synthetase by a metal-catalyzed system was previously shown to cause loss of catalytic activity and to render the protein susceptible to degradation by either the 20S proteasome (multicatalytic proteinase) or a specific bacterial protease (9, 16). The modifications "sensed" by the degrading proteases have not been firmly established, but an increase in surface hydrophobicity did correlate well with susceptibility to proteolysis (17). We found that the proteolytic susceptibility of the hydrogen peroxide-treated proteins also correlated with increased surface hydrophobicity (Fig. 2), consistent with the hypothesis that exposure of hydrophobic patches renders proteins susceptible to proteolytic degradation. No increase in proteolytic susceptibility nor in hydrophobicity occurred until ≈6 residues of methionine were oxidized. If this relationship also holds *in vivo*, then there exists a window of opportunity for reduction of the oxidized residues by methionine sulfoxide reductases without competition from proteases.

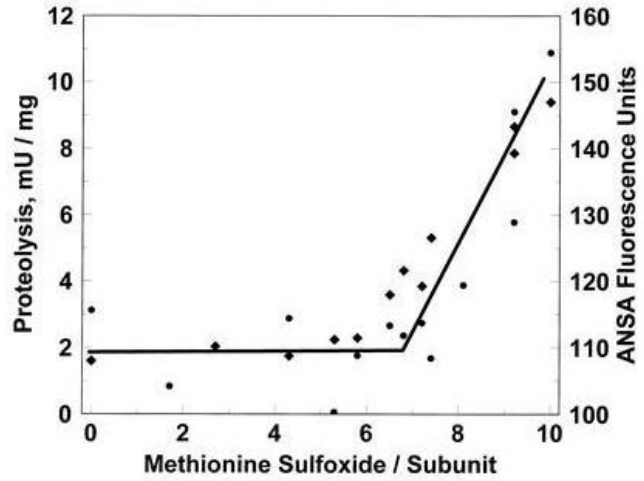

Figure 2 : The susceptibility of oxidatively modified glutamine synthetases to proteolysis by the proteosome (♦) correlated with increased surface hydrophobicity (•).

Identification of the Oxidized Methionine Residues.

The technique of simultaneous sequencing of the CNBr peptide collection allowed determination of the status of each methionine except Met-256 (Table 1, Fig. 3). Even at the highest concentration of peroxide, seven methionine residues remain intact, whereas eight are oxidized. We could not detect differences in susceptibility among the eight oxidized residues.

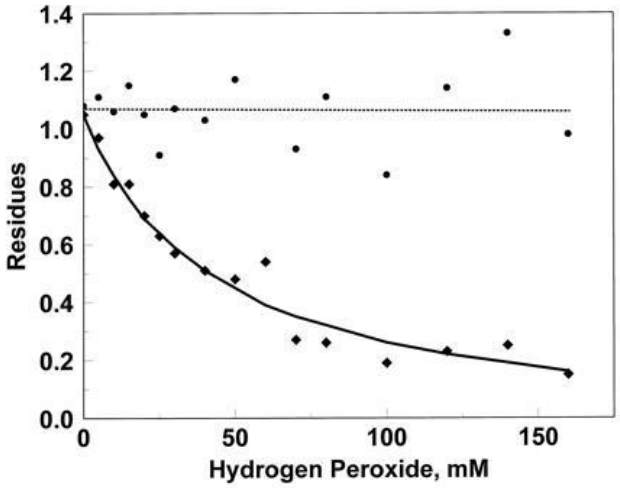

Figure 3 : Examples of results from simultaneous sequence analysis of CNBr peptide collections of glutamine synthetases exposed to hydrogen peroxide. (♦—-♦), aparagine in cycle 2, which monitors Met-8; (•- - - -•), lysine in cycle 4, which monitors Met-272.

The three-dimensional structure of the 12-subunit glutamine synthetase has been determined by Eisenberg and colleagues (18), and the locations of the methionine residues were mapped onto their structure (Fig. 4). The oxidized residues are relatively exposed, whereas the intact residues are generally buried within the core of the protein. Furthermore, the susceptible residues are physically arranged in an intriguing formation. The active site is formed by two subunits that create a bay through which substrates enter and products leave. The eight oxidizable methionine residues are arrayed along the border of the bay, forming a phalanx that guards the active site.

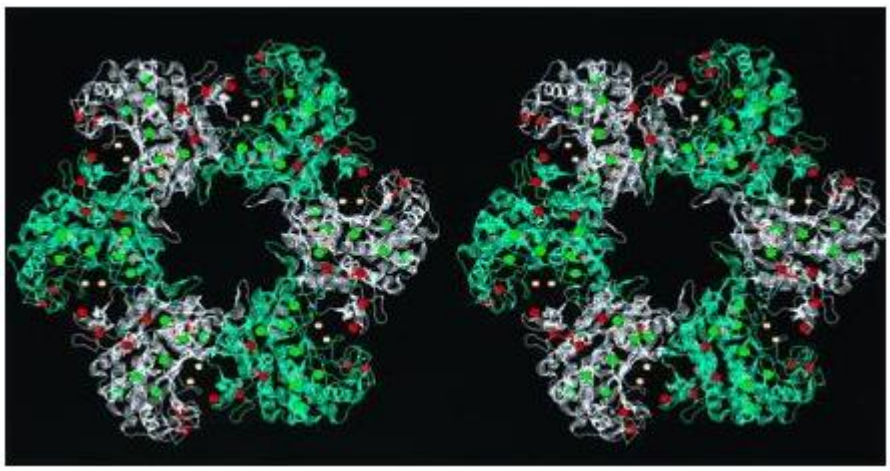

Figure 4 : Location of oxidized and intact methionine residues in glutamine synthetase. This stereo figure was created by the program rasmol (19) using the coordinates determined by Almassy *et al*. (18), deposited in the Brookhaven Data Base (reference 2GLS). For clarity, only one of the two hexamers is shown, and subunits are

alternately colored blue and white. The sulfur groups of methionine residues are shown as balls, with intact residues in green and the oxidized residues in red. The active sites are formed by two adjacent subunits, and the Mn^{2+} in the core of the active sites is shown in yellow. The oxidizable methionine residues appear to form an array about the entrance to the active site bay.

DISCUSSION

Methionine residues have long been known to undergo oxidation to methionine sulfoxide, often with a concomitant effect on an overall biological function, and the review by Vogt (20) provides a useful compilation of many examples. Recently, several investigators have noted that methionine residues within a protein exhibit variability in their susceptibility to oxidation. Susceptibility generally correlates with the surface exposure of the residue, although residues near the methionine can modulate its susceptibility (21). Hsu and colleagues (7) studied oxidation by hydrogen peroxide of recombinant human stem cell factor, which contains five methionine residues. The two surface-exposed residues, Met-1 and Met-159, were readily oxidized, but with negligible effects on biological activity. Met-27 was oxidized at about one-third the rate of the rapidly oxidized residues, again with little effect on activity. The remaining two residues, Met-36 and Met-48, were much less susceptible to oxidation and modification of either residue was accompanied by a substantial loss of biological activity. Similarly, Gitlin and colleagues (22) established that oxidation of Met-111 in interferon α-2b did not alter its biological activity. Nabuchi *et al*. (23) reported studies on hydrogen peroxide-mediated oxidation of the two methionine residues present in human parathyroid hormone. Oxidation of Met-8 slightly reduced biological activity, whereas oxidation of Met-18 substantially reduced activity. Keck showed that two surface-exposed methionine residues of interferon or three methionine residues of tissue plasminogen activation could be oxidized without loss of biological activity (24). A similar result with keratinocyte growth factor has been summarized recently (25). As noted in the introduction, α-2-macroglobulin will likely provide another example, although the location of the initially oxidized eight methionine residues is not yet known. Yao *et al.* (26) reported the rates of oxidation of methionine residues in calmodulin exposed to hydrogen peroxide. They were not able to obtain rates for each methionine residue, but the available results were consistent with the conclusion that susceptibility to oxidation was proportional to the surface exposure of the residue. These studies of various biologically active proteins support the hypothesis that surface-exposed methionine residues effectively scavenge oxidizing agents, while generally preserving the biological function of the molecule. In these cases, no other specific role of methionine has been ascertained.

While solvent-exposed methionine residues are likely to protect from environmentally proximate oxidizing agents, residues in or near active sites may protect enzymes from “auto-oxidation” by substrates or cofactors. For example, oxidation of a single methionine in rabbit 15-lipoxygenase was known to be mediated by substrates or products, and appearance of the methionine sulfoxide had been correlated with loss of catalytic activity. However, the studies of Gan and colleagues (27) established that replacement of the methionine residue by leucine did not prevent inactivation by substrates, demonstrating that formation of methionine sulfoxide was not the cause of inactivation. We suggest that oxidation of the active site methionine may actually retard the inactivation of the lipoxygenase. Whether the resulting methionine sulfoxide can be reduced back to methionine *in vivo* is unknown.

Methionine is readily oxidized by a variety of agents, as noted above. Indeed, a common laboratory method of scavenging chloramines, hypochlorous acid, or CNBr is simply to add a stoichiometric excess of methionine. In addition to their facile oxidation, surface-exposed methionine residues of proteins provide an enormously high concentration of antioxidant at the protein surface, an ideal combination for defense against oxidation of key residues within the protein or even for protecting other molecules against oxidation, for example at sites of inflammation. For simplicity, consider a spherical protein of diameter 60 Å and assume that eight methionine

residues are distributed within that volume. Then the concentration of methionine is ≈100 mM, and if one considered the residues restricted to a shell around the surface of the protein, then the effective concentration would be even higher.

It is notable that a significant number of methionine residues in glutamine synthetase may be oxidized without an increase in surface hydrophobicity or proteolytic susceptibility. Proteins within this window may be repaired by reduction of the methionine sulfoxide to methionine. The net effect is the catalytic scavenging of reactive species as shown in these schematic equations:

$$\text{Oxidation}: \text{Protein}_{\text{Met}} + \text{H}_2\text{O}_2 \rightarrow \text{Protein}_{\text{MetSO}} + \text{H}_2\text{O}$$

1

$$\text{Reduction}: \text{Protein}_{\text{MetSO}} + \text{NAD(P)H} + \text{H}^+ \rightarrow$$

$$\text{Protein}_{\text{Met}} + \text{NAD(P)}^+ + \text{H}_2\text{O}$$

2

$$\text{H}_2\text{O}_2 + \text{NAD(P)H} + \text{H}^+ \rightarrow \text{NAD(P)}^+ + 2\text{H}_2\text{O}.$$

This example points out that cyclic oxidation and reduction of methionine residues both scavenges H_2O_2 and drives an NAD(P)H oxidation reaction. Other reactive species may be similarly scavenged, including superoxide, ozone, hypochlorous acid, and chloramines. Other proteins such as thioredoxin and thioredoxin reductase would likely be important in these catalytic cycles.

Other investigators have pointed out that it may sometimes be desirable to inactivate molecules exposed to oxidizing conditions. For example, activated neutrophils release reactive oxygen species and proteases at sites of inflammation. The well-established oxidative inactivation of α-1-antitrypsin prevents this antiprotease from interfering with the action of elastase at the site. We would therefore expect that the oxidizable Met-358 would be surface exposed, whereas the other methionine residues would be relatively inaccessible, so that they do not react with the oxidizing agents before Met-358. The available structures are consistent with this expectation (28).

Returning to the general hypothesis that methionine residues function as endogenous antioxidants, it follows that mutants enriched at known positions in surface-exposed methionine residues should be more resistant to oxidative inactivation. These modified proteins may also have longer half-lives *in vivo*, especially if a methionine sulfoxide reductase is functional in the cell. Conversely, proteins engineered to decrease their exposed methionine residues would be more susceptible to oxidative inactivation. Increased oxidative inactivation would also be a consequence of impaired methionine sulfoxide reductase activity.

In summary, methionine residues may act as endogenous antioxidants. Surface-exposed residues react readily with oxidizing agents at physiological pH, and their effective concentration at the protein surface is very high. Other residues within the critical regions of the protein are thus protected, and the existence of a repair mechanism means that each methionine may scavenge many oxidizing molecules.

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Transport of Glutamate and Other Amino Acids at the Blood-Brain Barrier

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Abstract

In most regions of the brain, the uptake of glutamate and other anionic excitatory amino acids from the circulation is limited by the blood-brain barrier (BBB). In most animals, the BBB is formed by the brain vascular endothelium, which contains cells that are joined by multiple bands of tight junctions. These junctions effectively close off diffusion through intercellular pores; as a result, most solutes cross the BBB either by diffusing across the lipoid endothelial cell membranes or by being transported across by specific carriers. Glutamate transport at the BBB has been studied by both in vitro cell uptake assays and in vivo perfusion methods. The results demonstrate that at physiologic plasma concentrations, glutamate flux from plasma into brain is mediated by a high affinity transport system at the BBB. Efflux from brain back into plasma appears to be driven in large part by a sodium-dependent active transport system at the capillary abluminal membrane. Glutamate concentration in brain interstitial fluid is only a fraction of that of plasma and is maintained fairly independently of small fluctuations in plasma concentration. Restricted brain passage is also observed for several excitatory glutamate analogs, including domoic acid and kynurenic acid. In summary, the BBB is one component of a regulatory system that helps maintain brain interstitial fluid glutamate concentration independently of the circulation.

l-Glutamate is the most abundant free amino acid in brain and is the predominant excitatory neurotransmitter of the vertebrate central nervous system. Among its many functions, l-glutamate plays a critical role in synaptic maintenance and plasticity (McDonald and Johnston 1990); it also contributes to learning and memory through use-dependent changes in synaptic efficacy, such as long-term potentiation and long-term depression. Under pathologic conditions, excess release of l-glutamic acid and other excitatory amino acids can lead to excitotoxic lesions in brain from overexcitation of nerve cells. Excitotoxicity is thought to play an important role in the neural damage that occurs in diseases such as trauma, stroke, epilepsy and hypoglycemia. Comparable damage can be produced by direct administration of l-glutamate to the nervous system and, under select conditions, by peripheral administration of very high doses of l-glutamate to infant animals (Meldrum 1993).

Under normal conditions, most free l-glutamic acid in brain is derived from local synthesis from l-glutamine and Kreb's cycle intermediates. A considerable fraction is also derived from recycling from brain protein. In synaptic terminals, l-glutamate is stored in vesicles and released via a calcium-dependent mechanism. Once in the synaptic cleft, l-glutamate binds and activates postsynaptic glutamate receptors. Although many different glutamate receptor subtypes have been identified (Nakanishi 1992), the ionotropic glutamate receptors have been studied most extensively and are subdivided into three classes on the basis of sensitivity to the agonists, kainate, quisqualate or *N*-methyl-d-aspartate (NMDA).[2] The NMDA receptor functions as a gatekeeper for sodium and calcium, and has five separate binding sites, each of which is affected by different substrates capable of altering receptor affinity. The action of l-glutamate is terminated by removal from the synaptic cleft by neuronal presynaptic and glial high affinity reuptake systems, several of which have been cloned (Castagna et al. 1997, Kanai et al. 1994, Kanai 1997). These active sodium-dependent transport systems maintain a very large gradient of l-glutamate from the intracellular to the extracellular space (5000- to 10,000-fold) so that brain extracellular l-

glutamate concentrations are normally quite low, with cerebrospinal fluid concentration averaging <0.4 μmol/L (Ferrarese et al. 1993).

Given the critical role of l-glutamate in neural function, it is not surprising that a greater level of regulation is required of brain l-glutamate concentration than that observed in most other tissues. This regulation must include control of both extracellular as well as intracellular free l-glutamate concentrations because l-glutamate acts predominantly at the extracellular synaptic cleft. Plasma l-glutamate concentrations fluctuate during the day as a result of changes in diet, metabolism and protein turnover. If these changes were transferred directly to the brain interstitial space, they would have disrupting effects on neuronal synaptic communication.

The isolation and protection of the brain is accomplished in good part through the presence and function of the "blood-brain barrier" (BBB). The BBB is a system of tissue sites, including brain vascular endothelial cells, choroid plexus epithelial cells and arachnoid membrane; together, they restrict and regulate the flux of substrates between the circulation and the central nervous system (Pardridge 1998). The barrier at each site is formed by a single layer of cells that are joined together by multiple bands of tight junctions. These tight junctions seal off the paracellular diffusion space; thus, to cross the barrier, most solutes must either dissolve in and diffuse across the lipoid cellular membranes of the barrier cells or be transported across by selected BBB carriers. As a consequence, the passive influx of most polar solutes, such as l-glutamate, is quite limited at the BBB and is <1% of that occurring at the blood vessels of most other tissues. To compensate for the limited passive exchange, the cells of the BBB contain high levels of 20 or more specific transport systems that regulate the flux of key solutes from blood into brain interstitial fluid and cerebrospinal fluid and back out again.

In this paper, we summarize the current status of knowledge of amino acid transport at the BBB. Primary focus will be on the transporters at the brain capillary membranes because the capillaries, due to their large surface area, are the primary site of exchange for most solutes between brain interstitial fluid and the circulation. Further, the brain penetration of other acidic (or anionic) amino acid analogs will be discussed, as well as the changes that occur in brain permeation and transport during development.

BBB amino acid transport systems

Currently, nine amino acid transport systems have been reported to be present at the brain capillary endothelium of the BBB. **Figure 1** summarizes the current state of knowledge on their distribution and activity at the capillary luminal and abluminal membranes. These transport systems differ in substrate specificity, inhibition by model ligands (e.g., methyl-aminoisobutyric acid and 2-aminobicyclo[2,2,1]heptane-2-carboxylic acid [BCH]), and transport dependence on sodium (Smith and Stoll 1998).

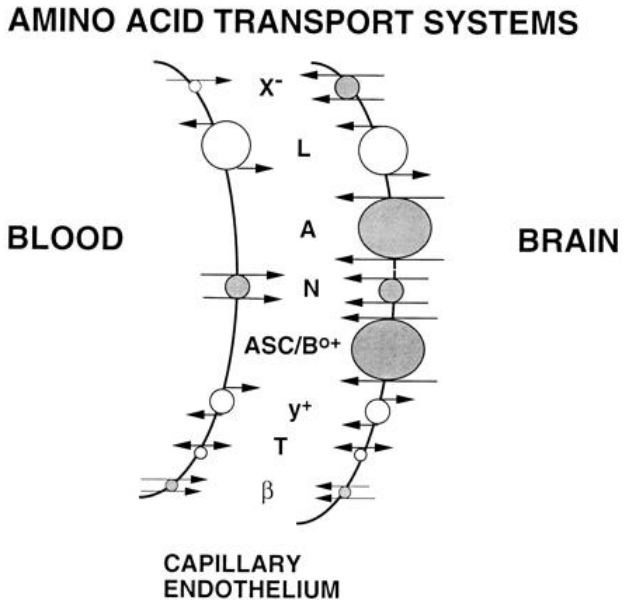

FIGURE 1 : Diagram of amino acid transport systems at the brain capillary endothelium and their localization to the capillary luminal (plasma-facing) or abluminal (brain-facing) membranes. Shaded systems are sodium dependent, whereas unshaded systems are sodium independent.

The first transport systems to be proposed for the BBB were identified on the basis of results from in vivo uptake studies (Oldendorf 1971, Oldendorf and Szabo 1976, Pardridge 1979). These transporters include the following: *1*) System L, which mediates high affinity, sodium-independent uptake of zwitterionic amino acids with "large, neutral" side chains, including l-leucine, l-phenylalanine, l-tryptophan, l-tyrosine, l-isoleucine, l-methionine and l-valine; *2*) System y^+, which mediates moderate affinity, sodium-independent uptake of amino acids with cationic side chains, including l-arginine, l-lysine and l-ornithine; *3*) System T, which mediates high affinity, low capacity transport of thyroid hormones (T_3 and T_4); and *4*) System x^-, which mediates sodium-independent, high affinity uptake of amino acids with anionic side chains, including l-glutamate and l-aspartate.

The System L and y^+ carriers are sodium independent and mediate facilitated exchange at both the capillary luminal and abluminal membranes. Their function is necessary to deliver dietary essential neutral and basic amino acids that cannot be synthesized within the brain (Betz and Goldstein 1978, Momma et al. 1987, Sánchez del Pino et al. 1992, and 1995, Smith et al. 1987, Stoll et al. 1993). In contrast, l-glutamate and l-aspartate, which can be synthesized readily in brain, show much lower rates of uptake into brain at the BBB (Al-Sarraf et al. 1995, and 1997b, Benrabh and Lefauconnier 1996). For these "dietary nonessential" amino acids, brain supply is governed more by intracerebral synthesis and breakdown.

Table 1 summarizes transport V_{max} and K_m values for amino acid uptake into brain at the BBB as measured with the in situ rat brain perfusion technique. Transport rates were determined for each amino acid in the absence of competitors. As shown in Table 1, Systems L, y^+ and x^- each mediate the uptake of two or more amino acids; thus there is the potential for competition among substrates. Actually, competition is quite important because, as shown in Table 1, the plasma concentration for most of the amino acid substrates equals or exceeds the corresponding transport K_m. As a consequence, each of the three transport systems is predicted to be nearly saturated with amino acid substrates as a group at normal plasma concentrations. Kinetic calculations for System L reveal a saturation percentage of >95% when all nine or so amino acid substrates are included. Due to transport saturation, individual amino acids must compete for transport, such that the apparent K_m for uptake from plasma is 3- to 20-fold greater than the true K_m from saline measured in the absence of competitors. The apparent K_m is

defined as $K_m(app) = K_m[1 + \Sigma(C_i/K_{mi})]$, where C_i is the plasma concentration of each competing amino acid and K_{mi} is the corresponding transport K_m for that amino acid. Transport saturation makes the brain amino acid delivery selectively vulnerable to large imbalances in plasma amino acid concentration such as those that occur in the hyperaminoacidemias, e.g., phenylketonuria and maple syrup disease (Smith and Stoll 1998).

Amino acid	Plasma concentration	K_m	V_{max}	K_m(app)	Influx
	μmol/L	*μmol/L*	*nmol/(m · g)*	*μmol/L*	*nmol/(min · g)*
Neutral amino acids (System L1)					
Phe	81	11	41	170	13.2
Trp	82	15	35	330	8.2[2]
Leu	175	29	59	500	14.5
Met	64	40	25	860	1.7
Ile	87	56	60	1210	4.0
Tyr	63	64	96	1420	4.1
His	95	100	61	2220	2.5
Val	181	210	49	4690	1.8
Thr	237	220	17	4860	0.8
Gln	485	880	43	19900	1.0
Basic amino acids (System y^+)					
Arg	117	56	24	302	6.7
Lys	245	70	22	279	10.3
Orn	98	109	26	718	3.1
Thyroid hormones (System T)					
T_3	≤0.002[3]	0.26	0.16		
Acidic amino acids (System x^-)					
Glu	30–50	24	0.21		
Asp	5–10	101	0.13		

TABLE 1 : *Blood-brain barrier transport constants for brain amino acid uptake as measured by the in situ rat brain perfusion technique*[1]

[1] Values are taken from Smith et al. (1987), Stoll et al. (1993), Al-Sarraf et al. (1997b), Filer et al. (1979), Refetoff (1989) and Hokari and Smith (unpublished data). V_{max} is the maximal saturable transport capacity; K_m is the half-saturation concentration in the absence of competitors; K_m(app) is defined as follows: $K_m(app) = K_m[1 + \sum (C_i/K_{mi})]$ where C_i is the plasma concentration of each competitor amino acid, and influx is the unidirectional amino acid flux rate from plasma to brain. Apparent K_m in vivo are much greater than true K_m because of transport saturation and competition (Smith et al. 1987).

[2] Estimated assuming ~70% of albumin-bound Trp contributes to brain uptake.

[3] Estimate of total triiodothyronine (T_3) concentration in plasma; the free T_3 concentration is ~1% that of total T_3.

Alhough l-glutamine shows measurable affinity for System L, Ennis et al. (1998) recently reported that glutamine is actually taken up into brain by a separate sodium-dependent mechanism at the BBB, which they identified tentatively as System N. In other tissues, System N mediates the sodium-dependent transport of l-glutamine, l-

histidine and l-asparagine. Lee et al. (1998) also found evidence for the presence of a separate glutamine transporter distinct from System L at the luminal membrane of the BBB with the use of isolated bovine brain endothelial cell membrane vesicles. However, in that study, the sodium-dependent System N transport was confined to the brain capillary abluminal membrane and was suggested to have a critical role in glutamine efflux from the central nervous system, not uptake. Further research is needed to clarify this discrepancy.

In vitro studies have also provided evidence for the presence of five other sodium-dependent, active transport systems for amino acids at the brain capillaries, including System A, System B^{o+}, System ASC, System β, and System X^- (Betz and Goldstein 1978, Lee et al. 1998, Sánchez del Pino et al. 1995, Tayarani et al. 1987, and 1989). Systems A, ASC, X^- and B^{o+} are proposed to be located primarily at the capillary abluminal membrane (Fig. 1) and actively transport amino acid substrates into the cerebrovascular endothelial cell for efflux from brain extracellular fluid. Systems A and ASC show preference for small neutral amino acids (e.g., l-alanine, l-serine, l-cysteine), whereas System B^{o+} expresses affinity for both neutral and basic amino acids (Guidotti and Gazzola 1992). β-Amino acids (e.g., β-alanine and taurine) are shuttled into brain capillaries by a low capacity, Na^+- and Cl^-- dependent transport carrier (System β) (Tamai et al. 1995, Tayarani et al. 1989). System X^- mediates sodium-dependent transport of anionic amino acids-l-glutamate and l-aspartate. Although the results are preliminary, there is clear evidence of amino acid transport polarity at the BBB with selective distribution of some carriers on the abluminal membrane.

Anionic amino acid transport systems

Several in vivo studies have demonstrated that l-glutamate and l-aspartate are taken up from plasma into brain by a low capacity, high affinity, sodium-independent transporter, tentatively labeled System x^-, which shows competitive interaction between glutamate and aspartate (System X^-; K_m = 2–40 μmol/L for l-glutamate)(Al-Sarraf et al. 1995, and 1997b, Benrabh and Lefauconnier 1996, Oldendorf and Szabo 1976). Because transport was measured from plasma into brain, it is presumed that the saturable carrier is located at the BBB capillary luminal membrane. The exact protein that mediates this uptake has not been identified. l-Glutamate uptake at the abluminal membrane of the capillary endothelial cell was shown by Hutchinson et al. (1985) and Lee et al. (1998) to be mediated by a sodium-dependent saturable mechanism similar to that of System X^-. A family of sodium-dependent anionic amino acid transporters have recently been cloned and identified in brain, but not localized to the BBB (Kanai et al. 1994). The molecular identification of the specific anionic amino acid transporters at the BBB remains to be determined.

The transport capacity for saturable influx of l-glutamate into brain is quite low compared with that of the System L and y^+ carriers (Table 1). This, together with the fact that the glutamate carrier is >80% saturated at normal plasma concentrations, predicts that anionic amino acid flux rates into brain are small (Al-Sarraf et al. 1997a, and 1997b, Segal et al. 1990). Hawkins et al. (1995) reported that the brain uptake “permeability-surface area” for l-[^{14}C]glutamate from normal plasma is ∼7 μL/(min · g), corresponding to an influx rate of 0.67 nmol/(min · g). This flux rate is 5- to 10-fold less than that of most of the large neutral and basic amino acids listed in Table 1. A portion of glutamate is mediated by a nonsaturable mechanism with a K_d of 2 μL/(min · g) (Pardridge 1979).

A number of studies have examined the influence of acute elevations in plasma l-glutamate concentration on brain l-glutamate content. Most have found that in brain regions with an intact BBB, l-glutamate content is fairly independent of plasma l-glutamate concentration (Price et al. 1981, and 1984). The brain intracellular l-glutamate pool, however, is quite large (4–15 mmol/kg wet weight), and may mask changes in brain extracellular glutamate, which is normally in the range of 0.2–5 μmol/L. With the use of microdialysis, Bogdanov and Wurtman (1994) found significant elevations in brain extracellular fluid l-glutamate concentration after large systemic doses of monosodium glutamate that would be expected to raise plasma l-glutamate concentration into the millimolar level. These results must be interpreted with caution, however, because Westergren et al. (1995) reported that

brain microdialysis compromises the integrity of the BBB, allowing greater passive leakage of glutamate into brain. It is likely that with large systemic dosing, some net uptake of l-glutamate occurs in brain. Evaluation of the relationship between cerebrospinal fluid and plasma glutamate may help resolve this controversy.

A second high affinity glutamate transport system has been demonstrated at the choroid plexus epithelium (K_m, 2–3 μmol/L), which may provide an alternate route for glutamate influx into brain (Preston and Segal 1992, Segal et al. 1990). Less is known of glutamate efflux from the central nervous system. Pardridge (1979) has speculated that an active efflux pump for glutamate exists at the blood-brain barrier and may contribute to the regulation of brain extracellular fluid glutamate concentration. Both Hutchinson et al. (1985) and Lee et al. (1998) detected sodium-dependent active components of glutamate transport with the use of in vitro brain endothelial preparations that allow evaluation of transport at the capillary abluminal membrane. Both suggested that the sodium-dependent carrier functions in vivo to transport glutamate from brain. However, more work on this is required to confirm the issue.

Al-Sarraf et al. (1997b) examined the kinetics of anionic amino acid uptake in 1-wk-old and adult rats and found that for the BBB V_{max} for both l-glutamate and l-aspartate is elevated in infant animals. This occurs even though the capillary density in brain is considerably lower in infant animals. No marked alteration was observed in passive BBB permeability, suggesting that if there were age-dependent changes in anionic amino acid transport at the BBB, they were due to alterations in carrier activity. The transport selectivity of the anionic amino acid carriers at the BBB has not been examined closely. BBB transfer rates for several amino acid analogs that have been studied (e.g., domoic acid, kynurenic acid, β-methyl-amino-alanine) are quite low and appear not to use the BBB anionic amino acid carrier (Fukui et al.1991, Preston and Hynie 1991, Smith et al. 1992).

Although the BBB helps protect most of the brain from changes in circulating plasma l-glutamate, there are a few brain areas that do not contain a BBB (**Fig. 2**) and do allow rapid l-glutamate uptake from the circulation (Hawkins et al. 1995). These are known collectively as “the circumventricular organs” and include the median eminence, area postrema, subfornical organ, subcommissural organ, pineal gland, neurohypophysis and organum vasculosum of the lamina terminalis (Gross and Weindel, 1987). Brain uptake rates for small solutes in these areas exceed those of normal brain by 10- to 1000-fold (Gross et al. 1987, Gross 1991, Hawkins et al. 1995, Shaver et al. 1992). Once within brain extracellular fluid, solutes can move into adjacent brain areas via intercellular diffusion or via flow along the Virchow-Robin spaces. Such movement has been documented for glutamate and aspartate in animals after high dose amino acid administration (Price et al. 1981, and 1984). The net result is that certain areas of the brain are vulnerable to acute fluctuations in plasma glutamate concentration of large magnitude as a result of “flooding” from the circumventricular organs.

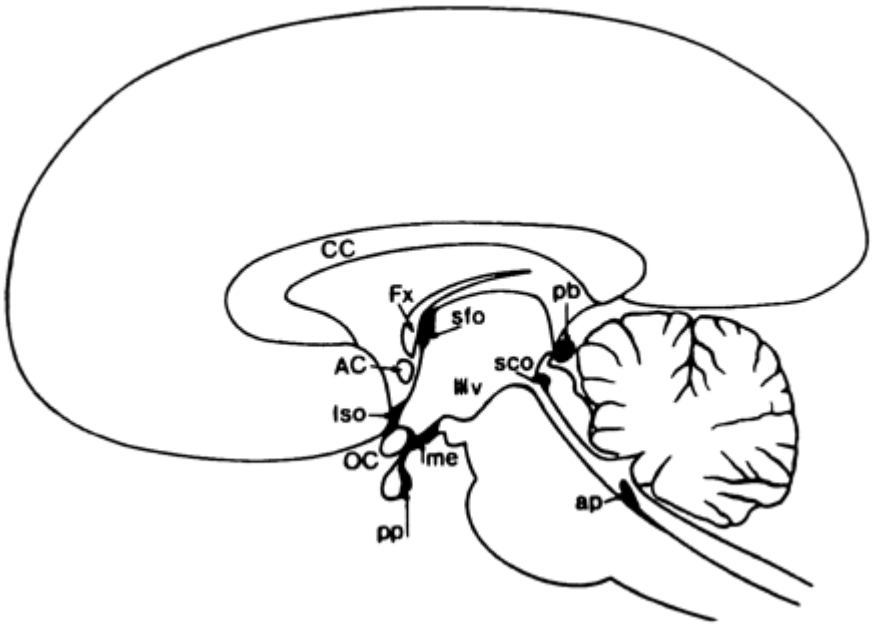

FIGURE 2 : Diagram showing the “nonbarrier” regions of the brain. Nonbarrier regions, shown in black, are as follows: ap, area postrema; pb, pineal body; sfo, subfornical organ; sco, subcommissural organ; me, median eminence; pp, posterior pituitary; and iso, lamina supraoptica. Other regions for reference: IIIv, third ventricle; AC, anterior commissure; and CC, corpus callosum (from Landas et al. 1985, with permission).

System y^+

Unlike the anionic amino acids, l-glutamate and l-aspartate, the essential cationic amino acids, l-lysine and l-arginine, cannot be synthesized by brain at adequate rates to meet the needs for brain metabolism and protein synthesis. Thus, the brain requires a steady and balanced supply of these “essential” amino acids from the circulation. In terms of molecular characterization, the cationic amino acid transporter, System y^+, was the first amino acid transport system at the BBB to be identified at the molecular and genetic level. The cDNA for the first System y^+ cationic amino acid transporter (CAT-1) was cloned serendipitously by Albritton et al. (1989) as part of the search for the host cell protein responsible for infection by the murine ecotropic leukemia virus. The normal physiologic function of this retrovirus receptor was unknown at the time. However, on the basis of a predicted structural homology with yeast histidine and arginine permeases, Kim et al. (1991) and Wang et al. (1991) independently injected the cRNA for the receptor into frog oocytes and demonstrated that it led to enhanced amino acid transport activity with characteristics closely matching those previously reported for System y^+. The deduced amino acid sequence of the retroviral receptor cDNA clone revealed a 622 amino acid glycoprotein with a predicted molecular mass of 67 kDa. After *N*-glycosidase treatment, however, the apparent molecular mass was 60–65 kDa. Hydropathy profiling predicted 12–14 transmembrane-spanning regions, similar to that of the previously cloned facilitative glucose transporter, GLUT1. Subsequently, the corresponding human and rat genes for CAT-1 were cloned and shown to share >88% sequence identity with the murine sequence (Puppi and Henning 1995, Stoll et al. 1993, Yoshimoto et al. 1992). The human gene maps to chromosome 13q12-q14 and consists of 10 introns and 11 exons (Albritton et al. 1992). The human cDNA sequence predicts a 629 amino acid protein with a comparable 12–14 transmembrane-spanning regions. In rats, the primary CAT-1 mRNA size is 7.9 kb, with a smaller band often seen at 3.4 kb in some tissues (Smith and Stoll 1998).

Stoll et al. (1993) demonstrated by RNase protection assay that CAT-1 is highly expressed at the BBB, with a >40-fold enhancement of mRNA for the protein in brain capillaries compared with whole brain or “capillary-depleted”

brain. mRNA for CAT-1 is not ubiquitous among tissues and varies significantly; the highest values are found in bone marrow, intestine, kidney, testes and brain, with essentially no expression in liver (Kakuda et al. 1993, Kim et al. 1991, Puppi and Henning 1995, Smith and Stoll 1998, Stoll et al. 1993). **Figure 3** shows a Northern blot of CAT-1 in brain capillaries, brain and other tissues.

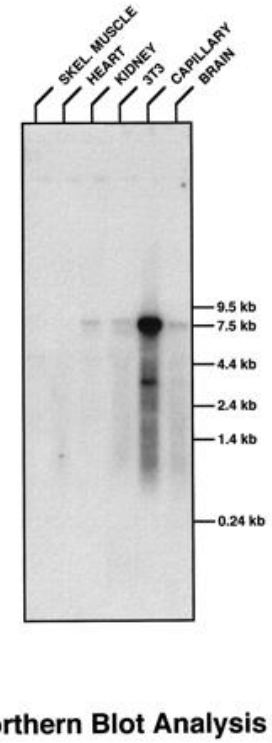

FIGURE 3 : Northern blot of cationic amino acid transporter (CAT)-1 mRNA in rat skeletal muscle, heart, kidney, NIH 3T3 cells, brain capillaries (isolated microvessels) and whole brain (from Smith and Stoll 1998, with permission).

Subsequent work has identified two additional genes for cationic amino acid transporters of the same family (CAT-2 and CAT-3). CAT-2 encodes for two transporter proteins of low and high affinity that are identical with the exception of an alternatively spliced region that differs in 20 amino acids over a 41 amino acid section within the predicted fourth extracellular loop (Closs et al. 1993a, and 1993b, Kakuda et al. 1993). CAT-2 is differentially expressed in tissues, including brain, but does not appear to be enriched at the BBB, where message levels are <10% of that of CAT-1 (Smith and Stoll 1999). CAT-3 is highly expressed in brain and readily discriminates between l-lysine and l-arginine on the basis of affinity (difference of approximately twofold).

A CAT-1 knockout mouse model has been developed in which targeted mutagenesis was used to alter the domain of the protein that is critical for virus binding and produce a germ line with null transport activity (Perkins et al. 1997). Homozygous pups with this mutation were 25% smaller than normal, very anemic and died on the day of birth. The results suggest a critical role in hematopoiesis and growth during development.

System L

The brain also requires a steady stream of essential large neutral amino acids (e.g., l-leucine, l-phenylalanine and l-tryptophan) to maintain metabolic function and protein synthesis. Many of these amino acids are shuttled into brain by the “large neutral amino acid” System L carrier. This carrier was characterized initially in Ehrlich cells by Oxender and Christensen (1963) and later shown by Oldendorf and colleagues to contribute to the brain uptake of 14 of the 16 primary neutral amino acids (Oldendorf 1971, Oldendorf and Szabo 1976, Pardridge 1983). The

carrier appears to operate principally via a sodium-independent, substrate-coupled antiport, although it can mediate net influx (Guidotti and Gazzola 1992).

System L at the BBB shares many of the characteristics of the L System transporter in other tissues, including inhibition by BCH (Aoyagi et al. 1988, Hargreaves and Pardridge 1988, Smith et al. 1987). The two carriers differ, however, in apparent transport "affinity" ($1/K_m$) for most substrates. For example, the K_m for l-phenylalanine uptake into brain (~10–20 μmol/L; Momma et al. 1987, Sánchez del Pino et al. 1995, Shulkin et al. 1995) is 100-1000 times less than that in other tissues. On the basis of this difference, it has been proposed that the blood-brain barrier L System represents a separate isoform, designated System L1.

Several genes have been identified that enhance neutral amino acid uptake when their mRNA is injected into cells. One encodes for a cell surface protein, 4F2hc, also known as CD 98, and has been demonstrated to enhance System L activity in cells (Bertran et al. 1992, Bröer et al. 1995, and 1997, Palacín, 1994). Recently, a System L cDNA was cloned by both Kanai et al. (1998) and Mastroberardino et al. (1998) and shown to encode for a ~41-kDa protein (LAT) with multiple transmembrane-spanning regions, which operates in conjunction with 4F2hc. The two proteins are reportedly linked by a disulfide bridge. The 4F2 complex was identified first in a human T-cell line as a heterodimer composed of an 85-kDa glycosylated heavy chain (4F2hc) with a single transmembrane-spanning region with a disulfide linkage to a 45-kDa nonglycosylated light chain. This smaller subunit was proposed by Palacín (1994) to be a transporter. The cDNA isolated by Kanai encodes for a protein that mediates BCH-sensitive neutral amino acid transport, when expressed in *Xenopus* oocytes (K_m for l-phenylalanine is 20 μmol/L). Message for the protein was found in multiple tissues including brain. The functional relation between the transporter and the 4F2hc is not precisely understood. It has been suggested, however, that 4F2hc is a required regulatory or modulator subunit for the transporter complex.

Consistent with the proposed role of 4F2hc in System L transport into brain, message for 4F2hc was found in mRNA from isolated rat brain capillaries, as well as in whole rat brain (Smith and Stoll 1999). Similarly, Boado et al. (1999) have demonstrated high level expression of the mRNA for the light chain of the L transporter (LAT) at the blood-brain barrier. Expression of 4F2hc also decreased during postnatal development in the rat, as expected from L-System transport studies.

SUMMARY

Over the past 25 years, significant progress has been made in the identification and characterization of blood-brain barrier amino acid transport systems, primarily with the use of physiologic methods. However, with the advent of the cloning and identification of the first blood-brain barrier amino acid transport protein and gene (System y^+) in 1993, a new era has been ushered in for the investigation of barrier amino acid transport systems. It is hoped that these new approaches will provide novel, more selective tools and probes with which to study the transport systems and evaluate their regulation. With these new molecular biological approaches, it may be possible ultimately to modify blood-brain barrier amino acid transport expression to treat human disease.

Footnotes

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for the symposium publication were John D. Fernstrom, the University of Pittsburgh School of Medicine, and Silvio Garattini, the Mario Negri Institute for Pharmacological Research.

[2] Abbreviations used: BBB, blood-brain barrier; BCH, 2-aminobicyclo[2,2,1]heptane-2-carboxylic acid; CAT, cationic amino acid transporter; NMDA, *N*-methyl-d-aspartate.

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Polyphenols as Adaptogens – The Real Mechanism of the Antioxidant Effect?

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1. Introduction

It is well-established from numerous population-based observational studies, that consumption of polyphenol-rich foods, principally fruits and vegetables is beneficial to health, reducing mortality rates and the incidence of the major diseases of modern civilisation, cancer and cardiovascular disease (Stevenson & Hurst, 2007). Until relatively recently, it was widely believed that these health benefits were mediated by free radicalscavenging antioxidants, i.e., vitamins C and E and polyphenols, all compounds with high antioxidant capacity when measured by in vitro chemical tests such as "ORAC". A large body of research, however, has not found a conclusive link between the apparent health benefits of polyphenols and their antioxidant capacity. In addition, supplementation with vitamins C and E, which are thought to operate in the body by radical scavenging, has been the subject of intensive research and large-scale intervention trials. The overall conclusion of this work is that there is no consistent evidence that supplementation of these vitamins above normal dietary intakes is of any benefit to health (Bjelakovic et al., 2008). This suggests that the health benefits of vitamins C and E and polyphenols are not related to their antioxidant capacity. More recent research is, nevertheless, linking polyphenols to other biological effects that have the same end-result as chemical antioxidants were thought to have, i.e., a sustained decrease in free radicals in the body, resulting from enhanced endogenous antioxidant defences and/or reduced production in the mitochondria, the main source of free radical generation. In subsequent sections, the evidence for this is discussed.

2. Relevance of mitochondria to antioxidant effects of polyphenols

Mitochondria are the major producers of free radicals or reactive oxygen species (ROS) in the body and some of the adaptive effects of polyphenols that modulate oxidative stress appear to act through the mitochondria. It is beyond the scope of this review to cover mitochondrial biology in depth, but there is an excellent and comprehensive book on the subject (Scheffler, 2008). For the purposes of this review, an appreciation of the essentials of mitochondrial function will be sufficient to allow interpretation of studies on how polyphenols interact with mitochondria.

2.1 Mitochondria generate metabolic energy and ROS

Mitochondria are responsible for the bulk of cellular energy production (Scheffler, 2008), with only a small proportion being accounted for by the glycolytic pathway. The "electron transport chain" (ETC – Figure 1) oxidises NADH, one output from the TCA acid cycle (Brookes, 2005). This generates electrons, which are transferred through the various components of the ETC, ultimately reducing oxygen to water. In the process, a membrane potential (or proton gradient) is generated by the five ETC complexes pumping protons across the mitochondrial membrane. The return flow of protons through ATP synthase drives ATP synthesis from ADP. The ATP produced during this process is the main energy source used by cells and tissues.

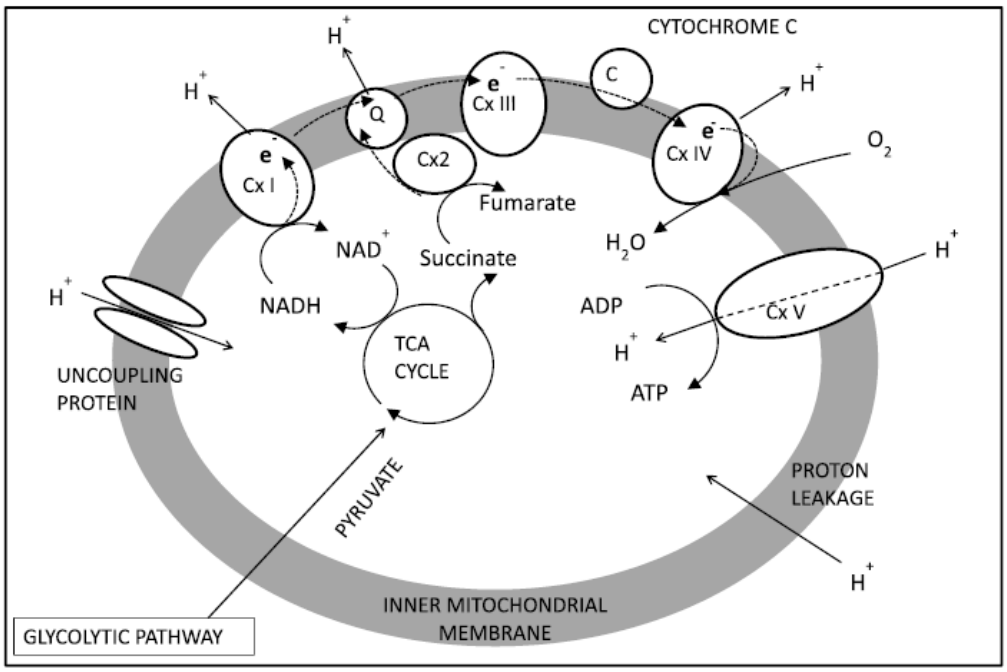

Fig. 1. Schematic summarising the main features of mitochondrial metabolism (Brookes, 2005). Electrons (e_) from the NADH to NAD+ transition are transferred between electron donors and acceptors, in the process generating energy to pump protons outside the inner membrane. The resulting proton gradient can leak back through the membrane or through uncoupling proteins, but most flow through Complex V (ATP synthase) and power ATP synthesis. Complex I (Cx I, NADH dehydrogenase) passes electrons to Complex III (Cx III, cytochrome bc1) via Q (ubiquinone). Complex II (Cx II, succinate dehydrogenase) delivers more electrons via Q, while Complex IV (Cx IV, cytochrome C oxidase) accepts electrons from Cx III, carried by cytochrome C and uses them to reduce oxygen to water.

The ETC is not 100% efficient and some oxygen molecules are incompletely reduced into the free radical (or reactive oxygen species – ROS) superoxide (Scheffler, 2008; Dorta et al., 2006). Superoxide can in turn generate other ROS species. The production of ROS by muscle cells is greatest during exercise, and the condition arising when ROS increase to damaging levels, is termed “oxidative stress” (Powers & Jackson, 2008). Mitochondria are the major source (approx. 90%, the remainder coming from immune system action and the environment) of ROS in the body (Ristow et al., 2009), but in healthy and especially, physically fit individuals, the mitochondria are also well-equipped with antioxidant enzymes to inactivate ROS before they can do more than minor damage to DNA or other vital cellular components (Hu et al., 2007).

The mitochondrial form of the antioxidant enzyme superoxide dismutase (SOD), Manganese , or MnSOD, converts superoxide into hydrogen peroxide, which is not in itself a radical, but easily forms one (Hu et al., 2007). This is, in turn, reduced to water by a mitochondrial form of another enzyme, glutathione peroxidase (GPX) (Dorta et al., 2006). The cell cytosol has a different form of SOD (Copper Zinc, or CuZnSOD) and a different enzyme, catalase, is primarily responsible for removing hydrogen peroxide (Dinkova-Kostova & Talalay, 2008). Under most circumstances, mitochondria in healthy cells leak only a tiny proportion of ROS into the cytoplasm. ROS generation and leakage increase markedly in unhealthy cells during e.g., exhaustive exercise, because the ETC becomes much less efficient when working close to its maximum capacity.

2.2 Mitochondrial adaptation to oxidative stress

It is well established that exercise increases the mitochondrial content of muscle fibres (known as mitochondrial biogenesis or MB) and consequently their respiratory capacity (Holloszy & Coyle, 1984; Hood et al., 2006; Huang & Hood, 2009). More recently, the increase in ROS generation during exercise has been found to be the primary signal for this adaptive effect of exercise (Ji et al., 2008). The benefit to the organism of MB is greater energy generation capacity and reduced ROS generation for a given energy output.

Although it might be expected that increases in mitochondrial/ETC density would lead to increased respiration and ROS production, the opposite actually appears to be the reality. ROS are primarily produced when the flow of electrons through the ETC is limited later in the chain (Barros et al., 2004; Kushnareva et al., 2002). Under these conditions, electrons back up and start to leak out of the complexes earlier in the chain, thereby generating ROS. Under conditions of rigorous exercise, for example, oxygen, the final electron acceptor, would be in limited supply, thus flow of electrons from Complex IV to oxygen would be limited and leakage would occur, primarily from Complex I (Brookes, 2005). There is strong supporting evidence for the link between limited electron flow and ROS generation. Compounds that inhibit any of the ETC complexes cause backing-up of electrons and increased ROS production (Cadenas & Boveris, 1980). The Complex III inhibitor antimycin A increased superoxide production in an in vitro cell model (Dairaku et al., 2004). Inhibition slows the flow of electrons through the ETC and increases the probability of incomplete reduction of oxygen, thereby making superoxide generation more likely. Moderate regular exercise is reported to induce a low level of ROS, which is thought to upregulate antioxidant/repair enzymes and consequently to reduce ROS-associated diseases (heart disease, type 2 diabetes, rheumatic arthritis, Alzheimer's and Parkinson's diseases, and certain cancers) (Radak et al., 2008; Ji et al., 2008; Jackson, 2008). Another complementary benefit is that increased mitochondrial respiration capacity and/or MB should reduce resting ROS generation and reduce the effects of aging (Lopez-Lluch et al., 2006). This comes about because ROS generation is highest when the flow of electrons through the ETC is limited. Increased respiratory capacity permits faster electron flow and lowers basal ROS generation. High basal ROS generation is thought to be the main factor causing aging (Cadenas & Davies, 2000), through damage to mitochondrial DNA. Whereas nuclear DNA is heavily protected from damage by ROS, mitochondrial DNA is located in the inner mitochondrial membrane, close to the ETC complexes and very exposed to damage from ROS. It is thought that damage to mitochondrial DNA leads to synthesis of defective ETC protein subunits and thereby, defective ETCs, which generate less energy and more ROS. A negative feedback loop then results in further increase in ROS generation and DNA damage, to both mitochondrial and nuclear DNA (Huang & Hood, 2009; Droge, 2002).

2.3 Mitochondrial adaptation - a new mechanism of antioxidant action?

It is reasonable to assume that, if exercise-induced oxidative stress is the primary signal leading to mitochondrial adaptation and consequently, increased respiratory capacity and decreased basal ROS production, then other sources of oxidative stress could generate similar adaptations. This is an excellent example of the principle of hormesis (Calabrese, 2008), i.e., a non-linear, adaptive, dose response to a toxin. High doses of ROS are clearly harmful, but low doses appear to be essential to initiate the signalling pathways that lead to beneficial adaptive responses. A hypothesis has been proposed recently to explain how oxidative stress induces mitochondrial adaptation to improve efficiency of energy generation, thereby improving physical fitness and general health, ameliorating health issues such as metabolic syndrome and diabetes and above all, increasing life span (Nunn et al., 2009, 2010). Oxidative stressors that are proposed to induce mitochondrial adaptation include exercise, calorie restriction, ionising radiation and most relevant to this discussion, phytochemical "pro-oxidants" (Nunn et al., 2009; Ristow & Zarse, 2010). The ways in which exercise and polyphenols generate oxidative stress are discussed below. Ionising radiation generates ROS directly, from any molecule encountered (Harman, 1956), thereby causing oxidative stress. Calorie restriction stimulates increased respiration that also leads to oxidative stress (Guarente, 2008; Tapia, 2006).

2.4 Signalling pathways that control mitochondrial adaptation

It is thought that mitochondrial adaptation to exercise is primarily brought about by a complex signalling cascade, which is initiated by increased generation of ROS (Droge, 2002) generated by the mitochondria during exercise. The most important aspect of this adaptation is MB i.e., an increase in the number and mass of mitochondria and/or an increase in the density of ETC complexes (Hoppeler et al., 1973; Baar et al., 2002; Holloszy & Coyle, 1984). Most mitochondrial proteins, including those that make up the ETC, are actually encoded by nuclear genes (Scheffler, 2008) and these are regulated by nuclear respiratory factors (Nrf) including Nrf-1 and Nrf-2 (Hawley & Holloszy, 2009). These Nrfs activate genes that encode mitochondrial respiratory chain proteins. Nrf-1 also up-regulates expression of mitochondrial transcription factor A (TFAM), which is transported into the mitochondria and regulates transcription of the mitochondrial genome (Hawley & Holloszy, 2009). Expression of mitochondrial fatty acid oxidation enzymes is regulated by peroxisomeproliferator coactivator 1-□ (PGC1-□) (Baar et al., 2002; Lopez-Lluch et al., 2006; Poderoso et al., 2000). Sirtuin1 (a regulatory protein deacetylase, is discussed further in Section 5.1) is thought to be involved in deacetylating and thus activating PGC-1□, which, in turn, coactivates peroxisome-proliferator activated receptor-□ (PPAR-□). PPAR-□ is primarily a receptor for fatty acids, which regulates fatty acid oxidation, but it can apparently also be activated by a number of natural products, including some polyphenols (Huang et al., 2005). The anti-diabetic drugs "glitazones" are known to activate PPAR-□ and have also been shown in vitro to induce MB and reduce mitochondrial ROS production (Fujisawa et al., 2009). Polyphenols isolated from red wine, particularly ellagic acid and epicatechin gallate (ECG), were able to activate PPAR□ in vitro with similar affinity to the reference pharmaceutical compound rosiglitazone (Zoechling et al., 2011). A number of studies on resveratrol, another polyphenol found in red wine, suggest that many of its purported beneficial effects are mediated by its stimulation of PGC1-□ signalling (Tan et al., 2008). Interactions with PPAR□ therefore appear to be a possible means for polyphenols to influence mitochondrial adaptation. These interactions may be mediated through direct binding to and activation of PPAR□, or indirectly through its co-activator PGC1-□.

2.5 How do ROS interact with signalling pathways?

One uncertainty in our knowledge of the MB signalling pathway is how an increase in ROS generation initiates signalling. One possible mechanism involves oxidised lipids and the Electrophile (or Antioxidant) Response Element (ERE, or ARE). Mitochondria are primarily composed of membranes into which the ETC complexes are embedded. The lipids in these membranes should be highly susceptible to peroxidation by superoxide generated in their immediate vicinity. One species of breakdown products known to arise from lipid peroxidation is a number of conjugated aldehydes such as 4-hydroxy-2-nonenal (HNE) (Uchida et al., 1999). Conjugated aldehydes and ketones are also potent activators of the ERE (Dinkova-Kostova & Talalay, 2008). The ERE itself is a regulatory region in nuclear DNA that controls the expression of a series of cytoprotective proteins, including a number of antioxidant enzymes, cytochrome P-450 xenobiotic hydroxylases and xenobiotic conjugative enzymes (Dinkova-Kostova & Talalay, 2008). The transcription factor that controls the ERE is Nrf2, which is normally bound to the sensory protein Keap1. Binding of an inducer, such as HNE, or phytochemicals such as curcumin, some polyphenols or sulphoraphane releases Nrf2, which activates the ERE and the proteins it regulates (Dinkova-Kostova & Talalay, 2008). It is possible that the ERE and Nrfs are a link between lipid peroxidation by ROS and the signalling pathway for MB. It appears from this discussion that one of the potential mechanisms for the adaptive effects of polyphenols and other phytochemicals is direct interaction with the MB signalling pathway, through direct activation of either PPAR receptors or the ERE, through interaction with its regulatory protein Keap1.

3. Effect of exercise on mitochondria

This area of science has been subjected to intensive research for at least two decades and is now well understood. The mechanisms of action of exercise in mitochondrial adaptation would reasonably be expected to be very similar to those of other adaptogens, such as polyphenols, if they work through generation of oxidative stress. Evidence that polyphenols can have a hormetic effect through generation of oxidative stress is discussed below. Exercise science should, therefore, be a good source of insights into the mechanistic details of how polyphenols should interact with mitochondria, as well as providing validated assays to monitor these effects both in vitro and in vivo.

3.1 Macroscopic effects of exercise

The well-known benefits of exercise in weight management have been explored by a comparison of mitochondrial metabolism between trained athletes and sedentary individuals (Befroy et al., 2008). The athletes had a 53% higher resting TCA cycle flux, but the same ATP synthesis rate as the sedentary individuals. Essentially, the athletes appeared to have much higher mitochondrial respiration capacity, which "wasted" energy in the resting state, thereby raising their metabolic rate. As discussed above (Section 3.2), it would be reasonable to expect that the athletes' mitochondria would generate less ROS than the sedentary controls in a resting state, because of greater mitochondrial capacity and efficiency, in spite of their higher metabolic rate.

3.2 Antioxidants may inhibit exercise-induced mitochondrial adaptation

If the mechanism underlying the adaptive effects of exercise is initiated by increased oxidative stress, i.e., ROS production, then logically, radical-scavenging antioxidants may be expected to at best have no effect, or at worst hinder adaptations. A number of studies on the use of radical-scavenging antioxidants, i.e., vitamins C and E, appear to support this hypothesis. In a comparison between "Ironman" triathletes and untrained controls, the triathletes had higher resting plasma levels of glutathione peroxidase (GPX), catalase, and superoxide dismutase (SOD), plus lower malondialdehyde (MDA, a biomarker of lipid peroxidation). Participation in the Ironman event then lowered the athletes' antioxidant enzymes and raised MDA. Triathletes who took antioxidant supplements had greater increases in MDA than those that did not (Knez et al., 2007). This suggests that training-level exercise up-regulates antioxidant defences, but competition-level exercise suppresses them.

Antioxidant supplements taken during training may cause further suppression of endogenous antioxidant defences. Supplementation of both trained and untrained subjects during a one-month training programme revealed that the supplements suppressed several early biomarkers of mitochondrial adaptation, including expression of PGC1-□, PPAR-□, SOD2 and GPX1 (Ristow et al., 2009). It appears, however, that there is no overall inhibition of exercise adaptation resulting from antioxidant supplementation because no effect on markers of oxidative stress or on increases in training-induced muscle performance was identified (Theodorou et al., 2011).

It appears reasonable to suggest that exercise is an antioxidant in itself, because it leads to significant up-regulation of antioxidant enzymes in humans (Gomez-Cabrera et al., 2006; Gomez-Cabrera et al., 2008). Similarly, SOD2, the specific mitochondrial form of the enzyme, was induced in rodents by exercise (Higuchi et al., 1985). Although antioxidant supplementation appears to be of no benefit to most sports training, it may be useful if applied in a well-planned and timely manner, to protect untrained individuals from the most damaging effects of high ROS production at the start of a fitness programme (Vincent et al., 2006). These findings are consistent with the hormetic hypothesis of polyphenolstimulated mitochondrial adaptation. It seems reasonable to conclude that if radicalscavenging antioxidants inhibit the signalling of mitochondrial adaptive effects, but polyphenols promote them, then polyphenols are not primarily acting as radical-scavenging antioxidants in vivo.

4. Evidence that polyphenols can induce mitochondrial adaptation

4.1 Polyphenols may be able to regulate Sirtuins

"Sirtuins" are a family of regulatory protein deacetylases, coded by SIRT genes. Sirtuin1 is thought to play a role in regulation of MB (see above). SIRT1 activation by a synthetic activator was found to up-regulate lipid oxidation (a pathway located in the mitochondria), suggesting potential in treatment of obesity, diabetes and metabolic disorders (Feige et al., 2008). Mice over-expressing SIRT1 showed a phenotype resembling calorie restriction, supporting the involvement of the SIRT1 pathway (and by implication, MB) in adaptations to calorie restriction (Bordone et al., 2007).

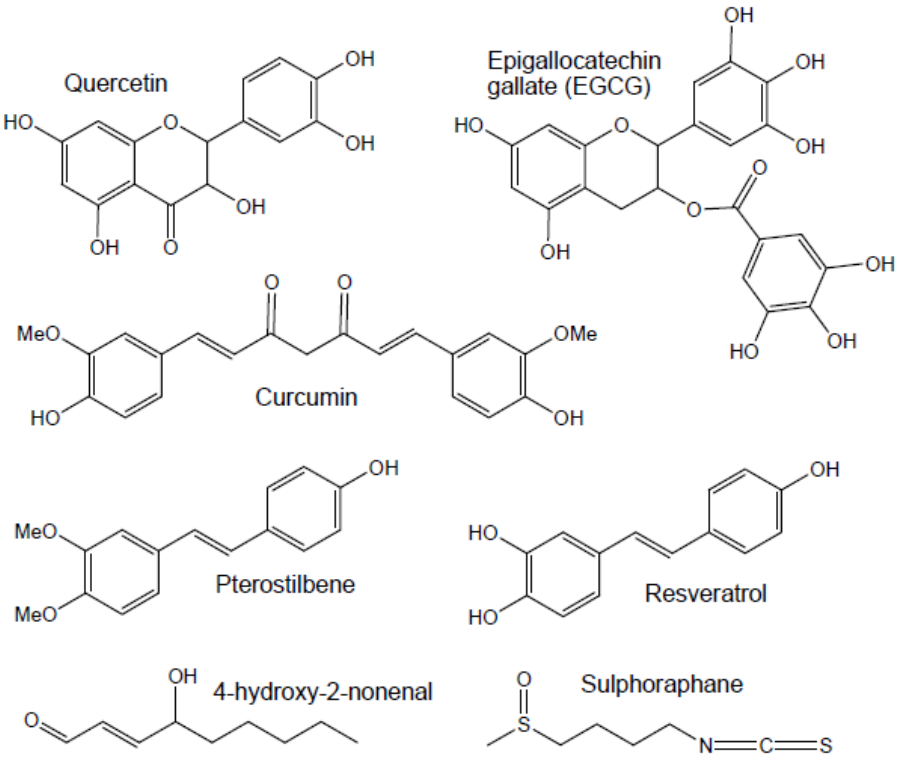

Fig. 2. Chemical structures of the compounds most often referred to in the text.

It has been demonstrated in vitro, that polyphenolics, particularly resveratrol, can enhance the activity of the recombinant human sirtuin coded by SIRT1, apparently by a conformational change to the enzyme. Resveratrol at 10 □M also extended the lifespan of yeast from ~23 to ~37 generations (Howitz et al., 2003). Chemical derivatives of resveratrol appear to be even more effective (Yang et al., 2007), suggesting that these compounds in some way decrease the DNA damage associated with aging. These enzyme-activation results have been questioned by subsequent studies (Grubisha et al., 2005; Kaeberlein et al., 2005) on the grounds that resveratrol required highly supra-physiological concentrations (a 3-fold activation at 20 □M) and a non-physiological substrate to have a measurable effect. Observations that the plasma concentration of resveratrol from a realistic dose is in the nanomolar range and that it exists in vivo almost entirely as conjugates, rather than as free resveratrol (Goldberg et al., 2003) cast further doubt on sirtuin activation as a key mechanism in vivo. It appears more likely that direct sirtuin activation is only a very minor mechanism of MB stimulation by polyphenols in vivo.

4.2 Inhibition of the ETC by polyphenols

It is well-established that inhibitors of the ETC increase ROS generation (Cadenas & Boveris, 980). It has been shown, in vitro, that flavonoids can inhibit specific mitochondrial functions, including NADH oxidase (Hodnick et al., 1986), F1-ATPase (Gledhill et al., 2007) and the membrane permeability transition (Santos et al., 1998). Other in vitro studies found that polyphenols inhibited overall mitochondrial respiration (Hodnick et al., 1987) and the closely related rate of ATP generation (Dorta et al., 2005). The former study also detected a burst of ROS generation associated with the inhibition of respiration. A wide range of compounds was tested in these studies, to the extent that structure-activity relationships were established. The two best classes of compound appeared to be stilbenes (e.g., resveratrol) and flavonols (e.g., quercetin). These findings suggest that polyphenols, if they were able to access the mitochondria in vivo, could directly but transiently increase ROS generation, thereby inducing beneficial adaptations in a similar way to exercise.

4.3 Can polyphenols access mitochondria to exert biological effects?

The ability of a compound to inhibit mitochondrial metabolism strongly suggests, but does not prove, that it can access the interior of the mitochondria. Several in vitro studies, however, have provided indirect evidence to support the potential of polyphenols to access the interior of mitochondria in vivo. In a study of the effects of treatment with EGCG on rat neuronal cells in vitro, in which the cells were fractionated to isolate the mitochondria, 90-95% of the detectable EGCG was present in the mitochondrial fraction (Schroeder et al., 2009). Similarly, quercetin was rapidly and extensively absorbed by Jurkat cells and their isolated mitochondria, as well as by the mitochondria of preloaded cells (Fiorani et al., 2010).

When isolated rat kidney mitochondria were treated with quercetin, various changes consistent with access of quercetin to the interior of the mitochondria were observed, including increased mitochondrial membrane permeability and oxygen consumption, but decreased membrane potential and oxidative phosphorylation (Ortega & Garcia, 2009). It thus appears that mitochondria would be easily able to absorb significant concentrations of polyphenols, provided the intracellular concentrations around them were high enough.

4.4 Bioavailability and access of polyphenols to mitochondria in vivo

Studies in vitro can only indicate potential for in vivo effects; to date there is very little direct evidence to support “mitochondrial bioavailability” in vivo. The many hundreds of polyphenol bioavailability studies done in vivo have only measured concentrations of polyphenols and their metabolites in plasma and/or urine; this proves only that the compound or compounds got as far as the circulatory system (Stevenson et al., 2008). A radioactive tracer approach is also unable to resolve the intracellular location of the radioactivity or the specific chemical compound involved. Studies on animals find radioactivity in all tissues in the body, but cannot distinguish between the original polyphenol, a conjugate or some much simpler breakdown product (Stevenson et al., 2008).

This approach is also unable to resolve the intracellular location of the radioactivity. Polyphenol feeding studies on pigs have found polyphenols at micromolar concentrations in a variety of tissues and organs (Bieger et al., 2008; Kalt et al., 2008; Kalt et al., 2007), but one concern with these studies is that entrained blood may not have been completely removed, thus casting doubt on the actual concentrations in the tissues. On balance, it seems probable that polyphenols can access tissues and therefore cells in vivo, but probably in the form of conjugated metabolites or breakdown products, rather than the unconjugated forms tested in vitro. We therefore have no solid evidence of the mitochondrial bioavailability in vivo of the polyphenols tested in vitro. A number of in vivo trials (discussed in later sections) have successfully detected physiological changes consistent with mitochondrial adaptation, linked to dosing with polyphenols, suggesting that polyphenols or their metabolites can in some way stimulate mitochondrial adaptation, but these trials give little information on how these effects could be mediated.

4.5 In vitro evidence for mitochondrial adaptation by polyphenols

The in vitro studies reported to date have nearly all been carried out on the polyphenols resveratrol and hydroxytyrosol. Resveratrol, at a supra-physiological concentration of 50 μM, was found to induce MB significantly and greatly up-regulate antioxidant enzyme synthesis including MnSOD in both mouse and human cell cultures (Robb et al., 2008). The critical importance of MnSOD to health and life itself has been demonstrated by several studies. Genetically modified mice that over-express this enzyme have a modestly extended lifetime (Hu et al., 2007), whereas MnSOD-knockout mice die within a few days of birth (Y. Li et al., 1995). Recombinant lactobacilli over-expressing the antioxidant enzymes SOD and CAT demonstrated greatly enhanced resistance to oxidative stress and significantly increased longevity compared with normal bacteria (An et al., 2011). Resveratrol treatment of isolated human vascular endothelial cells up-regulated many biomarkers of mitochondrial adaptation, including PPAR-□, Nrf1, TFAM, mitochondrial DNA, ETC proteins and MB. Endothelial nitric oxide synthase (eNOS) was also upregulated, but if NO synthesis was blocked, MB and the other adaptations were also blocked (Csiszar et al., 2009). These findings support the regulatory effects of eNOS/NO on mitochondria, at least in vascular cells. NO itself is well-established as an important factor in mitochondrial regulation (Cadenas et al., 2000).

Hydroxytyrosol (HT), a polyphenol found in olives and olive oil, at concentrations as low as 1 □M significantly stimulated MB and ETC complex synthesis, concomitant with upregulation of the MB-signalling molecules PGC1-□ and Nrf-1 and-2 (Hao et al., 2010). Very similar results were obtained from ARPE-19 cells, a human retinal pigment epithelial line, challenged with acrolein and in addition, HT increased expression of ERE-regulated phase II detoxifying enzymes (Lu et al., 2010). These in vitro studies provide considerable evidence that polyphenols could stimulate adaptive effects in vivo, provided they could accumulate in the cell or mitochondria at sufficient concentrations. Adaptations mediated by eNOS/NO would only require access to the vascular system, which is well-proven by numerous plasma bioavailability studies.

4.6 Other potential adaptogenic effects of polyphenols

Other adaptive effects, not necessarily related to mitochondria, have been associated with polyphenols. Quercetin glycosides stimulated glucose uptake in C2C12 mouse muscle cells in vitro (Eid et al., 2010), an effect that should be beneficial in treating type 1 diabetes, a condition thought to involve mitochondrial dysfunction (Fujisawa et al., 2009). The glycolytic pathway for ATP generation in the cytosol converts glucose to lactate (Scheffler, 2008). Lactate is converted to pyruvate, which is a major input into the TCA cycle in the mitochondria (Figure 1). Treatment with elevated concentrations of pyruvate stimulates MB in both L6E9 myoblasts and C2C12 cells (Duguez et al., 2004; Wilson et al., 2007). Induction of MB by increased pyruvate supply in vitro suggests that there could be a similar effect in vivo. Stimulation of glucose uptake by muscle cells may increase glycolysis and indirectly stimulate MB and other mitochondrial adaptations. In other words, this may be an additional mechanism for stimulation of mitochondrial adaptation by polyphenols.

4.7 Animal trials linking polyphenols with mitochondrial adaptation

Support for the in vivo effects of polyphenolics being closely related to those of exercise on mitochondria has been provided by two trials in mice, which found that high doses of dietary resveratrol (400 and 20 mg/kg/day respectively) reversed all the harmful biological changes (in particular, a shortened lifespan) induced by a high calorie diet, apart from weight gain (Lagouge et al., 2006; Baur et al., 2006). Both trials found increased activity of the mitochondrial signalling molecules SIRT1 and PGC1-□, as well as increased insulin sensitivity. Although 400 mg/kg/day is far from a practical intake for humans, the absence of any ill-effects to the test animals strongly suggests that this compound should have no toxicity in humans at any realistic dietary intake. A subsequent trial found significant increases in the major antioxidant enzymes in mice dosed with resveratrol as part of a high fat

diet (Robb et al., 2008). Of particular interest was a doubling of the activity of MnSOD in brain and kidney tissue, in agreement with the observations from in vitro trials of resveratrol.

Resveratrol and its methylated analogues were found to be effective in an in vitro cellular model of oxidative stress. One of these analogues, the naturally-occurring dimethylated derivative of resveratrol, pterostilbene, was subsequently found to reduce neurodegeneration, a major contributor to age-related cognitive decline, in a long-term rat trial (Joseph et al., 2008). Mitochondrial dysfunction has been implicated in and may be a primary cause of most common neurodegenerative conditions, including those related to aging (Scheffler, 2008). Although these trials do not provide a definitive link between the observed health benefits and resveratrol-induced MB, the results are consistent with such a link and certainly lend support to the link between polyphenols and human health benefits.

4.8 In vivo evidence for mitochondrial adaptation by polyphenols

Highly significant augmentation of exercise performance resulted from dietary supplementation with quercetin in a mouse model (Davis et al., 2009). Groups of mice were fed 0, 12.5 or 25 mg/kg/day of quercetin (approximately equivalent to a realistic dose of 1 or 2 g/day for an 80 kg human) for 7 days. Increases (relative to placebo) were observed in gene expression of PCG-1 and SIRT1 by up to 2 fold in muscle and brain, whilst levels of cytochrome C (a marker of mitochondrial mass) increased by 23% in muscle and 18% in brain. Mitochondrial DNA also increased up to 2 fold (Davis et al., 2009). This suggests that the numbers of mitochondria approximately doubled and their overall respiration capacity increased by around 20%. The importance of these results is the clear link between quercetin consumption, increases in biomarkers for mitochondrial adaptation, and MB itself. This trial is therefore, the first to demonstrate unequivocally mitochondrial adaptive effects of a common polyphenol, administered orally. Exercise trials were done on other, similarly dosed mice, in the same study. Forced treadmill running time to exhaustion increased by 37% at both doses of quercetin. Voluntary wheel running activity also increased, both in speed and in time spent. Total distance covered increased by ~45% by the end of the 7-day treatment period and was sustained for the following 7 days (Davis et al., 2009). Although these performance improvements appear spectacular, they are not unexpected. Laboratory animals are over-fed, chronically under-exercised and lack social interaction and environmental stimulation, being confined to single cages for most of their lives (Cressey, 2010). Although such animals may be poor models of human responses in most cases, they would be expected to be excellent models of obese, unfit and depressed humans who could benefit most from exercise and a healthy, high-polyphenol diet. In this light, the physical performance improvements observed in the trial undertaken by Davis and colleagues would not be expected to be reproduced in humans; much smaller changes in human fitness would be expected from the same treatment. The salient point here is that the performance enhancing effects of the quercetin are of minor relevance to human health, compared with the clear demonstration of its adaptive effects on mitochondria. In another study, resveratrol supplementation (0.2% w/w) for 12 weeks increased exercise performance in a mouse model of senescence, whereas performance declined in the control group (Murase et al., 2009). Polyphenols therefore, may have potential to ameliorate age-related physical decline in humans.

One aspect of the Davis et al. (2009) study that cannot be easily explained by mitochondrial adaptation is the large increase in voluntary wheel running. Davis and colleagues suggested that this resulted from an entirely different property of quercetin, namely that, in vitro, it is an adenosine A1 receptor antagonist, as are caffeine and some other common flavonoids (Alexander, 2006). Caffeine is both psycho-stimulatory and ergogenic and this may explain the apparently increased motivation for the mice to exercise. There is also solid evidence from human trials for adaptive effects of quercetin. A supplementary combination of quercetin (1 g), isoquercetin, EGCG and polyunsaturated fatty acids was tested on trained cyclists undergoing 3 hours of cycling on each of 3 days (Nieman

et al., 2009). Significant decreases in inflammatory biomarkers were detected relative to the control (placebo), but no change was observed in performance, or any marker of exercise or mitochondrial adaptation. The latter finding is not unexpected in trained athletes, who would be expected to have minimal capacity for increased performance or additional exercise-induced adaptation. In a further trial, 1 g/day of quercetin for two weeks was tested on untrained male test subjects (Nieman et al., 2010). Distance travelled in 12 minutes on a treadmill was determined. Relative to placebo, a small but significant increase in distance was observed, accompanied by slight increases in RNA coding for the MB biomarkers PGC-1, sirtuin 1, citrate synthase and cytochrome C oxidase (Complex IV) (Nieman et al., 2010). Similar results were obtained when Davis et al. (2009) undertook a human trial to examine whether they could replicate the effects previously observed in mice. Twelve untrained volunteers were given 500 mg of quercetin twice daily for 7 days, dissolved in a drink. After treatment, both VO2max and ride time to fatigue on an exercise bike were determined. The observed increases in VO2max and ride time to fatigue were 3.9% and 13.2%, respectively, compared with the control (placebo). Whilst no mitochondrial biomarkers were measured in this trial, the enhancement of endurance capacity observed, in the absence of any physical training, is entirely consistent with mitochondrial adaptation. One should bear in mind that the physical performance aspects of polyphenol-stimulated mitochondrial adaptation are of minor importance in the context of human health. In this respect, mitochondrial dysfunction is of much higher importance, given that it is implicated in some way in both the major diseases of modern civilisation (CVD, cancer and neurodegeneration) and the aging process (Huang & Hood, 2009). Mitochondrial adaptation, rather than antioxidant capacity, is emerging as the primary mode of action of the health benefits of dietary polyphenols. Whilst there is, to date, no evidence that polyphenol consumption can increase human lifespan, there is good evidence from animal trials (Baur et al., 2006; Lagouge et al., 2006). This suggests that humans may benefit similarly, even if only through reduction in the incidence of life-threatening diseases. It is unlikely that dietary polyphenols could have a major effect on the maximum life-span of humans, but they do appear to have great potential to increase the proportion of people who attain all or most of the maximum lifespan.

4.9 Polyphenolics, mitochondria and apoptosis

Mitochondria are the instigators of programmed cell death, or apoptosis (Dorta et al., 2006). If mitochondria are sufficiently damaged by oxidative stress and DNA damage to become dysfunctional and lose their capacity to adapt to oxidative stress, they initiate a signalling pathway leading to apoptosis, or programmed death, of the host cell and thus the demise of the defective mitochondria. This mechanism has been proposed to explain the "chemopreventive" effects of polyphenolics (Roy et al., 2009; Juan et al., 2008), based on the observation that cancer cells in vitro are more sensitive to mitochondrial-induced apoptosis than normal cells. Therefore, polyphenols may promote apoptosis of cancerous cells in vivo.

The ability to induce apoptosis was demonstrated in vitro for pterostilbene, a natural methylated derivative of resveratrol (Pan et al., 2007), resveratrol itself (Shakibaei et al., 2009), kaempherol, a flavonol similar to quercetin (Marfe et al., 2009), EGC (W. Li et al., 2009) and catechin (Iwasaki et al., 2009). This property of polyphenols may explain at least part of the putative anti-cancer effect of polyphenol-rich foods (see Introduction).

4.10 Does the antioxidant capacity of polyphenols have any role in health?

Polyphenol concentrations achieved in plasma from a realistic dietary intake are both transient and at most, ~2-4% of the small-molecule antioxidants normally present in the plasma; antioxidant enzymes contribute a large additional endogenous antioxidant capacity (Clifford, 2004; Stevenson & Lowe, 2009; Stevenson et al., 2009). In comparison, polyphenols are clearly insignificant in the overall context of resistance to oxidative stress. They could make a contribution at sites within the body where localised concentrations are much higher than the average. One example of this may be in the gastrointestinal (GI) tract, where polyphenol concentrations have been

demonstrated to be many times that achieved in plasma (Stevenson et al., 2009), a consequence of the low proportions of most polyphenols that are absorbed from the GI tract. The cells of the GI tract are thus exposed to concentrations that should be more than sufficient for a significant radical-scavenging antioxidant effect. Another possible example is in cell membranes, where in vitro studies found that up to 75% of polyphenols spiked into blood samples can end up bound to the membranes of the blood cells (Biasutto et al., 2010; Koren et al., 2010). This may allow them a significant and direct role in prevention of lipid peroxidation. If this membrane-binding phenomenon translates to the in vivo environment and is common to other cells (and to mitochondria) in the body, polyphenols may have a significant whole-body protective effect from lipid peroxidation.

5. The role of homeostasis in polyphenol action in the body

A good question to ask is why so many trials of antioxidants have failed to demonstrate any benefit and why polyphenol-induced mitochondrial adaptation appears only to be readily detected and manifested as an augmentation of exercise-induced adaptation. The answer may lie in the principle of homeostasis (van Ommen et al., 2009). Homeostasis is the normal state of a healthy cell or organism, where all biochemicals and enzymes are regulated to their optimal concentrations. When an organism is in homeostasis, dietary or pharmaceutical intervention has no particular benefit, because there is no “problem” to rectify. This may go some way towards explaining the apparent ‘failure’ of intervention trials with antioxidants. If the concentrations of endogenous antioxidants are tightly regulated, then addition of large amounts of exogenous antioxidants would result in downregulation of endogenous synthesis to restore homeostasis. Van Ommen and colleagues propose that any search for biomarkers of the effects of interventions must be undertaken with simultaneous perturbation of homeostasis, so the intervention can assist its restoration.

This is not a major consideration for pharmaceutical interventions, which are typically designed to treat disease, in which homeostasis has already been perturbed. Dietary interventions, in contrast, are usually aimed at optimisation of health, rather than treatment of disease. The search for health benefits of either dietary antioxidants or adaptogens will almost inevitably fail unless tested on subjects with pre-existing or applied oxidative stress. Oxidative stress may be applied to animals by treatment with toxins, enforced exercise, or the use of animal models of suitable disease states. For humans, the options are restricted to the use of test subjects with pre-existing conditions that elevate oxidative stress, such as metabolic syndrome, or the performance of endurance exercise by healthy subjects. If oxidative stress is applied during a trial, appropriate intervention reduces the magnitude of the perturbation (i.e., the stress) and accelerates the restoration of homeostasis.

6. Conclusion

A large body of evidence has now been accumulated to support the concept that polyphenols are primarily adaptogens rather than radical-scavenging antioxidants. This does not negate their potent capacity to reduce oxidative stress; rather it indicates that the mechanism is far more complex and subtle than previously realised. Several in vivo trials have clearly linked polyphenol interventions with actual mitochondrial adaptation, or macroscopic effects consistent with adaptation, specifically mitochondrial biogenesis (MB) and up-regulation of MB-signalling molecules and antioxidant enzymes as the main biomarkers established for detection of mitochondrial adaptation. In vitro trials have been entirely consistent in demonstrating up-regulation of the same biomarkers that provide clues to possible mechanisms of action for polyphenols. These are direct activation of the components of the MB signalling pathway (e.g., sirtuin 1 and PPAR□); direct activation of the ERE via binding to its regulatory protein Keap1; stimulation of glycolysis and glucose uptake, which increases the supply of nutrients to the mitochondria; stimulation of NO synthesis, which is a known signal for MB; and generation of a mild oxidative stress in the mitochondria through inhibition of the ETC or other mechanisms.

Since mitochondrial dysfunction is implicated in aging and major diseases such as cancer, CVD and neuro-degeneration, any means of improving mitochondrial function, or inducing destruction of the most dysfunctional mitochondria, should be highly beneficial to healthy aging and maintenance of good health. Dietary polyphenols are almost certainly a good means of achieving these ends.

7. List of abbreviations and definitions

ORAC : Oxygen radical absorbance capacity; an in vitro measure of relative antioxidant power. ROS: Reactive oxygen species; another term for free radicals. ETC: Electron transport chain; a group of mitochondrial proteins that generates ATP (adenosine triphosphate) by reduction of oxygen to water. NADH: Nicotinamide adenine dinucleotide; a biochemical reducing agent. MnSOD or SOD2: Mitochondrial form of the antioxidant enzyme, manganese superoxide dismutase. CuZnSOD or SOD1: Cytosolic form of superoxide dismutase. MB: Mitochondrial biogenesis; the increase of mitochondrial numbers within cells. Complex III: One of the five components of the mitochondrial electron transport chain (ETC). Nrf-1 and Nrf-2: Nuclear respiratory factors; transcription factors involved in control of adaptive responses. TFAM: Mitochondrial transcription factor A; transcription factor involved in control of adaptive responses. PGC-1□: Peroxisome proliferator-activated receptor gamma coactivator 1-alpha. PPAR-□: Peroxisome-proliferator activated receptor-□; receptor involved in control of adaptive responses. ERE/ARE: Electrophile (or Antioxidant) Response Element; gene promoter controlling antioxidant enzyme gene expression. HNE: 4-hydroxy-2-nonenal; product of lipid peroxidation and likely activator of the ERE. Keap1: Kelch-like ECH-associated protein 1; a sensing protein linked to the ERE. TCA cycle: Tricarboxylic acid cycle; ATP-generating metabolic pathway in mitochondria. eNOS: Endothelial nitric oxide (NO) synthase enzyme. GPX: glutathione peroxidase; antioxidant enzyme. MDA: malondialdehyde; a commonly used plasma biomarker of lipid peroxidation. SIRT: silent mating type information regulation 2 homolog; gene coding for a sirtuin, or regulatory protein deacetylase. EGCG: epigallocatechin gallate; a flavonoid polyphenol mainly found in green tea.

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Reference [9] Folia Veterinaria 2008 **52**, 3-4, 135-139

Faixova Z, Faix S

Biological effects of rosemary (Rosmarinus Officialis L.)

Many herbs and plant extracts are added to the diet not only for their aromatic properties but they have been identified as a source of various phytochemicals, many of which possess an important biological activity. Results of many experiments showed that rosemary essential oil had antimicrobial, antioxidant, anti-carcinogenic, cognition-improving and certain glucose level lowering properties which makes it useful as a natural animal feed additive. This review describe the most important biological activities of rosemary (Rosmarinus Officinalis L .) essential oil in animals and humans. I n vitro and in vivo effects of rosemary essential oil are discussed.

Reference [10] Life Sciences 2011 **7**, 89 19-20 708-16 21945192

Drira R, Chen S, Sakamoto K

Oleuropein and hydroxytyrosol inhibit adipocyte differentiation in 3 T3-L1 cell

AIMS Oleuropein and hydroxytyrosol, which are antioxidant molecules found in olive leaves and oil, have been reported to exert several biochemical and pharmacological effects. These polyphenols are able to prevent low-density lipoprotein oxidation and protect cells against several diseases. Here, we studied the effect of these compounds on adipocyte differentiation in 3 T3-L1. MAIN METHODS To perform this study, 3 T3-L1 preadipocytes viability was analysed via Trypan blue and MTT assays, and triglycerides were stained with Oil Red O. Adipogenesis related genes expression were checked by RT-PCR and qRT-PCR. Also, cells counting and flow cytometry were used to analyse the mitotic cell cycle during the adipogenesis clonal expansion phase. RESULTS Oleuropein and hydroxytyrosol dose-dependently suppressed intracellular triglyceride accumulation during adipocyte differentiation without effect on cell viability. PPARγ, C/EBPα and SREBP-1c transcription factors and their downstream targets genes (GLUT4, CD36 and FASN) were down-regulated after treatment by oleuropein and hydroxytyrosol. At 200 and 300 μmol/L oleuropein or 100 and 150 μmol/L hydroxytyrosol, the greatest effect on the adipogenesis process was observed during the early stages of differentiation. Flow cytometry revealed both polyphenols to inhibit the division of 3T3-L1 preadipocytes during mitotic clonal expansion and cause cell cycle delay. Furthermore, oleuropein and its derivate hydroxytyrosol decreased the transcriptional activity of SREBP-1c in a stable transfected 3T3-L1 cell line. SIGNIFICANCE These findings indicate that both compounds are able to prevent 3T3-L1 differentiation by inhibition of the mitotic clonal expansion and downregulation of the adipogenesis related genes.

Reference [11] Biosci Trends 2011 **5**(1) 23-9

Tian FF, Zhang FF, Lai XD, Wang LJ, Yang L, Wang X, Singh G, Zhong JL
Nrf2-mediated protection against UVA radiation in human skin keratinocytes

Ultraviolet A (UVA, 320-400 nm) radiation is an oxidizing agent that causes significant damage to cellular components and that leads to photoaging and cancer. It strongly induces NF-E2-related factor 2 (Nrf2) expressions in cultured FEK4 human skin fibroblasts but weakly induces it in transformed HaCaT skin keratinocytes. Nrf2 silencing increases cell damage at a moderate dose of UVA irradiation (250 kJ•m(-2)) in FEK4 fibroblasts, but whether a decrease in Nrf2 sensitizes HaCaT keratinocytes to a moderate to high dose (250-500 kJ•m(-2)) of UVA irradiation (i.e., 400 kJ•m(-2), peak emission 365 nm) is currently unknown. A moderate to high dose of UVA irradiation only slightly increased Nrf2 expression in HaCaT skin keratinocytes. Knockdown of Nrf2 by specific silencing of Nrf2 (siNrf2) strongly increased cell damage as gauged by membrane damage (LDH) and cell viability (MTT assay) following this dose of UVA irradiation. These results suggest that decreased Nrf2 significantly increased UVA irradiation-induced cell damage in skin keratinocytes. Nrf2 may play a role in protecting human skin keratinocytes from UVA radiation-induced damage.

Reference [12] European Journal of Clinical Nutrition 2009 **63**, 1387-1393

Razquin C, Martinez JA, Martinez-Gonzalez MA, Mitjavila MT, Estruc R, Marti A
A 3 years follow-up of a Mediterranean diet rich in virgin olive oil is associated with high plasma antioxidant capacity and reduced body weight gain

The aim of this study was to analyze the influence of a Mediterranean dietary pattern on plasma total antioxidant capacity (TAC) after 3 years of intervention and the associations with adiposity indexes in a randomized dietary trial (PREDIMED trial) with high cardiovascular risk patients.

187 subjects were randomly selected from the PREDIMED-UNAV center after they completed 3-year intervention program. Participants were following a Mediterranean-style diet with high intake of virgin olive oil or high intake of nuts, or a conventional low-fat diet. Adiposity indexes were measured at baseline and at year 3. Plasma TAC was evaluated using a commercially available colorimetric assay kit.

Results: sma TAC in the control, olive oil and nuts groups was 2.01±0.15, 3.51±0.14 and 3.02±0.14 mM Trolox, respectively after adjusting for age and sex. The differences between the Mediterranean diet and control groups were statistically significant (P<0.001). Moreover higher levels of TAC were significantly associated with a reduction in body weight after 3 years of intervention among subjects allocated to the virgin olive oil group (B=−1.306; 95% CI=−2.439 to −0.173; P=0.025, after adjusting for age, sex and baseline body mass index).

Mediterranean diet, especially rich in virgin olive oil, is associated with higher levels of plasma antioxidant capacity. Plasma TAC is related to a reduction in body weight after 3 years of intervention in a high cardiovascular risk population with a Mediterranean-style diet rich in virgin olive oil.

Reference [13] Biochemical Pharmacology 1994 **47**(11), 2063–2068

Zhang K, Das NP

Inhibitory effects of plant polyphenols on rat liver glutathione S-transferases

Several novel naturally occurring flavonoids and other polyphenols exerted varying degrees of concentration-dependent inhibition on uncharacterized rat liver glutathione S-transferase (EC 2.5.1.18, GST) isoforms. The order of inhibitory potencies of the five most potent polyphenols was tannic acid > 2-hydroxyl chalcone > butein > morin > quercetin, and their IC50 values were 1.044, 6.758, 9.033, 13.710 and 18.732 μM, respectively. Their inhibitions were reversible, as indicated by dialysis experiments. The optimum pH for the inhibitions by four of the compounds (tannic acid, butein, 2-hydroxyl chalcone and morin) was in the range of pH 6.0 to 6.5, but for quercetin the optimum pH was 8.0. These potent inhibitors possess one or more of the following chemical structural features: (a) polyhydroxylation substitutions, (b) absence of a sugar moiety, (c) for the chalcones, the presence of an open C-ring and hydroxylation at either the C-2 or C-3 position, (d) for the flavonoids, the attachment of the B-ring to C-2, and (e) a double bond between C-2 and C-3. Butein exhibited a non-competitive inhibition toward both glutathione (GSH) and 1-chloro-2,4-dinitrobenzene (CDNB). Interestingly, tannic acid showed a non-competitive inhibition toward CDNB but a competitive inhibition toward GSH. The inhibitory potency of tannic acid on rat liver GSTs was concentration and substrate dependent. Using CDNB, p-nitrobenzyl chloride, 4-nitropyridine-N-oxide, and ethacrynic acid as substrates, the IC50 values for tannic acid were 1.044, 11.151, 20.206, and 57.664 μM, respectively.

Reference [14] Food Chemistry 2000 **68**(4), 457-462

Benavente-Garcia O, Castillo J, Lorente J, Ortuno A, Del Rio JA

Antioxidant activity of phenolics extracted from Olea europaea L. leaves

The purpose of this study was to identify the main phenolic compounds present in an oliveleaf extract (OL) in order to delineate the differential antioxidant activities of these compounds through the extent of their abilities to scavenge the $ABTS^{\bullet+}$ radical cation and to clarify the structural elements conferring antioxidant capacity in aqueous systems. The results show that the relative abilities of the flavonoids from oliveleaf to scavenge the $ABTS^{\bullet+}$ radical cation are influenced by the presence of functional groups in their structure, mainly the B-ring catechol, the 3-hydroxyl group and the 2,3-double bond conjugated with the 4-oxo function. For the other phenolic compounds present in OL, their relative abilities to scavenge the $ABTS^{\bullet+}$ radical cation are mainly influenced by the number and position of free hydroxyl groups in their structure. Also, both groups of compounds show synergic behaviour when mixed, as occurs in the OL.

Reference [15] Biochemical and Biophysical Research Communications 2009 **382**(3), 549-554

Takahashi T, Tabuchi T, Tamaki Y, Kosaka K, Takikawa Y, Satoh T

Carnosic acid and carnosol inhibit adipocyte differentiation in mouse 3T3-L1 cells through induction of phase2 enzymes and activation of glutathione metabolism

In the previous studies, we reported that carnosic acid (CA) and carnosol (CS) originating from rosemary protected cortical neurons by activating the Keap1/Nrf2 pathway, which activation was initiated by *S*-alkylation of the critical cysteine thiol of the Keap1 protein by the "electrophilic" *quinone*-type of CA or CS. Here, we found that CA and CS inhibited the *in vitro* differentiation of mouse preadipocytes, 3T3-L1 cells, into adipocytes. In contrast, other physiologically-active and rosemary-originated compounds were completely negative. These actions seemed to be mediated by activation of the antioxidant-response element (ARE) and induction of phase2 enzymes. This estimation is justified by our present findings that only CA and CS among rosemary-originated compounds significantly activated the ARE and induced the phase2 enzymes. Next, we performed cDNA microarray analysis in order to identify the gene(s) responsible for these biological actions and found that phase2 enzymes (Gsta2, Gclc, Abcc4, and Abcc1), all of which are involved in the metabolism of glutathione (GSH), constituted 4 of the top 5 CA-induced genes. Furthermore, CA and CS, but not the other compounds tested, significantly increased the intracellular level of total GSH. Thus, we propose that the stimulation of GSH metabolism may be a critical step for the inhibition of adipocyte differentiation in 3T3-L1 cells and suggest that pro-electrophilic compounds such as CA and CS may be potential drugs against obesity-related diseases.

Reference [16] Mutation Research/Genetic Toxicology and Environmental Mutagenesis 2011 **723**(2), 165–170

Anter J, Fernandez-Bedmar Z, VIllatoro-Pulido M, Demyda-Peyras S, Moreno-Millan M, Alonso-Moraga A, Munoz-Serrano A, Luque de Castro MD

A pilot study on the DNA-protective, cytotoxic, and apoptosis-inducing properties of olive-leaf extracts

Leaves of olive trees are an abundant raw material in the Mediterranean basin. They contain large amounts of potentially useful phytochemicals and could play beneficial roles in health care. In the present study, the principal bioactive phenols in olive-leaf extracts (OLEs) have been identified and quantified, and their genotoxic/antigenotoxic, cytotoxic and apoptotic effects have been assessed.

The Somatic Mutation and Recombination Test (SMART) in wing imaginal discs of Drosophila melanogaster has been performed to test the possible genotoxicity of overall OLE and the individual components oleuropein and luteolin at different concentrations. The same assay was able to detect antigenotoxic activity against hydrogen peroxide as oxidative genotoxicant. None of the extracts/phenols tested showed significant mutagenic activity. This fact, together with the antigenotoxic activity against H2O2 detected for all these extracts/phenols, confirmed the safety of OLE, oleuropein and luteolin in terms of DNA protection.

HL60 human promyelocytic leukemia cells were used to assess the cytotoxic effects of the extracts/phenols. OLE, oleuropein and luteolin showed a dose-dependent cytotoxic effect with different IC50 (10 µl/ml, 170 µM, and 40 µM, respectively). DNA fragmentation patterns and cell staining with acridine orange and ethidium bromide indicated that the mechanism for the cytotoxic effect of OLE, oleuropein and luteolin was the apoptotic pathway, with DNA laddering and cytoplasmic and nuclear changes.

These results could help explain the mechanism of action that underlies the beneficial effect of OLE, proposed as a nutraceutical in the prevention of human cancer.

Reference [17] Trends in Food Science & Technology 2000 **11**(9-10), 357-363

Saija A, Uccella N

Olive biophenols: functional effects on human wellbeing

With increasing interest in novel descriptors of hedonic-sensory (HS) and functional (F) quality, scientific documentation of the dietary habits associated with the Mediterranean Aliment Culture (MAC) lifestyle, shows low risk for many chronic diseases. This has been interpreted as the F effect of widespread plant antioxidant intake. The antioxidant and antimicrobial activity of some of the most typical biophenols (BPs) contained in table olives (TOs) and olive oil, such as extra virgin olive oil (EVOO), was revealed through biomimetic experiments on the scavenging effects of chain-propagating lipid peroxyl radicals within membranes, and for human skin protection. Dietary intake of TO and EVOO BPs might lower the risk of degenerative diseases and microbial infections for consumers, *Homo consumans* (*Hc*). MAC foodstuffs, also referred to as 'life-stage foods', could emerge as 'F products', engineered to tackle the specific dietary requirements of the aged population.

Reference [18] Cancer Letters 1996 **100**(1-2), 139–144

Singletary KW

Rosemary extract and carnosol stimulate rat liver glutathione-S-transferase and quinone reductase activities

The effects of dietary intake and intraperitoneal (i.p.) administration of an extract of the spice rosemary and of the rosemary constituent carnosol on the liver activities of glutathione-*S*-transferase (GST) and NAD(P)H-quinone reductase (QR) in the female rat were evaluated. Rosemary extract at concentrations from 0.25 to 1.0% (by wt.) in the diet resulted in a significant 3.5- to 4.5-fold increase in liver GST and a 3.3- to 4.0-fold increase in liver QR activities compared to controls. Carnosol supplemented in the diet at levels from 0.01 to 1.0% did not enhance GST activity. When rosemary extract and carnosol were administered i.p. there was a significant increase in liver GST and QR activities. The injection of rosemary extract (200 mg/kg) was associated with 1.5-fold and 3.2-fold increases in GST and QR activities, respectively, compared to controls. The injection of carnosol at doses from 100

to 400 mg/kg was associated with 1.6- to 1.9-fold increases in GST activity and 3.1- to 4.8-fold increases in QR activity, compared to controls. These data indicate that rosemary extract in the diet or injected i.p. and carnosol administered i.p. are effective enhancers of the in vivo activity of liver GST and QR in the female rat.

Reference [19] Experimental Gerontology 2009 **44**(6-7) 383-389

Posadas SJ, Caz V, Largo C, De la Gandara B, Matallanas B, Reglero G, De Miguel E
Protective effect of supercritical fluid rosemary extract, Rosmarinus officinalis, on antioxidants of major organs of aged rats

Rosemary leaves, “Rosmarinus officinalis”, possess a variety of antioxidant, anti-tumoral and anti-inflammatory bioactivities. We hypothesized that rosemary extract could enhance antioxidant defenses and improve antioxidant status in aged rats.

This work evaluates whether supplementing their diet with supercritical fluid (SFE) rosemary extract containing 20% antioxidant carnosic acid (CA) reduces oxidative stress in aged rats.

Aged Wistar rats (20 months old) were included in the study. Rats were fed for 12 weeks with a standard kibble (80%) supplemented with turkey breast (20%) containing none or one of two different SFE rosemary concentrations (0.2% and 0.02%). After sacrifice, tissue samples were collected from heart and brain (cortex and hippocampus). Enzyme activities of catalase (CAT), glutathione peroxidase (GPX), superoxide dismutase (SOD) and nitric oxide synthase (NOS) were quantitatively analyzed. Lipid peroxidation and levels of reactive oxygen species (ROS) were also determined.

Rosemary decreased lipid peroxidation in both brain tissues. The levels of catalase activities in heart and cortex were decreased in the rosemary-treated groups. The SFE rosemary-treated rats presented lower NOS levels in heart and lower ROS levels in hippocampus than the control rats.

Supplementing the diet of aged rats with SFE rosemary extract produced a decrease in antioxidant enzyme activity, lipid peroxidation and ROS levels that was significant for catalase activity in heart and brain, NOS in heart, and LPO and ROS levels in different brain tissues. These observations suggest that the rosemary supplement improved the oxidative stress status in old rats.

Reference [20] Toxicology and Applied Pharmacology 2007 **225**(2) 214-220

Palmeira CM, Rolo AP, Berthiaume J, Bjork JA, Wallace KB
Hyperglycemia decreases mitochondrial function: The regulatory role of mitochondrialbiogenesis

Increased generation of reactive oxygen species (ROS) is implicated in “glucose toxicity” in diabetes. However, little is known about the action of glucose on the expression of transcription factors in hepatocytes, especially those involved in mitochondrial DNA (mtDNA) replication and transcription. Since mitochondrial functional capacity is dynamically regulated, we hypothesized that stressful conditions of hyperglycemia induce adaptations in the transcriptional control of cellular energy metabolism, including inhibition of mitochondrialbiogenesis and oxidative metabolism. Cell viability, mitochondrial respiration, ROS generation and oxidized proteins were determined in HepG2 cells cultured in the presence of either 5.5 mM (control) or 30 mM glucose (high glucose) for 48 h, 96 h and 7 days. Additionally, mtDNA abundance, plasminogen activator inhibitor-1 (PAI-1),

mitochondrial transcription factor A (TFAM) and nuclear respiratory factor-1 (NRF-1) transcripts were evaluated by real time PCR. High glucose induced a progressive increase in ROS generation and accumulation of oxidized proteins, with no changes in cell viability. Increased expression of PAI-1 was observed as early as 96 h of exposure to high glucose. After 7 days in hyperglycemia, HepG2 cells exhibited inhibited uncoupled respiration and decreased MitoTracker Red fluorescence associated with a 25% decrease in mtDNA and 16% decrease in TFAM transcripts. These results indicate that glucose may regulate mtDNA copy number by modulating the transcriptional activity of TFAM in response to hyperglycemia-induced ROS production. The decrease of mtDNA content and inhibition of mitochondrial function may be pathogenic hallmarks in the altered metabolic status associated with diabetes.

Reference [21] Mitochondria and Calcium in Health and Disease 2008 **44**(1), 24–35

Diaz F, Moraes CT

Mitochondrialbiogenesis and turnover

Mitochondrialbiogenesis is a complex process involving the coordinated expression of mitochondrial and nuclear genes, the import of the products of the latter into the organelle and turnover. The mechanisms associated with these events have been intensively studied in the last 20 years and our understanding of their details is much improved. Mitochondrialbiogenesis requires the participation of calcium signaling that activates a series of calcium-dependent protein kinases that in turn activate transcription factors and coactivators such as PGC-1α that regulates the expression of genes coding for mitochondrial components. In addition, mitochondrialbiogenesis involves the balance of mitochondrial fission–fusion. Mitochondrial malfunction or defects in any of the many pathways involved in mitochondrialbiogenesis can lead to degenerative diseases and possibly play an important part in aging.

8. CERTIFICATES

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No. 12097796001-01 1/1
October 05, 2012

CERTIFICATE OF ANALYSIS

Client: Mayor Invivo
Genopole, 5 rue Henri Desbrueres 91030 Evry cedex-France

Sample name: ORISOD

Received date: September 26, 2012

This is to certify that the following result(s) have been obtained from our analysis on the above-mentioned sample(s) submitted by the client.

Test Result(s)

Test Item	Result	QL	N	M
Arsenic (as As_2O_3)	Not detected	0.1 ppm		1
Lead	Not detected	0.05 ppm		1
Cadmium	Not detected	0.01 ppm		1
Mercury	0.01 ppm	.....		2

QL: Quantitation limit N: Notes M: Method
Method

JAPAN FOOD RESEARCH LABORATORIES

T. Arai
Takeko Arai
Principal Investigator

Oct. 05, 2012
Date

財団法人 日本食品分析センター

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