HYDROXYTYROSOL

A VERSATILE ANTIOXIDANT FROM OLIVE OIL

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A VERSATILE ANTIOXIDANT FROM OLIVE OIL

Proefschrift

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Mooi zijn de stromen
die komen uit de bron van het hart

(Bert Schierbeek)

CONTENTS

LIST OF ABBREVIATIONS

1RM	one repetition maximum
8-iso-$PGF_{2\alpha}$	8-isoprostane
ARE	antioxidant responsive element
AUC	area under the curve
CAT	catalase
CCh	carbachol
cGMP	cyclic guanosine monophosphate
CHD	coronary heart disease
CHP	cumene hydroperoxide
COPD	chronic obstructive pulmonary disease
CuZnSOD	copper-zinc superoxide dismutase
DTNB	5,5′-dithiobis-2-nitrobenzoic acid
ELISA	enzyme-linked immunosorbent assay
E_{max}	maximal effect
eNOS	endothelial nitric oxide synthase
GC	guanylyl cyclase
GPX	glutathione peroxidase
GSH	glutathione
GSSG	glutathione disulphide
GST	glutathione S-transferase
GTP	guanosine triphosphate
H_2O_2	hydrogen peroxide
HOCl	hypochlorous acid
HDL	high density lipoprotein
LDH	lactate dehydrogenase
IL-6	interleukin-6
IL-10	interleukin-10
LDL	low density lipoprotein
L_o	optimal length
LOOH	lipid hydroperoxide
LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinase
MDA	malondialdehyde
MnSOD	manganese superoxide dismutase
MUFA	mono-unsaturated fatty acid
NAC	N-acetylcysteine
NADPHox	nicotinamide adenine dinucleotide phosphate oxidase
NF-κB	nuclear factor kappa B
$NO^{\bullet}$	nitric oxide radical
$O_2^{\bullet-}$	superoxide radical

$OH^{\bullet}$	hydroxyl radical
OMWW	olive oil mill wastewater
ONOOH	peroxynitrite
PBMC	peripheral blood mononuclear cell
pD_2	negative logarithm of the molar concentration that produces half-maximal effect
PE	phenylephrine
$P_{o/w}$	octanol water partitioning coefficient
PUFA	poly-unsaturated fatty acid
RNS	reactive nitrogen species
RONS	reactive oxygen and nitrogen species
ROS	reactive oxygen species
RYR	ryanodine receptor
SOD	superoxide dismutase
SSA	sulphosalicylic acid
$t_{1/2}$	half life
TBARS	thiobarbituric acid-reacting substances
TBS	tris-buffered saline
TBS-T	tris-buffered saline containing Triton X-100
TCA	trichloroacetic acid
TEAC	trolox equivalent antioxidant capacity
TNFα	tumor necrosis factor alpha
X	xanthine
XO	xanthine oxidase

CHAPTER 1

General introduction

OLIVE OIL

Origin of olive oil

Olive oil is obtained from the fruits of Olea Europea, a traditional tree crop of the Mediterranean Basin. The annual worldwide olive oil production is estimated at about 2,000,000,000 kg (1, 2). It can be calculated that, when all bottles of olive oil annually produced are placed in a row, this would correspond with a distance of approximately 15 times the circumference of the earth.
The Mediterranean countries supply about 95% of the world olive oil production, 75% of which comes from the European Union (mostly Spain, Italy and Greece) (2). The production is now expanding to non-traditional producers such as the USA, South America and Australia.

Olive oil consumption

Greece, Spain and Italy have the highest per capita consumption of olive oil worldwide, ranging from 10-20 kg per capita per year (3). The average intake of olive oil is highest in Greece, i.e. approximately 54 g per capita per day. In contrast, the consumption of olive oil is much lower in Northern and Western European countries. For example, in Denmark the average consumption of olive oil is only 1 g per capita per day (3).
Consumption of olive oil is increasing in non-Mediterranean areas such as the USA and Australia. This is due to the growing interest in the health benefits of the Mediterranean diet, of which olive oil is the principal source of fat.

Composition and classification of olive oil

Olive oil is abundant in oleic acid, a mono-unsaturated fatty acid (MUFA) (18:1n-9), which ranges from 55 to 83% of total fatty acids, while the polyunsaturated fatty acid (PUFA) linoleic acid (18:2n-6) is present in concentrations between 3 and 21% (4).
Depending on its degree of acidity, olive oil is classified into different grades that serve as guidelines for the consumer in the choice of the preferred kind of oil. The oil's acidity, which is determined by its free oleic acid content, is a measure of the degradation of the oil.
The most valuable kind of olive oil is the extra-virgin one, obtained from intact olives that are quickly processed and cold-pressed without additional treatment. In this way, degradation of triglycerides is minimized. Extra-virgin olive oil has a maximum acidity of no more than 1% (1 g free oleic acid per 100 g oil). Virgin olive oil is a somewhat lower quality type of olive oil; its acidity

may not exceed 1.5%. Refined olive oil means that the oil has been treated to neutralize the acid content and is commonly regarded as a lower quality type of olive oil, compared to (extra) virgin olive oil. Extra-virgin olive oil is judged to have a superior taste, compared to virgin and refined olive oil.
(Extra) virgin olive oil contains several minor constituents such as vitamin E, β-carotene, flavonoids and other phenolic compounds.

OLIVE OIL PHENOLIC COMPOUNDS

Olive oil phenolic content

The amount of phenolic compounds in olive oil depends on several factors, including cultivar, climate, degree of maturation of the olives, manufacturing process, and storage conditions. The major phenolic compounds present in olive oil are hydroxytyrosol, tyrosol and oleuropein (Figure 1).

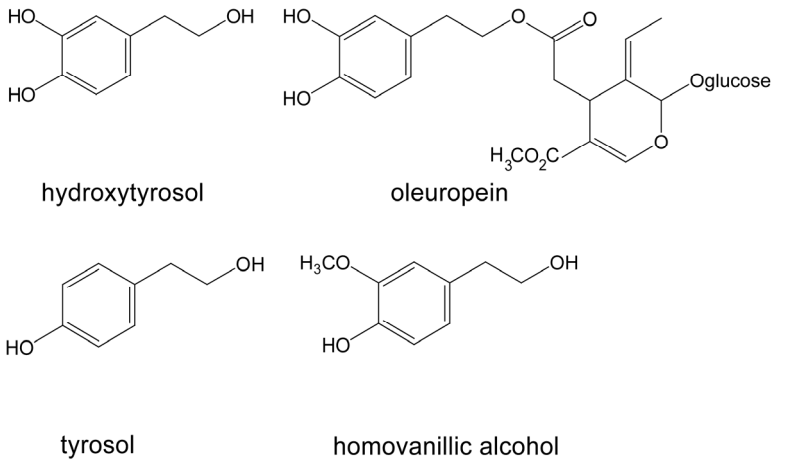

Figure 1. Molecular structure of hydroxytyrosol, oleuropein, tyrosol and homovanillic alcohol.

The total concentration of phenolic compounds in extra-virgin olive oil has been reported to vary between 50 and 800 mg/kg (5). Owen *et al*. reported a mean concentration of phenolic compounds of 230 mg/kg in extra virgin olive oil (6). In refined olive oil, the mean phenolic content is significantly lower (6). The amount of oleuropein in olive oil is relatively small. However, relatively high amounts of oleuropein are present in olives (4).

Olive oil mill wastewater (OMWW)

During the production of olive oil, large quantities of OMWW are produced. It is estimated that approximately 2,000,000,000 litre of OMWW is annually produced in Greece, currently disposed in evaporation tanks during the olive oil production season (1). This corresponds with approximately 40% of the annual domestic consumption of drinking water in Maastricht, the Netherlands (approximately 120,000 inhabitants).
OMWW has a higher content of phenolic compounds than olive oil itself. Concentrations up to 4,000 mg/l (7) and 11,000 mg/l (8) of total polyphenols in OMWW have been reported. The amount of antioxidants in olive oil is only 1-2% of the available pool of antioxidants in the olive fruit. The remainder is lost either in OMWW (approximately 53%) or in the pomace (approximately 45%) (1).
OMWW constitutes a serious environmental problem, as it has severe negative impact on soil and water quality (8, 9). In addition to the large volume of OMWW produced, the high content of phenolic compounds is regarded as one of the major factors of the environmental problems imposed by OMWW (1).

Recovery of phenolic compounds from OMWW

The recovery of phenolic compounds from OMWW is very valuable, not only from an environmental point of view, but also because these compounds might be useful in the pharmaceutical, food and cosmetic industry. Techniques are being optimized to extract phenolic compounds from OMWW (1, 8). Supplements with health promoting claims are currently being developed from OMWW. The content of phenolic compounds present in some of these extracts is 300-500 times higher than in olive oil itself. In this way, a waste pollutant is turned into a health promoting product.

Beneficial cardiovascular effects of olive oil phenolic compounds

Epidemiological evidence has shown that the incidence of coronary heart disease (CHD) is lower in Mediterranean countries than in Western-European and Northern-European countries (10). The Mediterranean diet is thought to significantly contribute to the protection against CHD. The main characteristics of the traditional Mediterranean diet include an abundance of plant foods (fruits, vegetables, whole-grain cereals, nuts and legumes), a high consumption of olive oil, a low-to-moderate consumption of fish and poultry, a low consumption of red meat, and a moderate consumption of wine (11) (Figure 2).

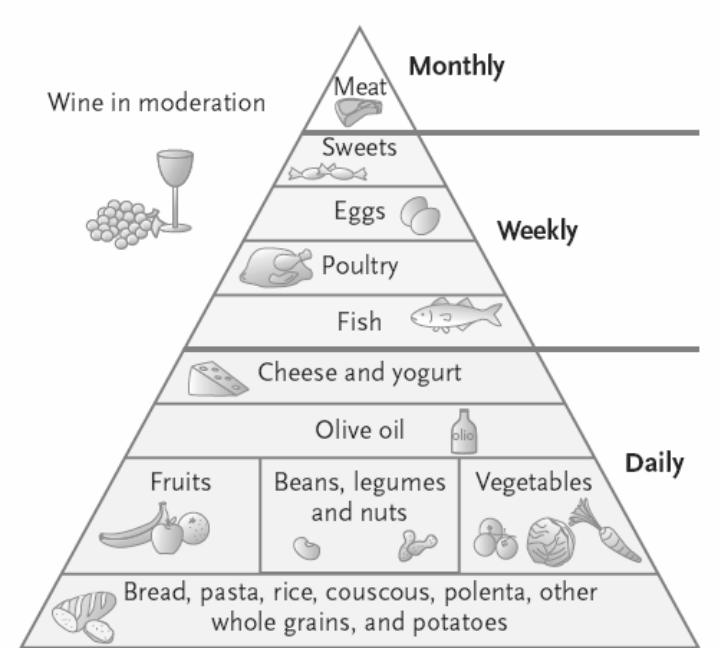

Figure 2. Olive oil is the principal source of fat and one of the key components of the traditional Mediterranean diet.
Adapted from Hu et al. N Engl J Med. 2003;348(26):2595-6.

Of the components of the Mediterranean diet, in particular olive oil has gained a lot of interest. The consumption of olive oil has been shown to provide cardiovascular health benefits such as favourable effects on cholesterol regulation and low density lipoprotein (LDL) oxidation (12). The beneficial effects of olive oil on CHD are often attributed to its high MUFA content. However, phenolic compounds present in virgin olive oil have been shown to provide additional beneficial cardiovascular effects (13-16).

ANTIOXIDANT PROPERTIES OF OLIVE OIL

Of the phenolic compounds present in olive oil and OMWW, especially hydroxytyrosol has gained a lot of attention. Research on hydroxytyrosol is particularly focused on its antioxidant properties, such as the scavenging potential of reactive oxygen and nitrogen species (RONS).

Reactive oxygen and nitrogen species (RONS)

RONS are continuously produced in the body and function as essential mediators in several vital processes including smooth muscle relaxation and the

respiratory burst to kill invading micro-organisms. Physiological important reactive oxygen species (ROS) include superoxide radical ($O_2^{\bullet -}$), hydroxyl radical ($OH^{\bullet}$), hydrogen peroxide (H_2O_2), and hypochlorous acid (HOCl). Examples of physiological important reactive nitrogen species (RNS) include nitric oxide ($NO^{\bullet}$) and peroxynitrite (ONOOH). The reactivity of H_2O_2 itself is relatively low. However, H_2O_2 can be converted to other reactive species, such as $OH^{\bullet}$.

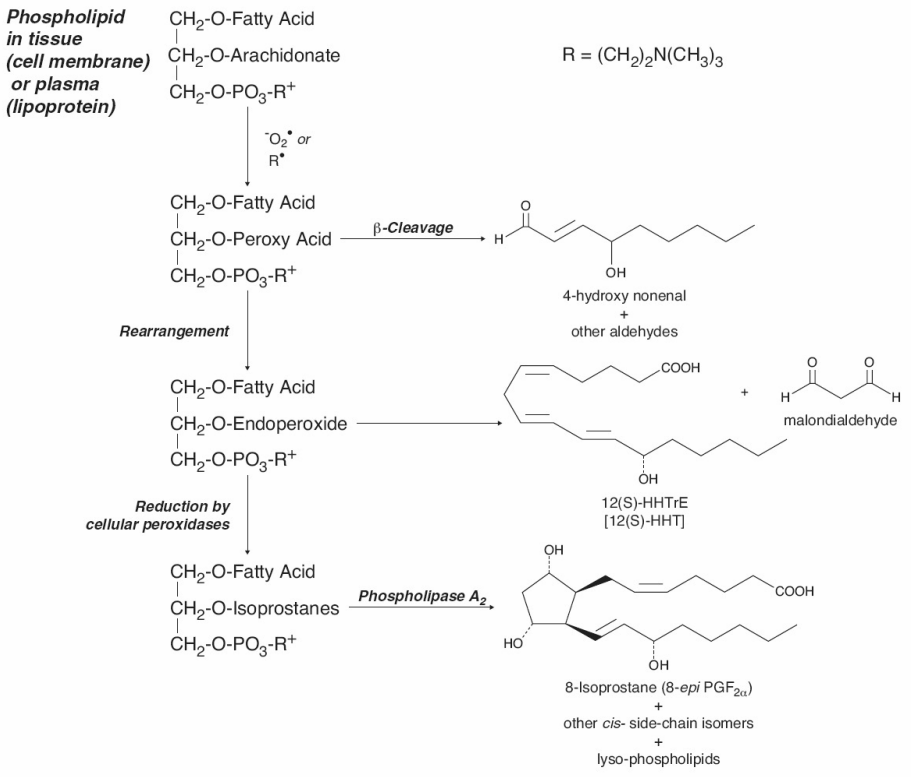

Figure 3. The formation of 8-isoprostane.
Adapted from Cayman Chemical.

PUFAs, present for example in cell membranes, are important targets of oxidation by RONS (17). Initiation of this reaction involves the subtraction of a hydrogen atom from the attacked PUFA, thereby leaving an unpaired electron on the lipid molecule. This newly-formed lipid radical will rapidly react with oxygen, creating a peroxyl radical. Subsequently, this peroxyl radical will be converted into a lipid hydroperoxide and generate a new lipid radical by subtracting a hydrogen atom from a second PUFA. This chain reaction, referred to as lipid peroxidation, takes place by continuously passing the unpaired

electron from one molecule to another. The free radical chain reaction will continue to propagate until quenched by an antioxidant. In Figure 3 is shown the formation of 8-isoprostane (8-iso-$PGF_{2\alpha}$) from arachidonic acid during peroxidation. It has been found that 8-iso-$PGF_{2\alpha}$ is a good biomarker for lipid peroxidation.
Besides lipid peroxidation, damage caused by RONS might also lead to DNA lesions, functional loss of enzymes, disturbed cell signalling, and, eventually, necrotic cell death or apoptosis.

Protection against RONS

Fortunately, the body comprises an elaborate antioxidant defence system which provides protection against RONS. The antioxidant system can be subdivided into enzymatic and non-enzymatic antioxidants. Important enzymatic antioxidants are superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX). SOD catalyzes the dismutation of $O_2^{\bullet-}$ to H_2O_2 and oxygen (O_2). CAT and GPX catalyze the conversion of H_2O_2 into H_2O. Important non-enzymatic antioxidants include the hydrophilic vitamin C and glutathione (GSH), and the hydrophobic vitamin E.
In case of a diet rich in vegetables and fruits, the endogenous antioxidant system provides sufficient protection against RONS (18). However, when the production of RONS overwhelms the protection provided by antioxidants, a situation called oxidative stress arises. Oxidative stress is associated with the pathophysiology of various diseases, e.g. cardiovascular diseases (19) and lung diseases such as chronic obstructive pulmonary disease (COPD) (20).
The supplementation of exogenous, dietary antioxidants might reduce oxidative stress and associated damage. Hydroxytyrosol might be an interesting compound to empower the antioxidant defence system in situations of oxidative stress. Important aspects that should be considered regarding the use of hydroxytyrosol as a supplement include not only its antioxidant potency, but also its bioavailability.

Antioxidant properties of hydroxytyrosol in vitro

Several studies on antioxidant properties of hydroxytyrosol have been performed. Deiana *et al.* have shown that hydroxytyrosol is able to scavenge ONOOH and therefore prevents ONOOH dependent DNA damage and tyrosine nitration (21). These results are in accordance with those of de la Puerta *et al.* who have also shown that hydroxytyrosol is able to scavenge ONOOH (22). Regarding the $O_2^{\bullet-}$ radical scavenging potential of hydroxytyrosol some controversies exist (23, 24). Hydroxytyrosol has been shown to provide protection against oxidative stress-induced damage to several types of

cells, such as intestinal epithelial cells (25), erythrocytes (26) and hepatocytes (27).

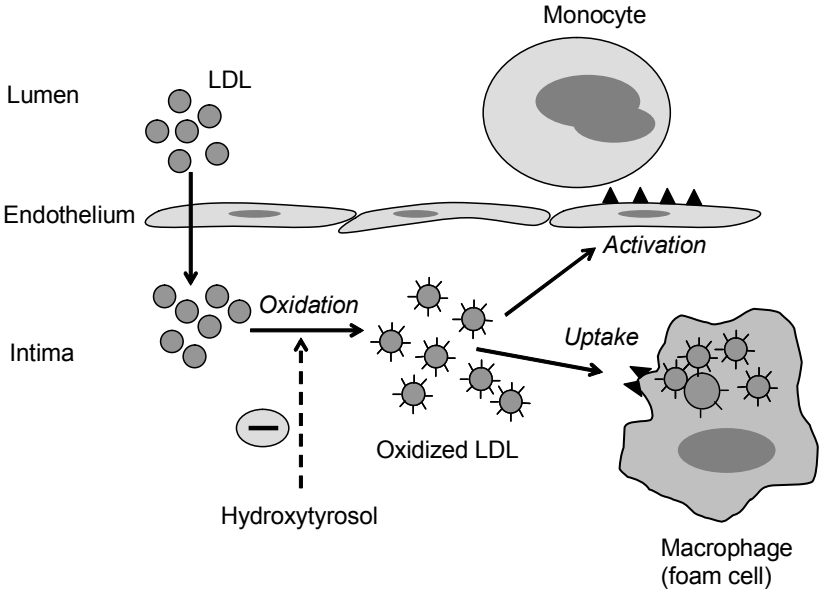

Figure 4. LDL oxidation and uptake of oxidized LDL in macrophage, one of the key steps in the initiation of atherosclerosis. Oxidation of LDL can activate endothelial cells to express leukocyte adhesion molecules. This causes blood cells such as monocytes rolling along the vascular surface to adhere to the site of activation. Monocytes enter the intima and turn into macrophages. The modified LDL particles are taken up by scavenger receptors of macrophages, which evolve into foam cells. Hydroxytyrosol prevents the oxidation of LDL and thereby inhibits the formation of foam cells.

Furthermore, it has been reported that hydroxytyrosol efficiently protects against lipid peroxidation *in vitro* (28). The protection against lipid peroxidation by hydroxytyrosol is also shown by the inhibition of the oxidation of LDL, which is regarded as one of the key steps in the initiation of atherosclerosis (29, 30). Excess LDL infiltrates the artery and is retained in the intima, particularly at sites of haemodynamic strain. Oxidation of LDL can activate endothelial cells to express leukocyte adhesion molecules. This causes blood cells such as monocytes rolling along the vascular surface to adhere to the site of activation. The modified LDL particles are taken up by scavenger receptors of macrophages, which evolve into foam cells (Figure 4). Activation of macrophages leads to an inflammatory reaction and tissue damage (31).

Antioxidant properties of hydroxytyrosol in vivo

For an antioxidant to have beneficial health effects *in vivo* it is of course essential that it is taken up. Several studies in humans and rats indeed report

that the uptake of hydroxytyrosol is good (32-35). Plasma concentrations of hydroxytyrosol after consumption of 25 ml of extra virgin olive oil range from 50 to 160 nM (16, 36). The consumption of supplements containing a relatively high amount of hydroxytyrosol might lead to an even higher hydroxytyrosol plasma concentration.
Hydroxytyrosol is metabolized in the body by the action of catechol-O-methyltransferase to homovanillic alcohol (Figure 1). Ingested oleuropein, one of the other phenolic compounds also present in virgin olive oil, is hydrolyzed in the intestine yielding additional hydroxytyrosol. Oleuropein itself hardly reaches the systemic circulation after ingestion (37, 38).
Visioli *et al*. have shown that hydroxytyrosol was able to inhibit passive smoking-induced oxidative stress in rats, as demonstrated by a reduced urinary excretion of isoprostanes (8-iso-$PGF_{2\alpha}$) (39). Moreover, a dose-dependent inverse correlation between the rate of 8-iso-$PGF_{2\alpha}$ excretion and increasing amounts of phenolic compounds ingested with olive oil was observed in human volunteers (40). Furthermore, hydroxytyrosol was able to increase plasma antioxidant capacity in rats (33).
Regarding the protective effect of hydroxytyrosol against the oxidation of LDL some controversies exist. Several human *in vivo* studies have shown that olive oil phenolic compounds efficiently protect against LDL oxidation (13-16, 41). However, no protective effect of hydroxytyrosol is usually found against LDL oxidation in *ex vivo* experiments (42-45).

HYDROXYTYROSOL: SCOPE AND OUTLINE OF THE THESIS

In general, hydroxytyrosol supplementation might be of interest in all kinds of situations of oxidative stress. The present thesis focuses on two specific aspects of hydroxytyrosol. Firstly, one of the 'traditional' fields of interest of hydroxytyrosol, i.e. its beneficial effects on the cardiovascular system, is further explored. Secondly, the possible beneficial effect of hydroxytyrosol is studied in an innovative, so far unexplored field of research, i.e. its protective effect against oxidative stress in skeletal muscle.

Effect of hydroxytyrosol on cardiovascular system

The molecular mechanism of the beneficial cardiovascular effects of olive oil antioxidants has not been fully elucidated. Studies investigating the antioxidant effects of olive oil phenolic compounds are conflicting (5). It has been shown in both *in vitro* and human *in vivo* studies that phenolic compounds efficiently protect against LDL oxidation (13-16, 29, 30, 41, 46). In contrast, no protective effect of hydroxytyrosol is generally demonstrated against *ex vivo* LDL oxida-

tion (42-45). In these experiments LDL is isolated from humans after hydroxytyrosol consumption, and subsequently the oxidizability of this LDL is determined *ex vivo*. Also regarding the antioxidant profile of hydroxytyrosol some controversies exist. For example, the reports on $O_2^{\bullet-}$ radical scavenging are ambiguous (23, 24). Chapter 2 is aimed at resolving the controversies on the antioxidant profile of hydroxytyrosol and on the protective effect of hydroxytyrosol against LDL oxidation. Moreover, the scavenging potential of hydroxytyrosol and related compounds is also further explored.

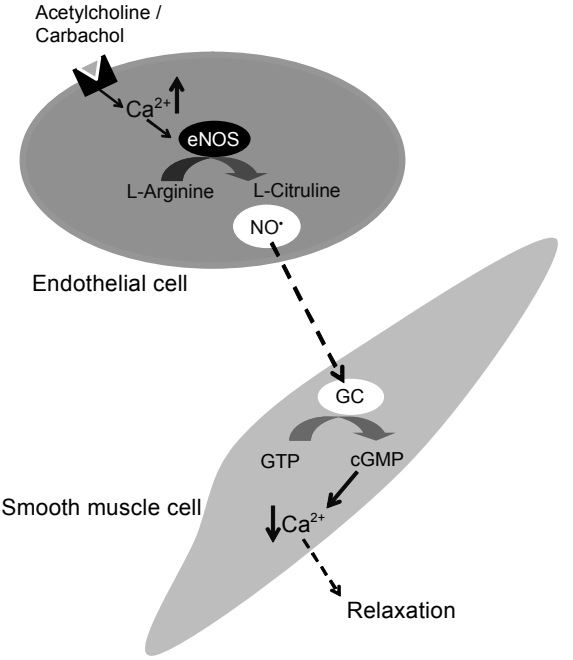

Figure 5. NO• mediated vasorelaxation. Biosynthesis of NO• is performed by the calcium dependent endothelial isoform of nitric oxide synthase (eNOS). The binding of an agonist triggers the conversion of L-Arginine by eNOS into NO•, with L-Citruline as byproduct. NO• diffuses into adjacent smooth muscle cells where it activates guanylyl cyclase (GC). GC converts guanosine triphosphate (GTP) into the second messenger cyclic guanosine monophosphate (cGMP), which activates protein kinases (PKG) leading to blood vessel relaxation.

Under physiological conditions, NO• is of pivotal importance in the regulation of the vascular tone. Endothelium-derived NO• stimulates soluble guanylyl cyclase (GC) activity in smooth muscle cells, finally leading to vasorelaxation (Figure 5). Oxidative damage to endothelial cells might lead to hypertension, which is a well-established risk factor for the development or progression of atherosclerosis (47, 48). Chapter 3 describes the protective effect of hydroxytyrosol against the oxidative stress induced impairment of the NO• mediated vasorelaxation of rat aorta.

Effect of hydroxytyrosol on skeletal muscle

The production of ROS is significantly enhanced during exercise. For example, superoxide radical ($O_2^{\bullet -}$) production by mitochondria is substantially increased (49). Other potential sources for ROS generation during exercise are plasma membrane NADPH oxidases and ischemia reperfusion (50-52). Moreover, ROS trigger the activation of pro-inflammatory pathways such as Nuclear Factor kappa B (NF-κB) and the invasion of the injured tissue with phagocytic cells. These pathways further promote the production of ROS in a feed forward process (Figure 6).

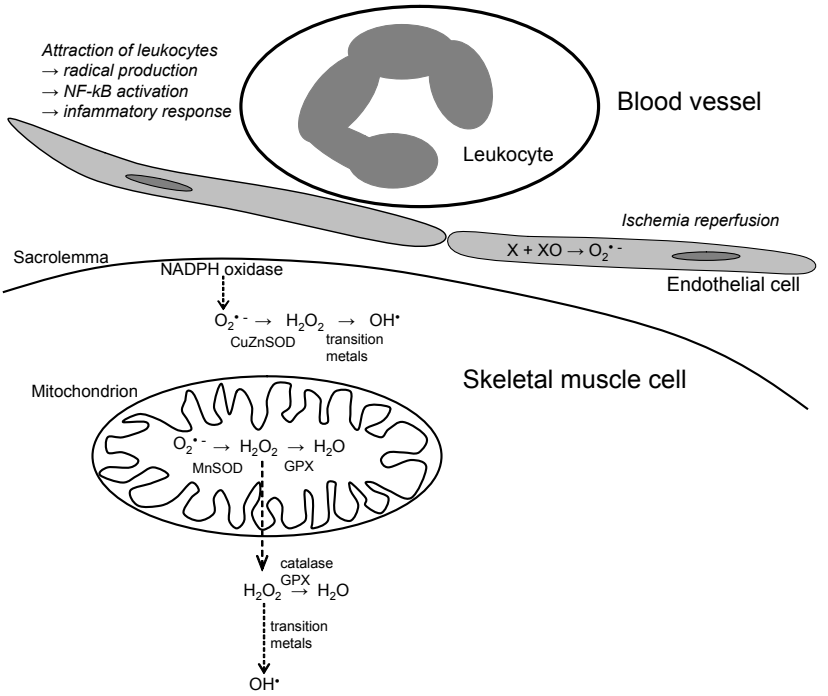

Figure 6. Important sources for ROS production during exercise in skeletal muscle. During exercise, the production of $O_2^{\bullet -}$ is considerably enhanced by mitochondria and membrane NADPH oxidases. Ischemia reperfusion further increases the production of radicals. During ischemia, xanthine dehydrogenase is converted into xanthine oxidase (XO), an enzyme producing $O_2^{\bullet -}$ especially during reperfusion. Moreover, ROS trigger the activation of NF-κB and the invasion of the injured tissue with phagocytic cells, which further promotes the production of ROS in a feed forward process.
X: xanthine; XO: xanthine oxidase; CuZnSOD: copper-zinc superoxide dismutase; MnSOD: manganese superoxide dismutase; GPX: glutathione peroxidase; NADPH oxidase: nicotinamide adenine dinucleotide phosphate oxidase.

Over the past decades, progress has been made in understanding both the beneficial and detrimental roles ROS play in skeletal muscle. Low levels of ROS are required for optimal muscle contractile function (53). However, a high and unbalanced production of ROS will lead to oxidative muscle damage, for example to proteins and lipids (18, 54). Important targets of oxidation by ROS are PUFAs present for example in the sacrolemma, which may lead to increased cell permeability and disturbed signalling over the cell. Moreover, ROS can affect skeletal muscle force production by damaging proteins that are important in regulating cytosolic free Ca^{2+}, such as the sarcoplasmic reticulum ryanodine receptor (RYR) and Ca^{2+} ATPases (55). Moreover, ROS are also able to modify an array of other regulatory proteins in skeletal muscle including myofibrillar proteins (56, 57), mitochondrial proteins (58), and proteins that regulate glucose metabolism (59) (Figure 7).

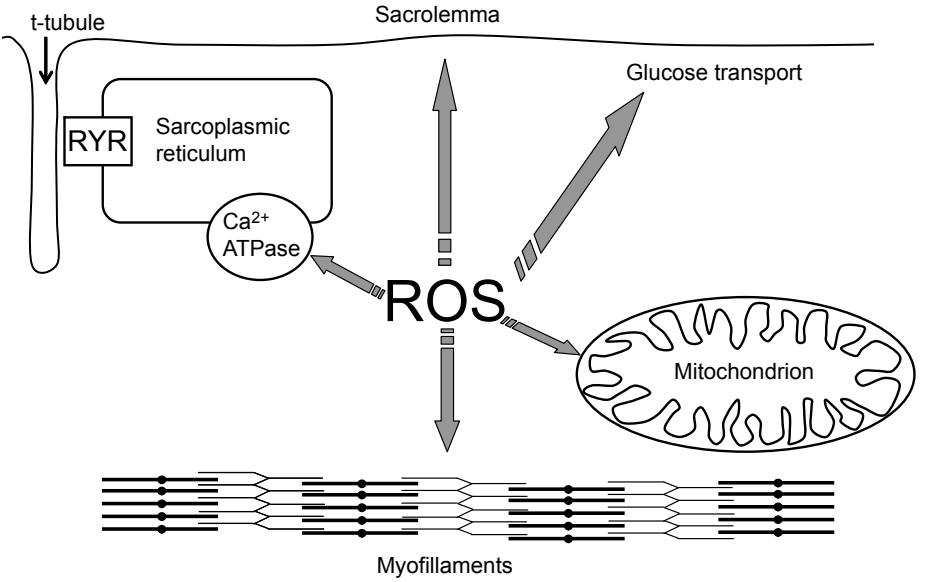

Figure 7. Targets of ROS in skeletal muscle. ROS can affect several cellular elements, such as myofillaments, mitochondria, and sarcoplasmic reticulum RYR and Ca^{2+} ATPases. Moreover, ROS can damage the sacrolemma and, moreover, affect glucose transport.
RYR: ryanodine receptor.

Fortunately, the body has an elaborate defence system of antioxidants that provides protection against ROS (18). In chapter 4, it was investigated whether the endogenous antioxidant defence system does provide sufficient protection against the increased radical flux induced by exercise. More specifically, the effect of exercise on oxidative damage, antioxidant capacity, and inflammation was investigated in untrained healthy volunteers.

Oxidative damage to skeletal muscle cells might result in compromised contractile function. Increasing the antioxidant capacity of the muscle cell could provide protection against ROS generated during intense muscular contractility. This might reduce oxidative muscle damage and possibly decrease the fatigability of muscle cells. Chapter 5 describes the protective effect of hydroxytyrosol against the oxidative stress- induced impairment of force production of isolated rat skeletal muscle.
The *in vivo* effect of hydroxytyrosol on antioxidant capacity, oxidative damage, inflammation and lactate levels during exercise in untrained healthy volunteers is presented in chapter 6. Finally, the results and the impact of the thesis are summarized in chapter 7 and 8.

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CHAPTER 2

New insights into controversies on the antioxidant potential of the olive oil antioxidant hydroxytyrosol

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ABSTRACT

In the present study, the antioxidant profile of olive oil antioxidants was investigated. Hydroxytyrosol and oleuropein are potent scavengers of hydroxyl radicals ($OH^{\bullet}$), peroxynitrite (ONOOH), and superoxide radicals ($O_2^{\bullet -}$). Homovanillic alcohol, one of the main metabolites of hydroxytyrosol, and tyrosol are less potent scavengers of these reactive species. None of the olive oil antioxidants are good hypochlorous acid (HOCl) or hydrogen peroxide (H_2O_2) scavengers.

Hydroxytyrosol efficiently protects against low density lipoprotein (LDL) oxidation *in vitro* and *in vivo*. However, no protective effect of hydroxytyrosol is usually demonstrated *ex vivo* against the oxidation of LDL isolated from humans after hydroxytyrosol consumption. The present study shows that this controversy is due to the isolation of LDL, which greatly reduces the protective effect of hydroxytyrosol against LDL oxidation. Hydroxytyrosol is an efficient scavenger of several free radicals. The physiological relevance of the high intrinsic antioxidant activity of hydroxytyrosol is illustrated by its protection against LDL oxidation.

INTRODUCTION

The role of olive oil in the protection against cardiovascular disease has been highlighted in several studies (1-5). Olive oil, the main source of fat of the Mediterranean diet, is rich in oleic acid, a mono-unsaturated fatty acid (MUFA). To date, most of the research on the protective effect of olive oil has been focused on its high MUFA content.

Recent studies show that olive oil is more than just a mono-unsaturated fat. Olive oil is a natural product that contains a wide range of ingredients. The composition depends, among others, on the type of olives, the manufacturing process, and the storage conditions. In general, extra virgin olive oil contains relatively high amounts of phenolic antioxidants (6), such as oleuropein, hydroxytyrosol, and tyrosol (Figure 1). These phenolic compounds have been shown to provide beneficial cardiovascular effects (1, 2, 7, 8). Consequently, supplements containing phenolic antioxidants from olive oil with health-promoting claims have been developed.

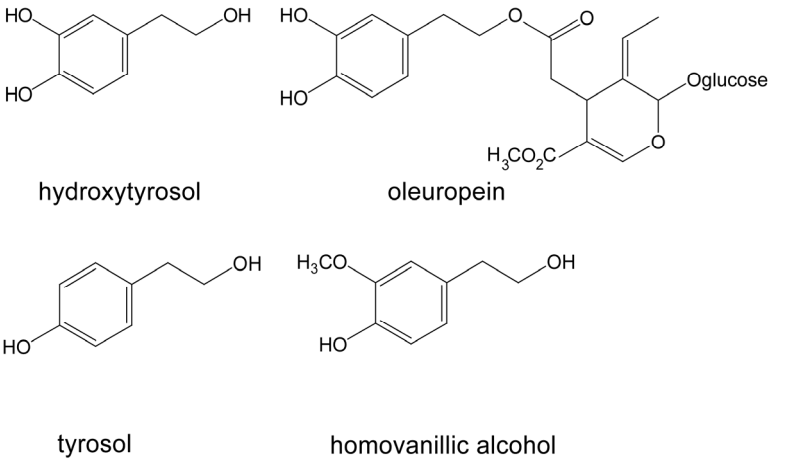

Figure 1. Molecular structure of hydroxytyrosol, oleuropein, tyrosol and homovanillic alcohol.

The molecular mechanism of the cardiovascular effects of olive oil antioxidants has not been fully elucidated. Studies investigating the antioxidant effects of olive oil phenolic compounds are conflicting (9). It has been shown in both *in vitro* and human *in vivo* studies that phenolic compounds efficiently protect against LDL oxidation (1, 2, 7, 8, 10-13). However, no protective effect of hydroxytyrosol is usually found against LDL oxidation in *ex vivo* experiments (14-17). In these experiments, LDL is isolated from humans after hydroxytyrosol consumption, and subsequently the oxidizability of this LDL is determined *ex vivo*.

The present study is designed to provide new insights into the controversy on the effect of phenolic compounds on LDL oxidation. Some controversies also exist regarding the antioxidant profile of phenolic compounds present in olive oil. For example, the reports on radical scavenging are ambiguous (4, 18). Therefore, an integral antioxidant profile of hydroxytyrosol and related compounds is determined by quantifying their scavenging potential of $OH^{\bullet}$, ONOOH, $O_2^{\bullet-}$, HOCl and H_2O_2. The intrinsic antioxidant activity forms the fundament for the physiological impact of olive oil phenolic compounds.

MATERIALS AND METHODS

Chemicals

Hydroxytyrosol was obtained from Cayman Chemical, Ann Arbor, MI, USA. Oleuropein was obtained from Extrasynthese, Lyon, France. Tyrosol was purchased from Fluka, Buchs, Switzerland. Dihydrorhodamine-123 (DHR-123), 2-deoxy-D-ribose, homovanillic alcohol, ascorbate, rutin, 2-methoxyphenol, phenol, HOCl, H_2O_2, dithionitrobenzoic acid, and mannitol were purchased from Sigma, St. Louis, MO, USA. Catechol was obtained from Janssen Chimica, Geel, Belgium. Lipoic acid was purchased from Asta Medica AG, Frankfurt, Germany. Reduced glutathione (GSH) was obtained from ICN Biomedicals Inc., Costa Mesa, CA, USA. Ebselen was obtained from Rhône-Poulenc Rorer, Köln, Germany. All other chemicals were of analytical grade purity.

Scavenging of reactive oxygen and nitrogen species

The antioxidant profile of hydroxytyrosol, oleuropein, tyrosol, and homovanillic alcohol was determined by quantifying their scavenging potential of reactive oxygen and nitrogen species. Scavenging of $OH^{\bullet}$ was determined by the deoxyribose method according to Halliwell *et al.* (19). Scavenging of $O_2^{\bullet-}$ was measured according to den Hartog *et al.* (20). The protection against ONOOH induced DHR-123 oxidation was measured as described by Kooy *et al.* (21). The HOCl scavenging potential of the compounds was measured according to Ching *et al.* (22). Scavenging of H_2O_2 was measured by adding 1 mM H_2O_2 to a 100 µM solution of the scavenger in a 150 mM sodium phosphate buffer (pH 7.4, 37°C). UV spectra were recorded before and during 30 min after the addition of H_2O_2.

The reactive oxygen and nitrogen species scavenging activities of the compounds tested were related to reference antioxidants that either have a high scavenging activity for one of these reactive species or were used for this purpose before. The reference compounds for $OH^{\bullet}$, ONOOH, $O_2^{\bullet-}$, HOCl, and

H_2O_2 scavenging were, respectively, mannitol (23), ebselen (24), rutin, lipoic acid (25), and GSH.

LDL isolation

Blood was collected from a healthy volunteer in tubes containing heparin and centrifuged at 3500 rpm at 4°C for 10 min to obtain plasma. This study was performed in compliance with the guidelines of the Medical Ethical Review Board of the Academic Hospital, Maastricht, The Netherlands. Potassium bromide was added to plasma to increase its density (0.325 g per ml plasma). LDL was isolated by density gradient ultracentrifugation at 32,000 rpm at 4°C for 17 h. The LDL fraction was isolated in a density range of 1.019 - 1.063 g NaCl/ml and diluted to a concentration of 1 mg/ml in a 50 mM sodium phosphate buffer at pH 7.4.

LDL oxidation

LDL oxidation was performed at 37°C in a 50 mM sodium phosphate buffer at pH 7.4 by adding 50 μM $CuSO_4$ to 50 μg/ml LDL in the presence of hydroxytyrosol or vehicle (ethanol). Conjugated diene formation was measured at 234 nm during 240 min.
The effect of the LDL isolation procedure on the protection by hydroxytyrosol against LDL oxidation was also determined. Hydroxytyrosol or vehicle (ethanol) was added to isolated LDL (1 mg/ml), vortexed, and incubated for 5 min. Subsequently, the LDL isolation procedure was repeated by creating the same density gradient. After ultracentrifugation, LDL was isolated and subsequently oxidized as described above. Mean lag times were calculated for all of the LDL experiments.
The addition of hydroxytyrosol (final concentration of 5 μM) to LDL that was isolated for a second time gave the same protection (data not shown) as hydroxytyrosol (5 μM) added to LDL after the first isolation procedure. This indicates that, with respect to the ability of hydroxytyrosol to protect, LDL has not been affected by the isolation procedure.

Lipophilicity

The lipophilicity of hydroxytyrosol was measured by determining the logarithm of the octanol water partitioning coefficient (log $P_{o/w}$). The octanol water partitioning coefficient ($P_{o/w}$) of hydroxytyrosol was calculated by dividing the concentration of hydroxytyrosol in the octanol layer by that in the aqueous layer (150 mM sodium phosphate buffer, pH 7.4) after mixing a 1 mM solution of hydroxytyrosol in buffer that was saturated with octanol with an equal

volume of octanol that was saturated with buffer. The concentration of hydroxytyrosol in each layer was determined spectrophotometrically at 283 nm.

Statistics

The results are expressed as means ± SEM (n=3). The LDL oxidation results are based on at least two separate experiments. For the results based on two experiments, the lag time is presented as mean ± half-range.

RESULTS

Both oleuropein and hydroxytyrosol were potent $OH^{\bullet}$, $O_2^{\bullet-}$, and ONOOH scavengers (Figure 2). Homovanillic alcohol, one of the main metabolites of hydroxytyrosol (Figure 1), was a relatively good scavenger of ONOOH and $OH^{\bullet}$. However, homovanillic alcohol was a less potent scavenger of $OH^{\bullet}$, $O_2^{\bullet-}$ and ONOOH than hydroxytyrosol. Compared to hydroxytyrosol, tyrosol was also a relatively poor scavenger of $OH^{\bullet}$, $O_2^{\bullet-}$, and ONOOH. None of the phenolic compounds from olive oil were good HOCl or H_2O_2 scavengers. The HOCl and H_2O_2 scavenging activity of the reference compounds was at least 10 to 100 times higher than that of the phenolic compounds.

To identify the functional group that is important in the antioxidant potential of the compounds studied, the scavenging activities of catechol, 2-methoxyphenol, and phenol were also determined (Figure 2). Catechol was a potent scavenger of $OH^{\bullet}$, $O_2^{\bullet-}$, and ONOOH. Phenol was less potent in scavenging $OH^{\bullet}$, $O_2^{\bullet-}$, and ONOOH compared to catechol. The compound 2-methoxyphenol was a relatively good scavenger of $OH^{\bullet}$, $O_2^{\bullet-}$, and ONOOH, although the scavenging activities were lower than that of catechol. Catechol, 2-methoxyphenol, and phenol did not display any relevant H_2O_2 or HOCl scavenging activity.

Hydroxytyrosol dose dependently protected against LDL oxidation. A representative experiment is shown in Figure 3. When 5 μM hydroxytyrosol was added to LDL, the lag time in the *in vitro* assay was more than 240 min, whereas the lag time of the control experiment was 30 min (Figure 3A).

To examine the effect of the isolation of LDL, the isolation procedure was performed for a second time. Hydroxytyrosol (5 μM) was added to isolated LDL, and subsequently, the LDL isolation procedure was repeated for a second time. The protective effect of hydroxytyrosol against LDL oxidation was drastically reduced by the second isolation procedure. The lag time of oxidation was shortened from more than 240 min before the second LDL isolation procedure to 60 min after the second LDL isolation procedure (Figure 3B).

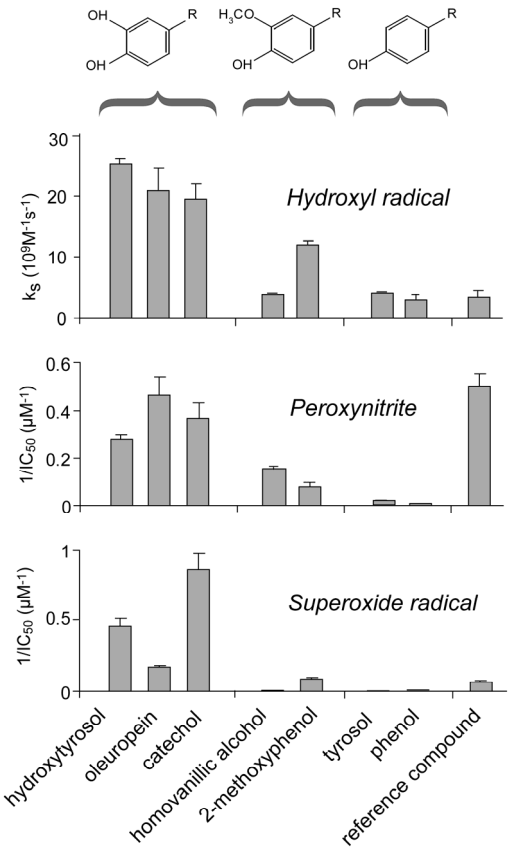

Figure 2. Antioxidant profiles of hydroxytyrosol, oleuropein, homovanillic alcohol, tyrosol, and related compounds. The $OH^{\bullet}$, ONOOH, and $O_2^{\bullet-}$ scavenging activities of the compounds were related to the scavenging activities of respectively mannitol (second-order rate constant = $3.4 \cdot 10^9$ $M^{-1} \cdot s^{-1}$), ebselen (IC_{50} = 2.0 μM), and rutin (IC_{50} =14.8 μM). The H_2O_2 and HOCl scavenging activity of the compounds was related to GSH (IC_{50} = 83 μM) and lipoic acid (second order rate constant = 0.1 $mM^{-1} \cdot min^{-1}$), respectively. None of the phenolic compounds displayed any relevant H_2O_2 or HOCl scavenging activity. The H_2O_2 and HOCl scavenging activity of the reference compounds was at least 10 to 100 times higher than that of the phenolic compounds.

The lipophilicity of hydroxytyrosol, which was measured by determining the log $P_{o/w}$ value, was found to be 0.04. This is similar to the $P_{o/w}$ value reported previously (26). This means that the concentration of hydroxytyrosol in biological systems is expected to be very similar in aqueous and lipid compartments.

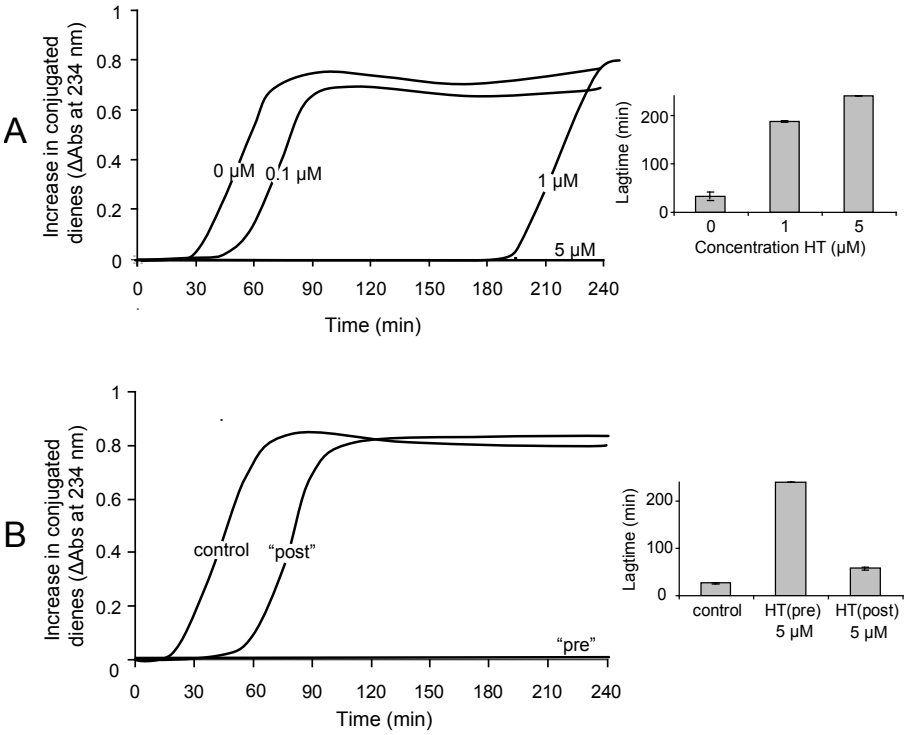

Figure 3. Protection by hydroxytyrosol (HT) against LDL oxidation. In panel A, the dose-dependent protection by hydroxytyrosol against LDL oxidation is shown. In panel B, the effect of the LDL isolation procedure on the protection by hydroxytyrosol against LDL oxidation is shown. Hydroxytyrosol is added to isolated LDL, and subsequently, the LDL isolation procedure is repeated. The protective effect of hydroxytyrosol before and after the second LDL isolation procedure is shown. The lag times of LDL oxidation are shown in the inserts. No conjugated diene formation occurred when a concentration of 5 µM hydroxytyrosol was used (before the second isolation procedure; panels A and B). For this reason, the lag time was set at 240 min, that is, the duration of the experiment.

DISCUSSION

The aim of the present study was to investigate the antioxidant potential of olive oil phenolic compounds. The fundament of the beneficial effect of an antioxidant is its intrinsic antioxidant activity. Therefore, the antioxidant profile of the olive oil antioxidants was established. Subsequently, the physiological impact of the antioxidant efficacy was evaluated by determining the protective effect against LDL oxidation.

In the present study, it was shown that both hydroxytyrosol and oleuropein have superior ONOOH, $OH^{\bullet}$, and $O_2^{\bullet-}$ scavenging activities, equal to or

surpassing that of the reference antioxidants used. The high potency of hydroxytyrosol and oleuropein for scavenging ONOOH observed in the present study is in accordance with previous reports (27, 28). Literature on the activity of hydroxytyrosol as an $O_2^{\bullet-}$ scavenger is contradictory (4, 18). Our results indicate that hydroxytyrosol and oleuropein do scavenge $O_2^{\bullet-}$.

In the present study, it was shown that none of the olive oil phenolic compounds were good H_2O_2 or HOCl scavengers. Previous reports have suggested that hydroxytyrosol is able to scavenge H_2O_2 (18) and HOCl (4). The reported scavenging of H_2O_2 (18) by hydroxytyrosol is most likely the result of the scavenging of reactive species that are formed out of H_2O_2. In the only report on the HOCl scavenging of hydroxytyrosol, the activity of hydroxytyrosol was related to poor HOCl scavengers, that is, vitamin C and vitamin E. Comparing the activity of hydroxytyrosol to a more relevant reference compound clearly shows that hydroxytyrosol hardly possesses any HOCl scavenging activity. The scavenging of the non-radical species H_2O_2 and HOCl involves a two-electron reaction. In the scavenging of these species, thiols appear to be superior to phenolic compounds.

Our results show that compounds having a catechol (orthodiphenolic) group in their molecular structure, that is, oleuropein and hydroxytyrosol, have superior antioxidant properties than compounds lacking this moiety, that is, homovanillic alcohol and tyrosol. As also reported previously, the present study shows that the antioxidant potency of the catechol group is superior to that of a resorcinol or phenol group. The relatively high antioxidant activity of catechol can be explained by the high electron donating effect of the second hydroxyl group (29, 30).

For an antioxidant to have beneficial health effects *in vivo,* it is of course essential that it is taken up. Several studies in humans and rats indeed report that the uptake of hydroxytyrosol is good (31-34). Plasma concentrations of hydroxytyrosol after consumption of 25 ml of extra virgin olive oil range from 50 to 160 nM (8, 35). The consumption of supplements containing a relatively high amount of hydroxytyrosol might lead to an even higher hydroxytyrosol concentration. Hydroxytyrosol is metabolized in the body by the action of catechol-*O*-methyltransferase to homovanillic alcohol. Oleuropein itself hardly reaches the systemic circulation after ingestion. However, most of the oleuropein is hydrolyzed in the intestine, yielding hydroxytyrosol (36, 37).

The physiological impact of the antioxidant efficacy of hydroxytyrosol was evaluated by determining its protective effect against LDL oxidation. LDL oxidation is one of the key steps in the initiation of atherosclerosis. The present study shows that hydroxytyrosol efficiently protects against LDL oxidation *in vitro* at relatively low concentrations. Review of the literature shows that our results are consistent with *in vitro* data from other studies on human LDL (Table 1). The present study also shows that the isolation procedure substantially

reduces the protective effect of hydroxytyrosol against LDL oxidation. This might explain the false negative results in *ex vivo* studies that usually show no protective effect of olive oil phenolic compounds in LDL isolated from humans having consumed a diet rich in olive oil (Table 1).

During the isolation procedure, a relatively small volume of LDL is added to a relatively large volume of sodium chloride solutions. In the present study, it was shown that the log $P_{o/w}$ of hydroxytyrosol is 0.04. This indicates that the reduction in the protective effect might be due to the loss of hydroxytyrosol from LDL during the LDL isolation procedure. Recently, the loss of hydroxytyrosol from LDL during the isolation procedure has also been addressed by de la Torre-Carbot *et al.* (38). This is in line with the results of our study. The loss of antioxidants other than hydroxytyrosol during the isolation procedure might also be involved in the underestimation of the protective effect of hydroxytyrosol in *ex vivo* experiments. In addition to the lipophilicity, also other properties determine the interaction of antioxidants with LDL, such as hydrogen binding to proteins. Possible structural LDL changes during the isolation procedure may affect this interaction, which can also result in an underestimation of the protective effect of hydroxytyrosol in *ex vivo* experiments.

The *ex vivo* protective effect of olive oil phenolic compounds reported by Ramirez-Tortosa *et al.* (39) might be due to the higher vitamin E content in the olive oil diet compared to the control diet. Vitamin E, an extremely lipophilic compound, will not be removed from LDL during the isolation procedure. Marrugat *et al.* also demonstrated a protective effect of olive oil phenolic compounds against LDL oxidation, measured *ex vivo* after hydroxytyrosol consumption (2). However, the observed increase in lag time of LDL oxidation was only marginal.

LDL oxidizability has also been evaluated *in vivo* in several studies (Table 1). Both long and short term ingestion of olive oil rich in phenolic compounds have been shown to lower plasma levels of oxidized LDL (1, 2, 7, 8). These effects are not attributable to the mono-unsaturated fatty acids of olive oil, as the fat compositions of the olive oil and the control oil were identical. These studies unequivocally proof that olive oil phenolic compounds provide protection against LDL oxidation.

In conclusion, hydroxytyrosol is an efficient scavenger of several free radicals. The physiological relevance of the high intrinsic antioxidant activity of hydroxytyrosol is illustrated by its protection against LDL oxidation.

Acknowledgements

We would like to thank Marie-José Drittij, Marc Fischer, and Mathijs Groeneweg for their valuable assistance.

Table 1. Overview of human studies on the protective effect of olive oil phenolic compounds against LDL oxidation

IN VITRO			
Concentration hydroxytyrosol (μM)		Effect	Reference
0.1 1 5		+ + + + + + + + + + +	Present study
0.3 [a,b] 1.1 [a,b] 1.7 [a,b]		+ + + + + + + + +	12
10 [b]		+ + +	10
10 [b,c]		+ + + +	11
EX VIVO			
Estimated daily intake (mg phenolic equivalents)			
Olive oil	Control	Effect	Reference
0.4	0	0	14
3.5 [d]	0 and 1.6 [d]	+	2
16 [e]	0 [e]	0	15
21	3	0	17
33	3	+ [f,g]	39
100	0	0	16
IN VIVO			
Estimated daily intake (mg phenolic equivalents)			
Olive oil	Control	Effect	Reference
3.5 [d]	0 and 1.6 [d]	+ + +	2
7.4 [d]	0.7 [d]	+	7
8.4 [d]	0.1 and 3.7 [d]	+ + +	1
11 [d]	0.2 and 3 [d]	+ + +	8
13.5 [d]	0.1 and 6 [d]	+ +	13

The effect shown is based on the percentage increase in the lag time of conjugated diene formation in isolated LDL (*in vitro* and *ex vivo* studies) or the reduction in the extent of LDL oxidation measured using ELISA (*in vivo* studies).+ corresponds with 0-10%, + + corresponds with 10-30%, + + + corresponds with 30-100%, and + + + + corresponds with >100%. 0 means no effect.

a. The concentration of phenolic compounds present in olive oil was expressed as μM caffeic acid equivalents; b. The protective effect was estimated from the figures presented the article; c. The protective effect of oleuropein against the formation of thiobarbituric acid-reacting substances (TBARS) and lipid hydroperoxides was presented; d. The daily intake was calculated using a density of olive oil of 0.92 kg/l; e. Estimated phenol intake (according to Vissers *et al*., ref 9); f. Susceptibility of LDL to oxidation was assessed by the rate of TBARS formation; g. Vitamin E intake was 12 vs. 8 mg (high vs. low phenolic equivalent diet).

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CHAPTER 3

The olive oil antioxidant hydroxytyrosol efficiently protects against the oxidative stress-induced impairment of the $NO^{\bullet}$ response of isolated rat aorta

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ABSTRACT

The Mediterranean diet, which is abundant in antioxidants, is associated with a relatively low incidence of coronary heart disease (CHD). Olive oil and olives, which contain the antioxidants hydroxytyrosol, oleuropein, and tyrosol, are important components of this diet. In this study, the effects of oxidative stress on the nitric oxide radical ($NO^{\bullet}$) mediated relaxation of rat aorta and the protection by these antioxidants were determined. Cumene hydroperoxide (CHP) was used to mimic oxidative stress induced by lipid hydroperoxides, which is mediated by the formation of hydroxyl radicals ($OH^{\bullet}$). CHP (300 μM) impaired the $NO^{\bullet}$ mediated relaxation of rat aorta by the acetylcholine receptor agonist carbachol (CCh) ($P<0.05$). This was due to a reduction in $NO^{\bullet}$ production. A diminished $NO^{\bullet}$ mediated relaxation disturbs the vascular tone and leads to a rise in blood pressure, which is a well-established risk factor for CHD. Hydroxytyrosol (10 μM) efficiently protected the aorta against the CHP induced impairment of the $NO^{\bullet}$ mediated relaxation ($P<0.05$). Oleuropein, tyrosol, and homovanillic alcohol, a major metabolite of hydroxytyrosol, did not show protection. Moreover, hydroxytyrosol was found to be a potent $OH^{\bullet}$ scavenger, which can be attributed to its catechol moiety. Because of its amphiphilic characteristics (octanol-water partitioning coefficient = 1.1), hydroxytyrosol will readily cross membranes and provide protection in the cytosol and membranes, including the water-lipid interface. The present study provides a molecular basis for the contribution of hydroxytyrosol to the benefits of the Mediterranean diet.

INTRODUCTION

Epidemiological evidence has shown that the incidence of coronary heart disease (CHD) is lower in Mediterranean countries than in Western European and Northern European countries (1). In the Mediterranean area, fruits, vegetables, fish, and olive oil are important constituents of the diet and especially these constituents are thought to contribute to the protection against CHD. The relatively high antioxidant content in this traditional Mediterranean diet is seen as a key factor in the lower incidence of CHD, a disease that is related to oxidative stress. Olives and olive oil are important sources of alimentary antioxidants. Olive oil is abundant in vitamin E, flavonoids, and other phenolic antioxidants. The major phenolic compounds in olive oil are hydroxytyrosol and tyrosol. Oleuropein is present in minor quantities in olive oil and is mainly found in the olive itself (2) (Figure 1).

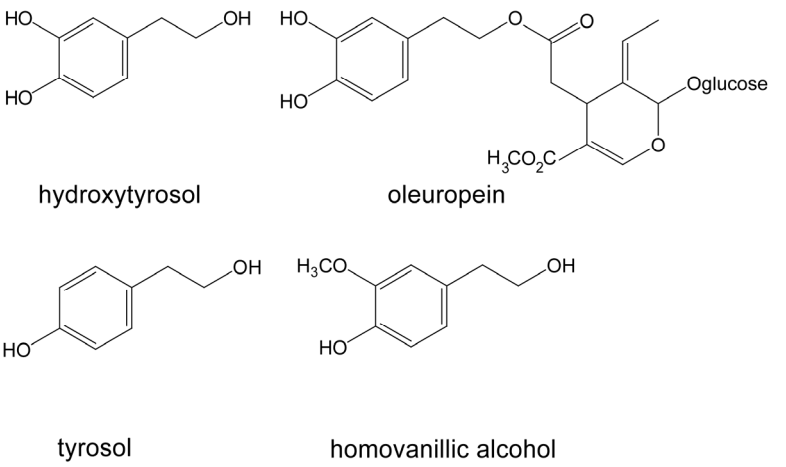

Figure 1. Molecular structure of hydroxytyrosol, oleuropein, tyrosol and homovanillic alcohol.

The aim of the present study is to investigate the effects of oxidative stress on the nitric oxide radical ($NO^{\bullet}$) mediated vasorelaxation of isolated rat thoracic aorta. Under physiological conditions, $NO^{\bullet}$ is of pivotal importance in the regulation of the vascular tone. Endothelium-derived $NO^{\bullet}$ stimulates soluble guanylyl cyclase (CG) activity in smooth muscle cells, finally leading to vasorelaxation. In the present study the lipophilic hydroperoxide cumene hydroperoxide (CHP) is used to induce oxidative stress in rat aorta. CHP is used as a model compound for lipid hydroperoxides (LOOH), which are formed in the process of lipid peroxidation during oxidative stress. The formation of hydroxyl radicals ($OH^{\bullet}$) by homolytic cleavage of LOOH induces oxidative stress. $OH^{\bullet}$ is one of the most reactive chemical species known.

The protection provided by compounds from olives and olive oil, i.e. hydroxytyrosol, oleuropein, and tyrosol, against the vascular effects induced by CHP is studied as well. Inasmuch as hydroxytyrosol is metabolized *in vivo*, also the protective effect of one of its main metabolites, homovanillic alcohol (Figure 1), is studied. To elucidate the molecular mechanism of the protection against CHP provided by the antioxidants, the potential to scavenge $OH^{\bullet}$ is determined.

MATERIALS AND METHODS

Chemicals

Hydroxytyrosol was obtained from Cayman Chemical Company, Ann Arbor, MI, USA. Oleuropein was purchased from Extrasynthese, Lyon, France. Tyrosol, homovanillic alcohol, CHP, carbachol (CCh), phenylephrine (PE), sodium nitroprusside, 2-deoxy-D-ribose, ferric chloride hexahydrate, butylated hydroxytoluene, and mannitol were obtained from Sigma-Aldrich, St Louis, USA. H_2O_2, ascorbate, 2-thiobarbituric acid, and trichloroacetic acid were purchased from Merck, Darmstadt, Germany. Anti-formaldehyde-fixed-cGMP serum was raised in sheep (3). Alexa fluor 488 donkey anti-sheep IgG conjugate was obtained from Molecular Probes, Leiden, the Netherlands. All other chemicals were of analytic grade.

Organ bath experiments

The experimental protocol was approved by the Ethics Committee for Animal Experiments of the University of Maastricht. Male Lewis rats (9 -11 wk old) were decapitated, the aorta was rapidly excised, and small rings (~2 mm long) were mounted in thermostated organ baths (37°C) containing Krebs buffer (pH 7.4) gassed with a mixture of 95% O_2 and 5% CO_2. Each aorta ring was connected to an isometric transducer, and the tension was adjusted to 5 mN. The composition of the Krebs buffer was (mM): NaCl (117.5), KCl (5.6), $MgSO_4$ (1.18), $CaCl_2$ (2.5), NaH_2PO_4 (1.28), $NaHCO_3$ (25), and glucose (5.5). During the experiment, the buffer was changed every 15 min. At the beginning of the experiment, the aortic rings were washed for 60 min.
To determine the $NO^{\bullet}$ mediated relaxation, the aortic rings were first precontracted using the α_1-adrenergic agonist PE. A dose-response curve of PE (from 10^{-8} to $3{\cdot}10^{-6}$ M) was constructed. Subsequently, the $NO^{\bullet}$ mediated relaxation using the muscarinic agonist CCh was determined in a dose-dependent manner. The concentration CCh was increased from 10^{-8} to 10^{-5} M. After the construction of a reference curve of PE and CCh in each aorta ring, the organs

were subjected to two 15-min washes. After they were washed, the organs were incubated for 5 min with different antioxidants (or vehicle). Hydroxytyrosol, oleuropein, and tyrosol were dissolved in ethanol, and homovanillic alcohol was dissolved in water. A relatively small volume of the compound (20 µl) was added to the organ bath (20 ml) in the direct proximity of the aortic ring. Subsequently, the organs were incubated with CHP (or vehicle) for 30 min. The vehicle ethanol (maximal volume of 20 µl in 20 ml of buffer) did not affect the PE and CCh response of the aorta.

After these incubations, the aorta was subjected to two 15-min washes, and a second dose-response curve of PE and CCh was constructed. The negative logarithm of the molar concentration that produces half-maximal effect (pD_2) and the maximal effects (E_{max}) of PE and CCh were calculated. The higher the pD_2, the more potent PE or CCh is. The difference in E_{max} and pD_2 between the first and second dose-response curve was calculated and expressed as E_{max} in percentage and pD_2. The effects of CHP and the antioxidants on the PE and CCh response were expressed as the percentage of the control PE and CCh curves.

cGMP-immunostaining of aortic tissue

Aortic rings of 9-wk-old male Lewis rats (n=2) were mounted in thermostated organ baths as described above. The organs were incubated with 100 µM hydroxytyrosol (or vehicle) for 5 min and then with 300 µM CHP (or vehicle) for 30 min. After two 15-min washes with Krebs buffer, the aortic rings were contracted by adding $3 \cdot 10^{-6}$ M PE to the organ baths. Subsequently, the aortic rings were dilated with $1 \cdot 10^{-5}$ M CCh or 100 µM sodium nitroprusside. One of the aortic rings mounted in the organ bath was left untreated during the entire experiment. This control aorta ring was not incubated with hydroxytyrosol or CHP, nor was it contracted or dilated.

After a 6-min incubation with CCh or nitroprusside, the aortic rings were fixed for 2h with ice-cold fixative solution of 4% freshly prepared depolymerised paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Thereafter, the aortic rings were fixed overnight at 4°C in 4% paraformaldehyde containing 20% sucrose. After the overnight fixation, the aortic rings were frozen in Tissue-Tek O.C.T. compound.

A cryostat was used to cut 8-µm sections, which were thawed onto chrome-alumn/gelatin-coated slides and then dried for 20 min at room temperature. After three 5-min washes with Tris-buffered saline (TBS), the sections were incubated overnight at 4°C with the primary antibody, i.e. sheep anti-formaldehyde-fixed cGMP (1:4000) diluted in TBS containing 0.3% Triton X-100 (TBS-T). The specificity of the cGMP antibody has been described elsewhere (3).

After the incubation with primary antibody, the sections were washed, once in TBS and then twice in TBS-T, each step lasted 15 min. For the visualization of the primary antibodies, the sections were incubated with Alexa Fluor-donkey anti-sheep IgG conjugate (1:100 dilution). Incubations with the secondary antibody lasted for 60 min in the dark at room temperature. Thereafter, sections were washed, once in TBS-T and twice in TBS, and mounted with TBS-glycerol. To check for non-specific binding of the second antibody to aortic tissue, a few slides were incubated only with the secondary antibody. These slides showed no cGMP-immunostaining.
Images were obtained at X40 magnification using a cooled CCD F-view camera on an Olympus AX70 microscope. Exposure time was held constant at 2s, except when sodium nitroprusside was used, where exposure time was 0.5 s to prevent overexposure. Two 8-µm sections from each aorta ring were analysed.

$OH^{\bullet}$ scavenging activity

$OH^{\bullet}$ scavenging activity was determined by the deoxyribose method according to Halliwell *et al*. (4). This method is based on the competition between the antioxidants and the detector molecule 2-deoxyribose for $OH^{\bullet}$ to derive the second-order rate constant for the interaction of the antioxidant with $OH^{\bullet}$. The degradation of 2-deoxyribose after reaction with $OH^{\bullet}$ in a complex mixture of products results, after heating, in the formation of thiobarbituric acid-reactive material which is quantified spectrophotometrically (5). The incubation mixture contains: H_2O_2 (2.8 mM), 2-deoxyribose (2.8 mM), ascorbate (100 µM), and ferric chloride (20 µM). In another experiment, CHP (2.0 mM), in stead of H_2O_2, was used as a source for radicals. The protective effect of hydroxytyrosol was determined.
The second-order rate constant of the scavenger (k_s) with $OH^{\bullet}$ is calculated using a rate constant of $3.1 \cdot 10^{-9}$ $M^{-1}s^{-1}$ for deoxyribose (k_{DR}) (4). The absorbance at 532 nm (A) depends on the concentration of the scavenger ([S]) and the absorbance without the scavenger (A_0). For calculation of k_s the following formula was used:

$$1/A = 1/A_0 \, (1 + k_s[S]/k_{DR}[DR])$$

where [DR] is the concentration of 2-deoxyribose (2.8 mM). The k_s value of the scavenger is obtained from the slope of the linear plot of the reciprocal value of A vs. [S]. The $OH^{\bullet}$ scavenging activity of the olive oil compounds was compared with that of the well-known $OH^{\bullet}$ scavenger mannitol.

Lipophilicity

The lipophilicity of hydroxytyrosol was measured by determining the octanol-water partitioning coefficient ($P_{o/w}$). $P_{o/w}$ of hydroxytyrosol was calculated by dividing the concentration of hydroxytyrosol in the octanol layer by that in the water layer after a 1 mM solution of hydroxytyrosol in water that was saturated with octanol was mixed with an equal volume of octanol that was saturated with water.

Statistics

Values are means ± SEM. Differences in the response of the aorta between the several conditions were statistically analysed performing the Student's t-test.
$P < 0.05$ was considered as statistically significant. Control experiments were performed on eight (no addition) and seven (CHP) rats. The experiments with hydroxytyrosol were performed on three to five rats per concentration. The experiments with the other compounds that did not show an effect were performed on two rats. For these compounds the data are presented by means ± half range.

RESULTS

Organ bath experiments

During the incubation of the aorta with 300 μM CHP for 30 min, no contraction was observed. Incubation of the aorta with 300 μM CHP resulted in only a small decrease in E_{max} of the PE response (Figure 2A) ($P<0.05$). The pD_2 of the PE response was not affected after incubation with CHP (Figure 2A). A similar minor effect on the PE response was also observed after incubation with 100 μM and 1000 μM CHP (data not shown).
The response of the aortic rings to CCh was only slightly diminished after incubation with 100 μM CHP, whereas it completely disappeared after incubation with 1000 μM CHP (data not shown). Incubation with 300 μM CHP significantly decreased the CCh response ($P<0.05$), E_{max} decreased ~ 50% and pD_2 decreased 0.71 (Figure 2B). Thus, oxidative stress, caused by incubation of the aorta with 300 μM CHP, drastically impaired the NO• mediated relaxation by CCh, whereas the PE response was hardly diminished.

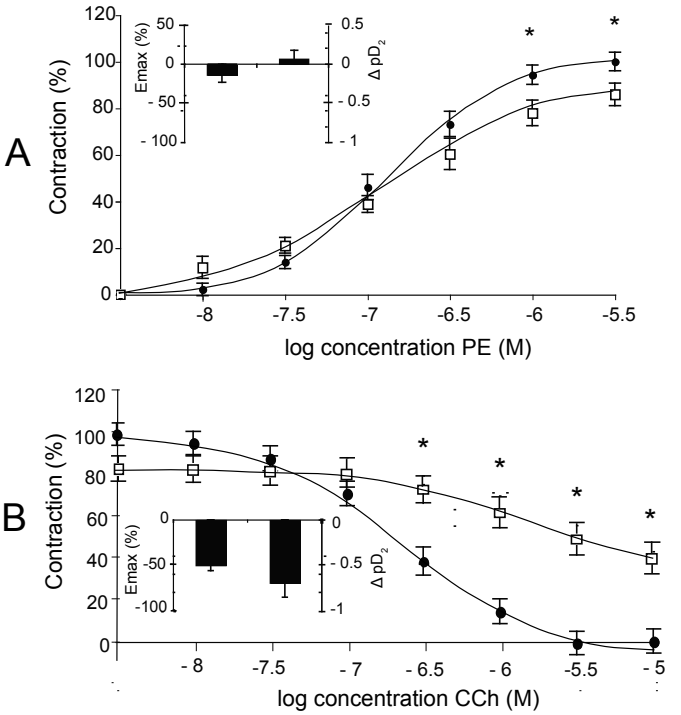

Figure 2. Effect of 300 μM CHP on the PE (panel A) and CCh (panel B) response. Aorta was contracted with PE (from 10^{-8} to $3{\cdot}10^{-6}$ M) and then dilated with CCh (from 10^{-8} to 10^{-5} M). Data points on y-axis show contraction of the aorta without PE (panel A) or CCh (panel B). Effects of CHP (white squares) are expressed as percentage of control (black circles) PE and CCh curves (no addition is set at 100%). Effects of CHP on E_{max} and pD_2 of the PE and CCh response are depicted in the inserts. * $P<0.05$

Hydroxytyrosol, oleuropein, tyrosol, and homovanillic alcohol (at 30 μM), had no effect on the PE and CCh response. These compounds also did not affect the minor effect of CHP on the PE response. Oleuropein, tyrosol, and homovanillic alcohol (at 30 μM) did not protect against the CHP induced reduction in the $NO^{\bullet}$ mediated relaxation by CCh (Figure 3).

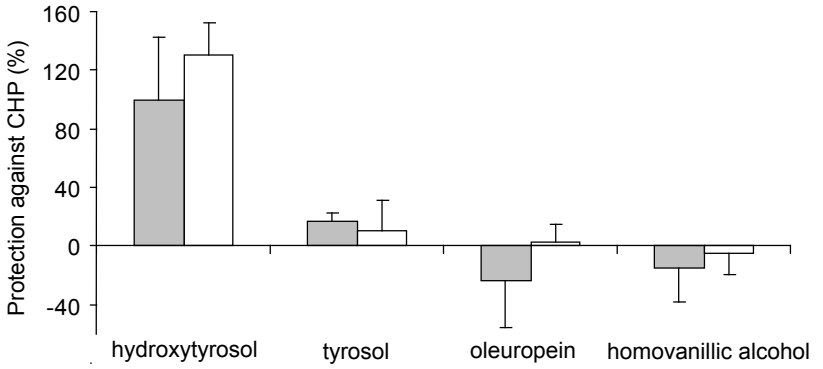

Figure 3. Protective effect of hydroxytyrosol, tyrosol, oleuropein, and homovanillic alcohol against CHP (300 μM) induced impairment of the NO• mediated aortic relaxation. Values represent protection of E_{max} (grey bars) and pD_2 (white bars) of CCh response. Final concentration of antioxidants was 30 μM.

Hydroxytyrosol did protect against the effects of 300 μM CHP on the NO• mediated relaxation (Figure 3). At 30 μM, hydroxytyrosol completely protected against the effect of 300 μM CHP on E_{max} of the CCh response ($P<0.05$), and pD_2 of the CCh response even slightly increased ($pD_2 = +0.21$) compared with control ($P<0.05$). The protection provided by hydroxytyrosol against the effects of CHP on the NO• mediated relaxation was dose dependent (Figure 4). Even relatively low concentrations of hydroxytyrosol (10 μM and 30 μM) protected against 300 μM CHP ($P<0.05$). Hydroxytyrosol at 3 μM resulted in only partial protection (Figure 4).

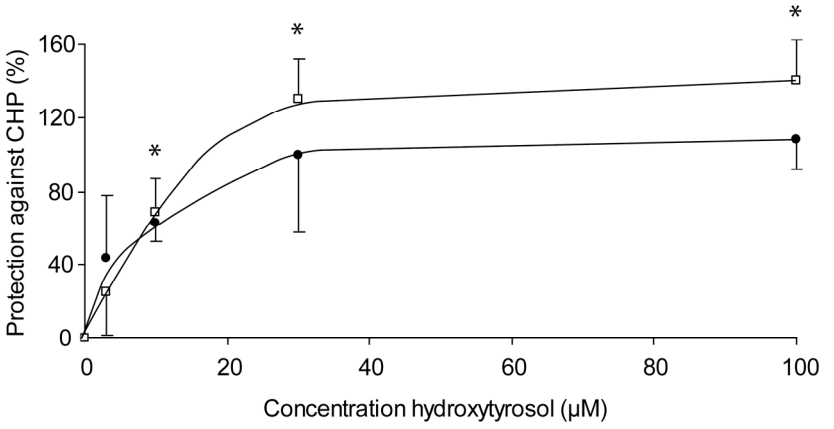

Figure 4. Dose-dependent protection of hydroxytyrosol against CHP (300 μM) induced impairment of NO• mediated relaxation. Values represent protection of E_{max} (black circles) and pD_2 (white squares) of CCh response. Control (without CHP and hydroxytyrosol) is set at 100%.
* $P<0.05$

cGMP-immunostaining of aortic tissue

The cGMP-immunostaining of aortic tissue that was pretreated with CHP and subsequently contracted by PE and dilated by CCh (Figure 5B) was similar to the cGMP-immunostaining of untreated aorta (Figure 5A).
In CHP-treated aortic tissue incubated with sodium nitroprusside, cGMP accumulates in smooth muscle cells (Figure 5C). cGMP immunostaining of the aorta by CCh is substantially greater when the tissue is preincubated with hydroxytyrosol before the addition of CHP than when the tissue is incubated with CHP alone (Figure 5D).

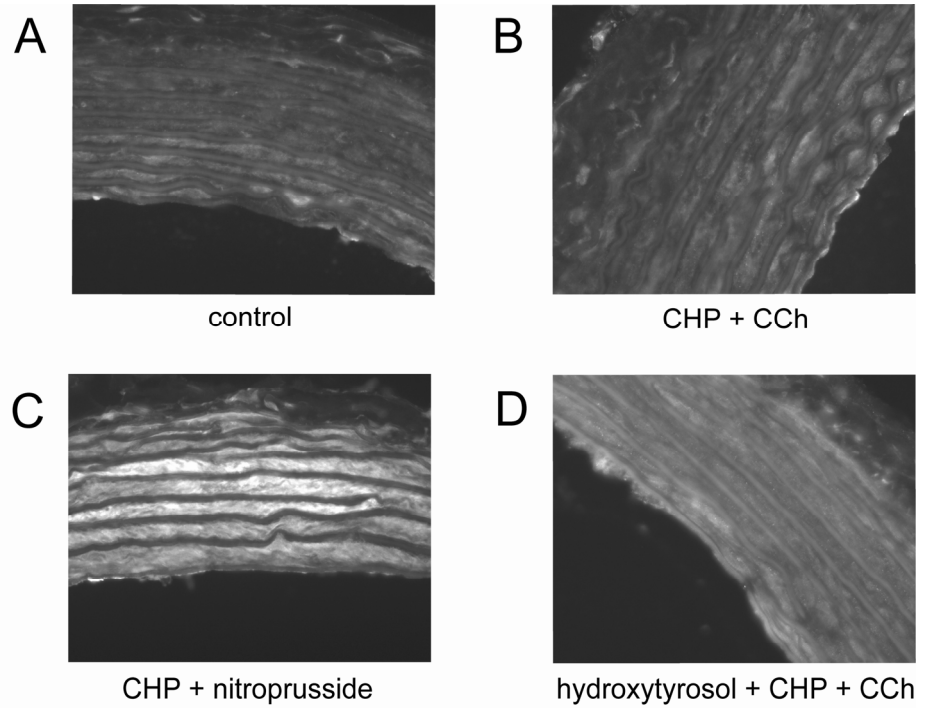

Figure 5. cGMP-immunostaining in rat aortic tissue. Panel A: untreated aorta (control). Aorta was incubated with 300 μM CHP and subsequently contracted by PE ($3 \cdot 10^{-6}$ M) and dilated by CCh (10^{-5} M) (panel B and D) or sodium nitroprusside (100 μM) (panel C). Panel D: cGMP-immunostaining of aorta incubated with 100 μM hydroxytyrosol before addition of CHP.

$OH^{\bullet}$ scavenging

To elucidate the mechanism of protection against CHP, the ability of the compounds from olive oil to scavenge $OH^{\bullet}$ was determined. Oleuropein and hydroxytyrosol were very potent $OH^{\bullet}$ scavengers. The $OH^{\bullet}$ scavenging activity

was nearly seven times higher than the activity of mannitol, a well-known OH• scavenger (Figure 6). Tyrosol and homovanillic alcohol were less effective than hydroxytyrosol in scavenging OH•.

When CHP was used as source for radicals in the 2-deoxyribose assay, the protective effect of hydroxytyrosol (k_s = 13.2 ·10^9 $M^{-1}s^{-1}$) is comparable to the protection observed when H_2O_2 is used as the radical source.

To identify the part of the molecule that is responsible for the scavenging of OH•, the scavenging activities of the compounds were compared with the activities of catechol, 2-methoxyphenol, and phenol (Figure 6). Catechol displayed an OH• scavenging activity comparable to that of hydroxytyrosol and oleuropein. 2-Methoxyphenol showed a somewhat higher OH• scavenging activity than homovanillic alcohol, whereas phenol and tyrosol were equipotent.

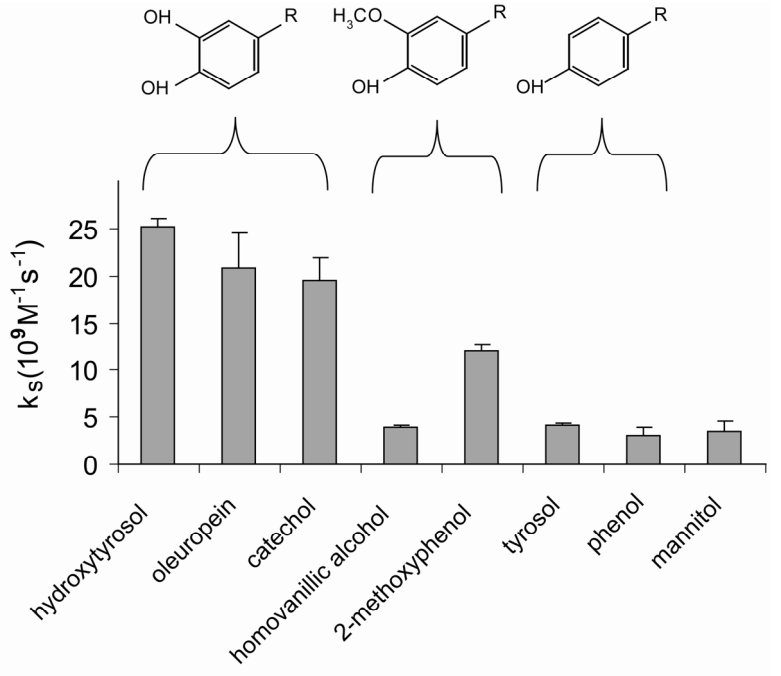

Figure 6. OH• scavenging activities of hydroxytyrosol, tyrosol, oleuropein, and homovanillic alcohol compared with mannitol and compounds sharing identical moieties. Scavenging activities are expressed as the second-order rate constant of the scavenger (k_s) with OH•.

Lipophilicity

Lipophilicity is a key factor in many biological effects of compounds. The lipophilicity of hydroxytyrosol was measured by determination of $P_{o/w}$, which

was found to be 1.1. This means that the concentration of hydroxytyrosol is expected to be very similar in the water and the lipid phase.

DISCUSSION

In the present study the effect of oxidative stress on the $NO^{\bullet}$ mediated relaxation of rat aorta was determined. During oxidative stress, polyunsaturated fatty acids (PUFAs), present in, e.g., membranes of endothelial and smooth muscle cells in blood vessels, are oxidatively damaged. This process is called lipid peroxidation. During this process, LOOH are formed. These LOOH amplify the oxidative stress, because they readily form radicals, e.g., the extremely reactive $OH^{\bullet}$ by homolytic cleavage of the O-O bond.

In the present study, it was shown that CHP, a model compound for LOOH, drastically impaired the $NO^{\bullet}$ mediated relaxation by CCh. pD_2 decreased 0.7 after incubation with CHP, which means that the acetylcholine receptor-mediated response decreased 80%. The E_{max} decreased ~50% after incubation with CHP, which implies that the smooth muscle itself is also affected. Endothelium-derived $NO^{\bullet}$ is an important regulator of vascular tone. Damage to the $NO^{\bullet}$ regulating mechanism is involved in hypertension and the development or progression of atherosclerosis (6, 7). CHP hardly decreased the PE response, which means that the α_1 adrenoceptor mediated contraction is not affected.

Hydroxytyrosol very efficiently protected the aorta of the rat against the CHP induced impairment of the $NO^{\bullet}$ mediated relaxation: 1 molecule of hydroxytyrosol is able to provide protection against 30 molecules of CHP. Therefore, hydroxytyrosol has to be considered as an excellent antioxidant.

To further study the inhibitory effect of CHP on the CCh induced relaxation, we investigated the effect of CHP on cGMP levels in aortic tissue. The addition of sodium nitroprusside to CHP-treated aortic tissue significantly increased cGMP levels in smooth muscle cells. Therefore, the inhibitory effect of CHP on the CCh induced relaxation of the aorta cannot be explained by inactivation of GC activity in smooth muscle cells. This indicates that CHP most likely impairs the release of $NO^{\bullet}$ from the endothelium.

We demonstrated that preincubation with hydroxytyrosol before the addition of CHP results in substantially more cGMP-immunostaining in aortic tissue by CCh than does incubation with CHP alone. These results support the hypothesis that hydroxytyrosol protects against the oxidative stress induced impairment of the $NO^{\bullet}$ mediated vasorelaxation.

Hydroxytyrosol is metabolized in the body by the action of catechol-O-methyltransferase, an enzyme also involved in catecholamine catabolism (8). One of its major metabolites, homovanillic alcohol, provided no protection

against the CHP induced effects on the NO• mediated relaxation. Also, oleuropein and tyrosol were not able to protect the aorta against oxidative stress.

CHP induces oxidative damage by the generation of OH•. To explain the difference in protection against oxidative stress between hydroxytyrosol, oleuropein, homovanillic alcohol, and tyrosol, the ability of the compounds to scavenge OH• was determined. Hydroxytyrosol and oleuropein were potent scavengers of OH• compared with homovanillic alcohol and tyrosol. The high OH• scavenging activity of hydroxytyrosol and oleuropein can be attributed to the presence of a catechol moiety in their molecular structure.

In catechol, each ortho OH group has a high electron-donating effect on the other OH group. Weakening of the OH bond by this electron donation facilitates transfer a hydrogen atom to OH•, converting the OH• into water (9, 10). The electron-donating effect of an ortho-methoxy group is also relatively high but significantly lower than that of an ortho-hydroxy group (9). Subsequently, methylation of an OH group in a catechol moiety will reduce the electron-donating effect. This explains the lower OH• scavenging activity of homovanillic alcohol. Tyrosol lacks a potent electron-donating substituent, explaining its poor OH• scavenging activity.

The finding that hydroxytyrosol very efficiently protects the aorta against CHP is consistent with its high OH• scavenging activity. The observed second- order rate constant of hydroxytyrosol (k_s) with OH• in our assay is even higher than the diffusion rate constant. A rate constant higher than the diffusion rate constant can be explained by iron chelation. By chelating iron, hydroxytyrosol is present exactly at the site of OH• generation. This phenomenon has been called site-specific scavenging (5). Iron is also involved in the generation of OH• from CHP in the organ bath model. Apparently, in these experiments, hydroxytyrosol is also present at exactly the right place, i.e. at exactly the site where the radical is formed.

Hydroxytyrosol has an amphiphilic structure ($P_{o/w}$ ~1), which means that its concentration in cytosol and membranes is practically the same. $P_{o/w}$ of 1.1 indicates that hydroxytyrosol will readily cross membranes. The amphiphilic nature also indicates that hydroxytyrosol will provide protection in the cytosol and membranes, including the water-lipid interface. From the difference in protection of the aorta against CHP between hydroxytyrosol and oleuropein, it appears that the presence of a catechol moiety does not guarantee an efficient antioxidant activity. The hydrophilic sugar moiety in oleuropein probably prevents oleuropein from crossing membranes. Therefore, it cannot protect against CHP within smooth muscle or endothelial cells. The sugar moiety also explains its poor bioavailability. The relative amount of orally administered oleuropein that reaches the systemic circulation unchanged is small. *In vivo*, oleuropein can be metabolized into the aglycon hydroxytyrosol in the intestine or the liver (11).

Amphiphilic compounds, such as hydroxytyrosol, are generally well absorbed. Several studies indeed report that the uptake of hydroxytyrosol is good (12-14). The consumption of olive oil in the Mediterranean area is high compared with Western European and Northern European countries. For instance, in Greece the mean yearly intake is ~15 kg per person. Consumption of a real-life dose of 25 ml of olive oil per day (containing ~1 mg of hydroxytyrosol) leads to a plasma concentration of 50 nM (15) to 160 nM (16).

The hydroxytyrosol concentrations tested in our study are higher than those obtained with consumption of olive oil. A relatively high concentration of CHP (300 μM) was required to induce oxidative damage in a relatively short time span (17). This acute *in vitro* model is used to mimic long-lasting oxidative stress *in vivo*, sometimes lasting even more than a decade. A relatively low concentration of hydroxytyrosol (10 μM) protected well against the excess of CHP (300 μM).

The level of oxidative stress in the body is far lower than that generated in the organ baths. This indicates that less hydroxytyrosol than that used in our in vitro study will be needed *in vivo* to protect against oxidative stress. Thus hydroxytyrosol levels reached by the Mediterranean diet are expected to protect against oxidative stress *in vivo*. Moreover, it is quite possible that, *in vivo*, hydroxytyrosol has additive or synergistic effects with endogenous antioxidants or other antioxidants that are abundantly present in the Mediterranean diet.

Various reports on beneficial effects of hydroxytyrosol have been published (18, 19). For example, it has been shown that hydroxytyrosol is able to protect against the oxidation of low-density lipoprotein (LDL) (20), one of the key steps in the initiation of atherosclerosis. In a recent nutritional trial in humans, it was shown that partial substitution of carbohydrate with olive oil products lowered blood pressure, improved lipid levels, and reduced the estimated cardiovascular risk (21). These effects were attributed to the high content of monounsaturated fatty acids in the diet. The results of the present study indicate that phenolic compounds present in olive oil might also contribute to the blood pressure-lowering effect. The beneficial effect of phenolic compounds on endothelial function is supported by a recent study of Ruano *et al.*, in which they showed that the intake of high-phenolic olive oil, when compared to low-phenolic olive oil, improved endothelial function in patients with hypercholesterolemia (22).

In conclusion, hydroxytyrosol is very efficient in protecting the aorta against the oxidative stress induced impairment of the $NO^{\bullet}$ mediated relaxation. $NO^{\bullet}$ is of pivotal importance in the regulation of the vascular tone. The high potency of hydroxytyrosol can be explained by 1) its amphiphilic nature, which results in a good absorption and the presence of hydroxytyrosol in membranes and cytosol, and 2) its efficient radical-scavenging activity, which involves site specific

scavenging. The present study provides a molecular basis for the contribution of hydroxytyrosol to the benefits of the Mediterranean diet.

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CHAPTER 4

A single session of resistance exercise induces oxidative damage in untrained men

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ABSTRACT

During exercise, the production of reactive oxygen and nitrogen species significantly increases. The aim of the present study was to investigate the effects of a single session of resistance exercise on antioxidant capacity, oxidative damage, and inflammation. Muscle biopsies, urine, and blood samples were collected from seven healthy men before and after a single bout of resistance exercise. A single session of resistance exercise was found to induce oxidative damage, as shown by a 40% increase in the concentration of urinary $F_{2\alpha}$-isoprostanes ($P<0.05$). Total antioxidant capacity (TEAC) of plasma increased 16% ($P<0.05$). This increase seemed to be predominantly attributable to an increase in plasma uric acid concentrations of 53% ($P<0.05$). Similar to uric acid, but to a relatively much smaller extent, vitamin C and vitamin E levels in plasma were also elevated ($P<0.05$). Moreover, the erythrocyte glutathione (GSH) concentration increased 47% during exercise ($P<0.05$). Also in skeletal muscle, uric acid levels were found to increase after exercise ($P<0.05$). Moreover, thirty minutes after exercise, skeletal muscle glutathione S-transferase (GST) and glutathione reductase activity increased 28% and 42%, respectively ($P<0.05$). Skeletal muscle reduced glutathione (GSH) and glutathione disulphide (GSSG) concentrations were not affected by exercise. The Nuclear Factor kappa B (NF-κB) activity in peripheral blood mononuclear cells (PBMCs) was not increased by exercise, indicating that a NF-κB mediated inflammatory response does not occur. We conclude that a single session of resistance exercise induces oxidative damage, despite an adaptive increase in antioxidant capacity of blood and skeletal muscle.

INTRODUCTION

The production of reactive oxygen species (ROS) and reactive nitrogen species is significantly enhanced during exercise. For example, superoxide radical ($O_2^{\bullet-}$) production by mitochondria is increased (1). Other potential sources for ROS generation during exercise are phospholipase A_2 dependent processes, plasma membrane nicotinamide adenine dinucleotide phosphate (NADPH) oxidases, and ischemia reperfusion (2-4). During ischemia, xanthine dehydrogenase is converted into xanthine oxidase, an enzyme producing $O_2^{\bullet-}$ especially during reperfusion. Injury to skeletal muscle tissue, such as that induced by exercise, is accompanied by invasion of the injured tissue with phagocytic cells. This further promotes the production of ROS and activates pro-inflammatory pathways such as NF-κB.

Fortunately, the body has an elaborate antioxidant defence system that provides protection against these reactive species. The antioxidant defence system can be subdivided into enzymatic antioxidants, such as glutathione reductase and glutathione S-transferase (GST), and non-enzymatic antioxidants, such as uric acid, glutathione (GSH), vitamin C and vitamin E.

Nonetheless, when the production of reactive species exceeds the protection provided by antioxidants, oxidative stress occurs. This can result in oxidative damage to biomolecules such as proteins and lipids. For example, several studies have shown that exercise induces lipid peroxidation, as demonstrated by an increase in thiobarbituric acid reactive substances (TBARS), isoprostanes and exhaled pentane (5). In nearly all of these studies, endurance exercise was applied (5). Far less information is available on resistance exercise-induced oxidative stress (6-13).

The aim of the present study is to investigate whether the antioxidant defence system is able to cope with the increased radical flux induced by resistance exercise. More specifically, we investigated in untrained healthy males the diverse effects of a single session of resistance exercise on antioxidant activity, oxidative damage, and inflammation.

MATERIALS AND METHODS

Subjects

Eight healthy male volunteers with no history of participating in any regular exercise program were recruited for the present study. One of the subjects was excluded from the statistical analysis because he could not comply with the exercise protocol and had to stop before all the repetitions were performed.

Subjects' characteristics are shown in Table 1. All subjects were informed on the nature and possible risks of the experimental procedures before their written informed consent was obtained. This study was approved by the Medical Ethical Review Board of the Academic Hospital, Maastricht, the Netherlands.

Table 1. Subjects' characteristics

	Mean ± SEM
Age (yrs)	22.4 ± 2.0
Body mass (kg)	74.4 ± 3.3
Height (m)	1.82 ± 0.03
BMI ($kg \cdot m^{-2}$)	22.3 ± 0.8

Values are expressed as mean ± SEM.

Pretesting

Two weeks before the experimental trial, the subjects performed a short exercise session to become familiarized with the exercise protocol and the equipment. Proper weightlifting technique was demonstrated and practiced for each of the two lower-limb exercises (leg press and leg extension). Subsequently, maximum strength was estimated using a standard multiple repetitions testing procedure (14). After warming up, the load was gradually increased, and the successful lifts until failure were determined. A five-minute resting period between subsequent attempts was allowed.
In another session, at least one week after the first session and at least one week before the experimental trial, subjects' one repetition maximum (1RM) for each of the two lower-limb exercises was determined (15). The 1RM of the leg extension was determined initially, followed by that of the leg press.
After warming up, the load was set at 90% of the estimated 1RM, and was increased after each successful lift until failure. Thereafter, the load was increased by 2.5-5% and the successful lifts until failure were determined. A 5-min resting period between subsequent attempts was allowed. A repetition was valid if the subject was able to complete the entire lift in a controlled manner without assistance. 1RM was typically reached within three attempts.
Approximately 16 and 8 repetitions were performed during the first and second exercise sessions, respectively. The two sessions were kept as short as possible to prevent training effects.

Standardization of diet and activity prior to testing

Subjects were instructed not to consume any products rich in antioxidants, such as fruit juices, chocolate, olive oil, wine, or antioxidant supplements during three days before testing, on the test day itself, and on the day after the test day until 24 h after exercise. The low-antioxidant diet was chosen firstly to

minimize a potential protective effect of high antioxidant intake and, secondly, to reduce variation in the baseline level of antioxidants, such as vitamin E and vitamin C. The volunteers consumed a carbohydrate-rich noodle dish, containing approximately 10 g of the vegetable leek, at 8.00 pm in the evening before the test day. Subjects were asked to record their food intake for three days before testing, on the test day itself, and on the day after the test day until 24 h after exercise. Food choices and quantities were recorded. By reviewing the recorded food intake, it was verified that the subjects' diet complied with the instructions. The volunteers were instructed to refrain from any sort of heavy physical exercise during the entire period, except for the resistance exercise session.

Experimental trial

The subjects arrived at the laboratory at 8.00 am, in an overnight fasted state. Then, they performed a 5-min low-intensity warm up using a Stairmaster (Jimsa Benelux BV, Rotterdam, the Netherlands). Thereafter, the resistance exercise session targeted the legs, with eight sets of ten repetitions on the horizontal leg press machine (Technogym BV, Rotterdam, the Netherlands) and eight sets of ten repetitions on the leg extension machine (Technogym). The leg press and leg extension were performed in random order so that two subjects could be tested simultaneously. The starting workload applied during resistance exercise session was 75% of the individual 1RM for both the leg press and leg extension with 2-min rest intervals between sets. In total, the exercise regime required 41 min to complete. This resistance exercise protocol has been published previously and is routinely used as a resistance type of exercise in our institute (14, 15).
If subjects could not finish all ten repetitions at full weight, this was reduced to 65 or 55% of the individual 1RM. Only one of the evaluated volunteers was able to finish the entire protocol at 75%. The average intensity level of the exercise regime was 70%. All subjects were verbally encouraged during the test to complete the entire protocol.

Blood sampling

A Teflon catheter was inserted in an antecubital vein for venous blood sampling. Blood was collected before the start of the exercise (t=0), during exercise (t=20 min), 2 min after cessation of exercise (t=43 min) and during subsequent recovery (t=60 min, 90 min, 120 min, 150 min, 180 min, and 24 h after exercise). Blood samples were collected in tubes containing heparin and placed on ice. Blood was aliquoted for the vitamin C and glutathione and glutathione disulphide (GSH/GSSG) analysis. To preserve the samples, 5%

trichloroacetic acid (TCA) was added to the former, and 1.3% sulphosalicylic acid (SSA) was added to the latter. After centrifugation at 1000·g at 4°C for 5 min, aliquots of plasma were stored at -80°C until analysis.

Blood analyses

Plasma lactate concentrations were analysed spectrophotometrically (340 nm), measuring NADH formation using lactate dehydrogenase (LDH) (16).
The total antioxidant capacity (TEAC) was determined in plasma that was deproteinized with a final concentration of 5% TCA (17). The samples were incubated with the ABTS radical solution for 5 min and subsequently the absorbance at 734 nm was measured. The TEAC is expressed as µM trolox equivalents.
Uric acid was determined in plasma that was deproteinized with a final concentration of 5% TCA, using HPLC. A Hypersil BDS C-18 end-capped column, 125 x 4 mm, particle size 5 µm (Agilent, Palo Alto, CA, USA), was used, with a mobile phase of 0.1% trifluoroacetic acid (v/v) in water. UV detection was performed at 292 nm. The relative contribution of uric acid to the TEAC is calculated using the TEAC value of 1 for uric acid (18).
Vitamin C was determined in plasma by HPLC using the following method. Vitamin C was oxidized by ascorbate oxidase to dehydroascorbate. The latter was condensed with o-phenylene diamine to its quinoxaline derivative. This derivative was quantified on the same system used for uric acid with a mobile phase of 0.08 M phosphate buffer (pH 7.8) and methanol, with fluorimetric detection (355-425 nm).
Vitamin E was extracted using hexane after adding vitamin E nicotinate as internal standard. HPLC analysis was performed on the same system used for uric acid with a mobile phase of methanol. UV detection was used (295 nm).
The erythrocyte non-protein thiol concentration, representing mainly GSH, was measured using 5,5′-dithiobis-2-nitrobenzoic acid (DTNB). The erythrocyte GSSG concentration was measured using 2-vinylpyridine pre-treatment.

NF-κB activity in peripheral blood mononuclear cells (PBMCs)

PBMCs were isolated from the blood samples (collected into leucosep tubes at t=0, 43 min, 90 min, and 24 h after exercise) using Lymphoprep (Bio-Connect, Huissen, the Netherlands). Nuclear extracts were immediately prepared and stored at -80°C until analysis. The protein concentration was determined according to the method of Bradford (BioRad, Veenendaal, the Netherlands).
NF-κB activity was measured using the TransAM NF-κB p50 Activation Assay (Active Motif, Rixensart, Belgium) and expressed as µg Jurkat NF-κB equivalents per µg nuclear protein.

Urine collection

Morning urine was collected on the test day and on the morning of the day after the test day. Aliquots of urine were stored at -80°C until analysis of $F_{2\alpha}$-isoprostanes and creatinine.

Urine analysis

$F_{2\alpha}$-isoprostanes (8-iso $PGF_{2\alpha}$) in urine were analysed by an enzyme immunoassay (Cayman Chemical, Ann Arbor, MI, USA) and were related to creatinine values.

Muscle biopsies

Muscle biopsy samples were taken 30 min before the start of the exercise and 30 min after exercise, to determine the acute effects of exercise. Muscle biopsies were obtained from the middle region of the m. vastus lateralis (15 cm above the patella) and approximately 3 cm below entry through the fascia using the percutaneous needle biopsy technique (19). The second muscle biopsy was taken from the contra lateral leg. Muscle samples were freed from any visible nonmuscle material and were rapidly frozen in liquid nitrogen. Muscle biopsies were stored at -80°C until analysis.

Preparation of muscle homogenates

Frozen muscle biopsies were weighed and subsequently ground with a liquid nitrogen cooled mortar. The powder was resuspended in demineralised water to a final concentration of 250 mg tissue/ml. SSA (final concentration of 1.3%) was added to a small part of the homogenates, for the analysis of GSH and GSSG. The homogenates were directly frozen in liquid nitrogen and were subsequently stored at -80°C until analysis. The homogenates were centrifuged at 14,000·g at 4°C for 3 min. The muscle biopsy supernatant was used for analysis.

Muscle biopsy analyses

GST activity, glutathione reductase activity, uric acid and protein levels in muscle biopsies were determined according to the methods of Julicher *et al*. (20). GST activity was determined by the enzymatic reaction of chlorodinitrobenzene with GSH. The increase in the formation of the coloured product of the reaction was measured at 340 nm for 2 min. Glutathione reductase activity was measured by determining the consumption of NADPH in the enzymatic reduction of GSSG to GSH. The decrease in absorption at 340 nm was meas-

ured for 2 min. Skeletal muscle GSH and GSSG concentrations were measured according to the method for the determination of erythrocyte GSH and GSSG concentrations, as described above.
Uric acid concentrations were determined in muscle biopsies of six subjects. In general, all analyses were performed within a month after sample collection.

Statistics

All data are expressed as means ± SEM. The non-parametric, repeated-measures ANOVA of Friedman was applied to determine differences over time in plasma antioxidant concentrations, lactate levels, and NF-κB activity. In cases of significant differences over time, a post hoc test, described by Siegel and Castellan (21), was used to locate the differences in time (vs t=0). The Wilcoxon signed-rank test was used to compare pre- (t=0) versus post-exercise values (muscle biopsies, urine and 24h plasma). Statistical significance was set at $P<0.05$.

RESULTS

Resistance exercise

Mean 1RM measured during pretesting was 195 ± 11 kg on the horizontal leg press and 117 ± 6 kg on the leg extension. The starting workload applied during resistance exercise averaged 146 ± 8 kg and 88 ± 5 kg (75% of 1RM) for the leg press and leg extension, respectively. For most of the participants, this workload had to be reduced, as specified in the material and method section. The average intensity level of the exercise regime was 70%.

Lactate

Plasma lactate levels markedly increased during exercise ($P<0.05$) and subsequently decreased during recovery (Figure 1).
Plasma lactate concentrations during exercise and subsequent recovery were identical to those reported by Koopman *et al.* (15), who applied the same exercise protocol. Twenty-four hours after exercise, lactate levels were slightly elevated compared with baseline levels (1.39 ± 0.21 mM at 24 h vs 0.86 ± 0.08 mM at t=0; $P<0.05$).

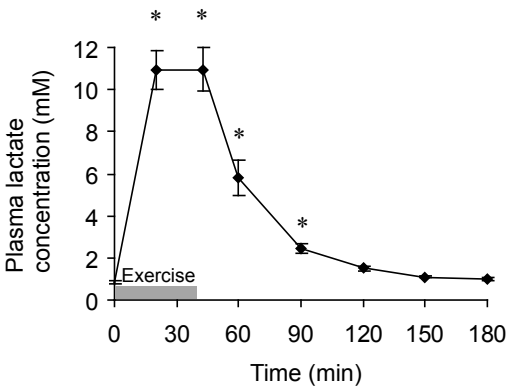

Figure 1. Plasma lactate levels at baseline, during resistance exercise (duration is indicated by the grey bar) and subsequent recovery. Data provided are means ± SEM.
* Significantly different from pre-exercise values at baseline (P<0.05).

Plasma antioxidant capacity

Plasma TEAC increased during exercise and reached maximal values 50 min after exercise (maximal increment of 16 ± 4%; Figure 2A). Twenty-four hours after exercise, TEAC values remained elevated compared with baseline values (784 ± 19 μM at 24 h vs. 743 ± 19 μM trolox equivalents at t=0; P<0.05). Plasma uric acid concentrations gradually increased during exercise. After exercise, the uric acid concentration increased up to 153 ± 13% (t= 90 min; P<0.05), after which it slightly decreased (Figure 2B). The uric acid concentration remained elevated even up to 24 h after exercise (366 ± 32 μM at 24 h vs. 295 ± 21 μM at t=0; P<0.05). The antioxidant uric acid significantly contributes to the TEAC. Subtracting the contribution of uric acid from the TEAC gives the 'uric acid-subtracted TEAC' value. The 'uric acid-subtracted TEAC' markedly decreased during exercise with a maximum decline of 14 ± 2% after exercise (Figure 2C). Twenty-four hours after exercise, the 'uric acid-subtracted TEAC' remained below baseline (419 ± 19 μM at 24 h vs. 449 ± 7 μM trolox equivalents at t=0; P<0.05).
Plasma vitamin C concentrations elevated from 15.3 ± 0.6 μM at baseline to 16.4 ± 0.7 μM after exercise (t=43 min; P<0.05). Also at t=150 and 180 min vitamin C concentrations were elevated compared to baseline (P<0.05) (Figure 3A). Twenty-four hours after exercise, plasma vitamin C concentrations were equal to those at baseline (15.3 ± 0.5 μM at 24 h vs. 15.3 ± 0.6 μM at t=0). Plasma vitamin E concentrations increased from 10.0 ± 0.4 μg/ml at baseline to 10.9 ± 0.5 μg/ml during exercise (t=20 min) (P<0.05) and returned to baseline levels during recovery (Figure 3B). Plasma vitamin E concentrations were slightly elevated 24 h after exercise (10.5 ± 0.5 μg/ml at 24 h vs. 10.0 ± 0.4 μg/ml at t=0; P<0.05).

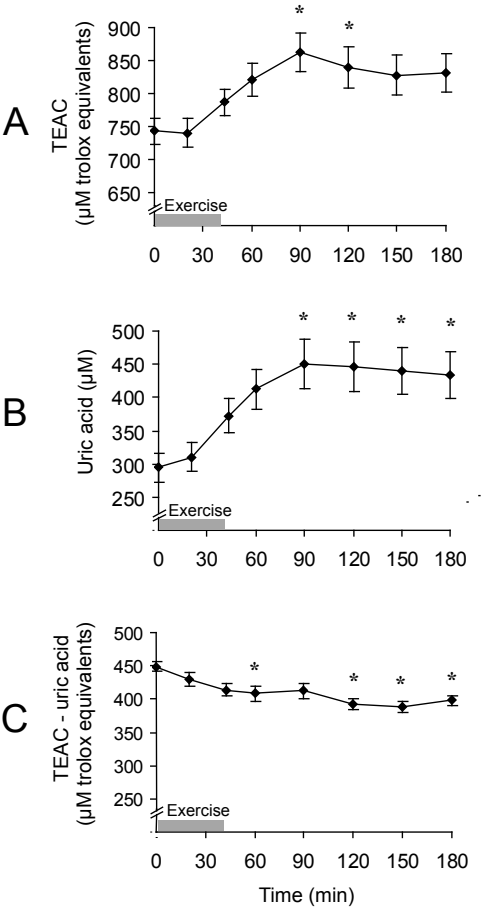

Figure 2. Plasma TEAC values (panel A), uric acid concentrations (panel B) and TEAC values, corrected for uric acid (panel C), at baseline, during resistance exercise (duration is indicated by the grey bar) and subsequent recovery. Data provided are means ± SEM.
* Significantly different from pre-exercise values at baseline ($P<0.05$).

NF-κB activity

Exercise did not induce changes in NF-κB activity in PBMCs. The NF-κB activity at t=43 min, t=90 min, and 24 h (respectively 0.49 ± 0.01, 0.52 ± 0.02 and 0.53 ± 0.04 µg Jurkat equivalents/µg nuclear protein) after exercise was similar to the NF-κB activity at baseline (0.53 ± 0.04 µg Jurkat equivalents/µg nuclear protein).

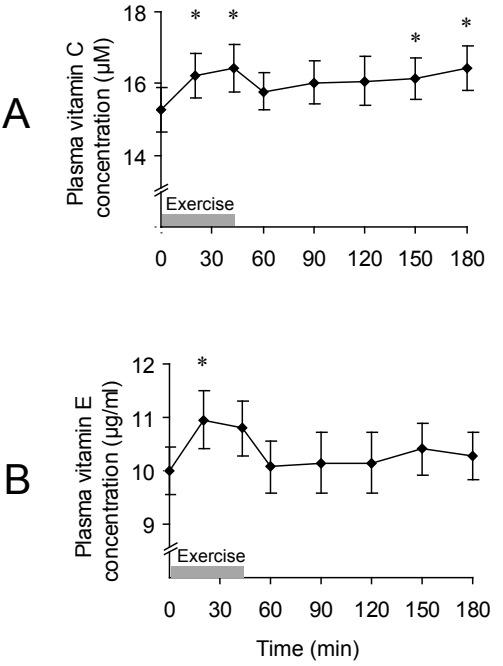

Figure 3. Plasma vitamin C (panel A) and vitamin E (panel B) concentrations during resistance exercise (duration is indicated by the grey bar) and subsequent recovery. Data provided are means ± SEM. * Significantly different from pre-exercise values at baseline (P<0.05).

8-iso $PGF_{2\alpha}$ levels in urine

The concentration 8-iso $PGF_{2\alpha}$ in urine increased from 0.117 ± 0.021 nmol/mmol creatinine pre-exercise to 0.164 ± 0.030 nmol/mmol creatinine post-exercise (P<0.05). $F_{2\alpha}$-isoprostanes are prostaglandin-like biomarkers specific for ROS-mediated peroxidation of arachidonic acid, a poly unsaturated fatty acid (PUFA) present, for example, in membranes (22).

Antioxidant activity in skeletal muscle

Uric acid concentrations in skeletal muscle increased from 82 ± 6 µmol/kg muscle pre-exercise to 106 ± 9 µmol/kg muscle 30 min after exercise (P<0.05). GST activity in skeletal muscle increased from 0.224 ± 0.032 U/mg protein at rest to 0.286 ± 0.014 U/mg protein 30 min after exercise (P<0.05; Figure 4). Glutathione reductase activity in skeletal muscle increased from 10.4 ± 1.8 mU/mg protein at rest to 14.8 ± 2.4 mU/mg protein 30 min after exercise (P<0.05; Figure 4).

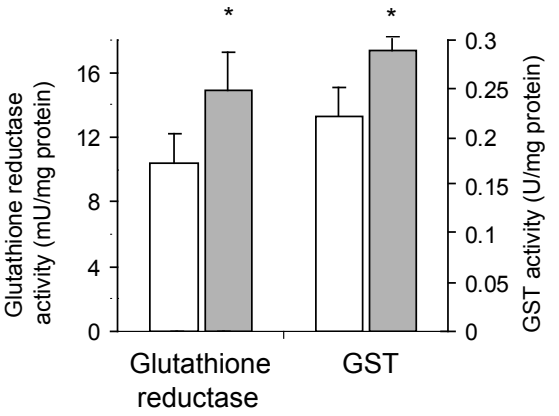

Figure 4. Glutathione reductase and GST activities in skeletal muscle at baseline (white bars) and 30 min after exercise (grey bars). Data provided are means ± SEM.
* Significantly different from pre-exercise values at baseline ($P<0.05$).

GSH and GSSG concentrations in skeletal muscle were not affected by exercise. In contrast, erythrocyte GSH concentrations increased 47% during exercise, from 10.9 ± 2.4 nmol/mg Hb at baseline to 16.0 ± 1.9 nmol/mg Hb during exercise (t=20 min; $P<0.05$), and returned to baseline values during recovery (Figure 5). The erythrocyte GSSG concentration remained at baseline value.

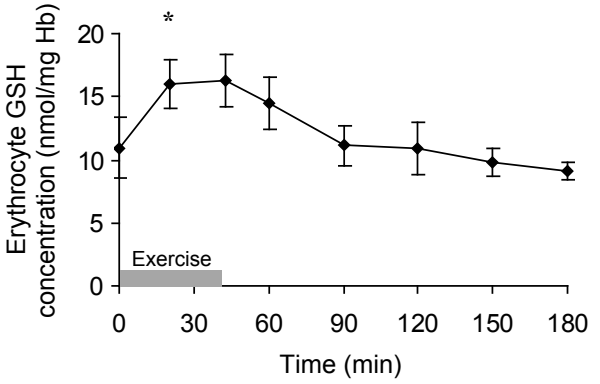

Figure 5. Erythrocyte GSH concentrations at baseline, during exercise (duration is indicated by the grey bar) and subsequent recovery. Data provided are means ± SEM.
* Significantly different from pre-exercise values at baseline ($P<0.05$).

DISCUSSION

Exercise enhances the production of ROS. The aim of the present study was to investigate whether the body's antioxidant defence system is able to cope with the increased radical flux induced by a single session of resistance exercise.

Antioxidants protect against radicals by converting them to chemically less reactive species. In this scavenging reaction, the antioxidant becomes oxidized or chemically modified in another way. Therefore, it was expected that antioxidant levels would be reduced by increased radical production during exercise. However, in the present study, a single session of resistance exercise was found to increase the concentration of several antioxidants.

The increase in the total antioxidant capacity (TEAC) of plasma appeared to be predominantly due to elevated uric acid concentrations. A similar increase in plasma uric acid by exercise has been reported previously (23). Also in skeletal muscle, uric acid levels were found to increase after exercise.

The increase in uric acid might be caused by the exercise-induced breakdown of ATP, as well as the activation of xanthine oxidase that forms, besides $O_2^{\bullet-}$, also uric acid. The inhibition of renal clearance of uric acid by lactate accumulation may also be responsible for the rise in uric acid level (24). In the present study it was shown that lactate levels rose significantly during resistance exercise. The elevated uric acid levels persisted a long time after exercise. This can be explained by the relatively long half-life of uric acid of approximately 20 hours (25). The rise in uric acid levels might be a physiological mechanism to counteract an increased radical production by exercise.

Similar to uric acid, vitamin C and vitamin E levels in plasma were also elevated during exercise. In the case of vitamin E, this has previously been reported (8). This relatively small increase in the concentrations of vitamin C and vitamin E has a negligible effect on the plasma TEAC value.

In contrast to vitamin C and vitamin E, uric acid substantially contributes to the TEAC. Subtracting the contribution of uric acid from the TEAC gives the 'uric acid-subtracted TEAC' value. Resistance exercise induces a decrease in this 'uric acid-subtracted TEAC', which indicates that other antioxidants responsible for the TEAC are consumed during exercise. This consumption of antioxidants confirms that radicals have been produced by the applied resistance exercise regime.

Exercise induced a large increase in the concentration of GSH in erythrocytes. Similar to the rise in uric acid, this might be an adaptive response to increased radical production. The origin of the increase in GSH is unclear. In skeletal muscle, no increase in GSH was found.

The activity of the antioxidant enzymes GST and glutathione reductase in skeletal muscle was shown to significantly increase by a single session of resistance exercise. In rodents, antioxidant enzymes in skeletal muscle have

also been reported to increase, especially after repetitive prolonged bouts of exercise (26). The enhancement in skeletal muscle antioxidant enzyme activity probably involves the activation of the antioxidant responsive element (ARE) pathway by exercise-induced radical formation (27). Similar to the rise in uric acid and erythrocyte GSH levels, this can also be regarded as an adaptive response to increased radical production.

The rise in urinary $F_{2\alpha}$-isoprostanes indicates that resistance exercise induces oxidative damage. This indicates that the adaptive responses in blood and skeletal muscle do not provide full protection against the increased radical flux induced by the applied resistance exercise.

Injury to skeletal muscle tissue is accompanied by invasion of the area with phagocytic cells, which further promotes the production of ROS and activates pro-inflammatory pathways such as NF-κB. The results of the present study indicate that the oxidative damage induced by a single bout of resistance exercise does not induce a NF-κB mediated inflammatory response in untrained healthy volunteers.

No consensus exists on the effect of resistance exercise on markers of oxidative stress (6). This topic has been reviewed in detail by Bloomer and Goldfarb (6). Some studies show that resistance exercise induces oxidative damage (6, 8, 10-12), by demonstrating increased levels of malondialdehyde (MDA) or protein carbonyls, while other studies show no increase in oxidative damage (6, 7, 9). This discrepancy might be explained by a number of factors, such as differences in biomarkers studied, the type and duration of resistance exercise or whether or not subjects were trained. For example, chronic anaerobic training might enhance the endogenous antioxidant defence (6), which might attenuate exercise-induced oxidative damage. Nevertheless, in trained individuals, resistance exercise has also been reported to induce oxidative damage to proteins or lipids (10, 11, 28).

A negative health effect of a high radical flux induced by exercise is expected to be more pronounced in individuals with a compromised antioxidant defence system (29, 30), such as in COPD or type 2 diabetes. Besides an impaired antioxidant defence, these patients have a relatively high production of radicals even under basal conditions (31, 32). Interestingly, exercise is recommended in the treatment of these patients. Antioxidant supplementation during exercise might especially be relevant for these patients, as it strengthens the antioxidant defence against ROS generated during exercise. Antioxidant supplementation should not affect the positive health effects of exercise. Although free radicals are typically considered as destructive species, current evidence indicates that in specific situations some reactive species might have a beneficial health effect (2).

Despite the exercise-induced adaptive increase in antioxidant capacity of blood and skeletal muscle, a single session of resistance exercise induces oxidative

damage in healthy men. The challenge is 1) to unravel the mechanisms that underlie both the positive and negative health effects of ROS generated during exercise and 2) to selectively block those pathways that have negative health effects without affecting the beneficial processes. Undoubtedly, antioxidants will come into play.

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CHAPTER 5

The olive oil antioxidant hydroxytyrosol protects against oxidative stress-induced reduction in skeletal muscle force production

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Submitted

ABSTRACT

The aim of the present study was to investigate the protective effect of the olive oil antioxidant hydroxytyrosol against oxidative stress-induced impairment of skeletal muscle contractile function. The isolated rat diaphragm was used to study skeletal muscle function *in vitro*. Hydroxytyrosol concentration-dependently protected the muscle strips against the H_2O_2-induced impairment in force production during short-term stimulation at a wide range of stimulation frequencies (10-100 Hz). A relatively low concentration of hydroxytyrosol (25 μM) protected well against 1 mM H_2O_2. Hydroxytyrosol also protected the muscle strips against the H_2O_2-induced impairment of force production during especially the initial stage of a long-term fatigue protocol. The protection provided by hydroxytyrosol was not based on a direct H_2O_2 scavenging effect. H_2O_2 can be converted to other, much more reactive species, such as the highly reactive hydroxyl radical ($OH^{\bullet}$). The excellent antioxidant efficacy of hydroxytyrosol is illustrated by its potent $OH^{\bullet}$ scavenging activity. The *in vivo* impact of the high antioxidant potency of hydroxytyrosol might not only lie in the field of muscle protection during strenuous exercise, but also in conditions such as ventilatory insufficiency or chronic fatigue syndrome.

INTRODUCTION

Skeletal muscle fibres continuously generate reactive oxygen species (ROS) at a slow rate (1). This rate increases during muscle contraction. Over the past decades, progress has been made in the understanding of both the beneficial and detrimental roles that ROS play in skeletal muscle. Low levels of ROS have been implicated in optimal muscle contractile function (1). However, a high and unbalanced production of ROS will lead to oxidative damage (2-4). ROS act indiscriminately. Some ROS are able to damage virtually any cellular component, including proteins, lipids and DNA.

ROS can affect skeletal muscle force production by damaging proteins that are important in regulating cytosolic free Ca^{2+}, such as the sarcoplasmic reticulum ryanodine receptor (RYR) and Ca^{2+} ATPases (5). Moreover, ROS are also able to modify an array of other regulatory proteins in skeletal muscle including myofibrillar proteins (6, 7), mitochondrial proteins (8) and proteins that regulate glucose metabolism (9). The detrimental effects of ROS provide the rationale for antioxidant supplementation to reduce oxidative muscle damage and the fatigability of muscle cells.

Hydroxytyrosol (Figure 1), a phenolic compound present in virgin olive oil, has been shown to be a very effective antioxidant (10). For example, it has been reported that hydroxytyrosol efficiently protects vascular tissue against oxidative stress (11). Moreover, hydroxytyrosol is able to reduce oxidative damage in intestinal epithelial cells (12), hepatocytes (13), and erythrocytes (14).

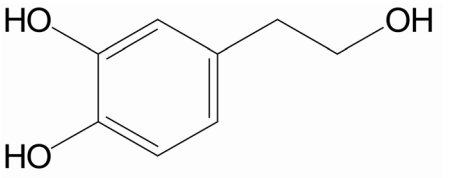

Figure 1. Molecular structure of hydroxytyrosol.

The aim of the present study is to investigate the protective effect of hydroxytyrosol against the oxidative stress-induced reduction in skeletal muscle force production. H_2O_2 is used to induce oxidative stress in skeletal muscle. In the present study we use the *in vitro* rat diaphragm preparation, which is a well-established model to study skeletal muscle function *in vitro* (15).

MATERIALS AND METHODS

Chemicals

Hydroxytyrosol was obtained from Cayman Chemical Company, Ann Arbor, MI, USA. Mannitol, 2-deoxy-D-ribose, H_2O_2 and ferric chloride hexahydrate were purchased from Sigma-Aldrich, St Louis, USA. Ascorbate was obtained from Merck, Darmstadt, Germany. All other chemicals were of analytical grade purity.

Isolated diaphragm muscle strips

The experimental protocol was approved by the Ethics Committee for Animal Experiments of the University of Maastricht before the start of the study. Male Lewis rats (12-14 wk old) were decapitated, the diaphragm was rapidly excised, and small strips (around 2 mm width and 1 cm long) were mounted in thermostated organ baths containing Krebs buffer (pH 7.4) gassed with 95% O_2-5% CO_2. Each diaphragm strip was connected vertically to an isometric transducer. The composition of the Krebs buffer was (mM): NaCl (117.5), KCl (5.6), $MgSO_4$ (1.18), $CaCl_2$ (2.5), NaH_2PO_4 (1.28), $NaHCO_3$ (25) and glucose (5.5). Field stimulation was created along the entire length of each muscle strip with platinum electrodes.
At the beginning of the experiment, the strips were washed during 45 min at room temperature. Fifteen minutes before the start of the experiment, the temperature of the baths was increased to 37°C. Each strip was first adjusted to its optimal length (L_o) using twitch contractions. After the adjustment to L_o, the strips were washed during 15 min.

Measurement of contractile function

Firstly, the response of the diaphragm strips to single pulse stimulation, i.e. single twitch, was determined. Thereafter, the response of the diaphragm muscle strips to increasing stimulus frequencies was assessed by the application of 10, 20, 33, 50 and 100 Hz-pulses applied in 500 ms trains. A 2-min recovery period was used between contractions. After the completion of this short-term stimulation protocol, a fatigue protocol was applied. During the fatigue protocol, the strips were stimulated for 6 min at 5 Hz. The pulse duration was 10 ms during the entire experiment. Subsequently, the strips were washed during 15 min.

Incubations

When hydroxytyrosol was used, it was added 5 minutes before H_2O_2 was added. Hydroxytyrosol remained in the organ bath during the incubation with H_2O_2. In some experiments, the muscle strips were incubated only with hydroxytyrosol, i.e. without the subsequent addition of H_2O_2. In control experiments neither H_2O_2, nor hydroxytyrosol was added to the organ baths.
Ten min after the addition of H_2O_2, the strips were washed for 15 min. After washing the diaphragm, the contractile function of the muscle strips (short-term stimulation and fatigue) was determined for a second time as described above. In this way, each muscle bundle served as its own control (Figure 2).

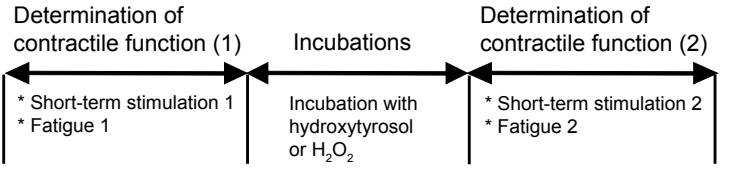

Figure 2. Schematic illustration of the various procedures of the study in time. Details on the protocol are given in the Materials and Methods section.

Calculation of the effects of hydroxytyrosol and H_2O_2 on contractile function

The contractile function of the muscle strips was determined before ('stimulation 1') and after incubation ('stimulation 2') with hydroxytyrosol and H_2O_2 (Figure 2). The force production of the muscle strips after incubation with H_2O_2 and hydroxytyrosol was compared with the initial force production before the incubations ('stimulation 1'). The effects of H_2O_2 and hydroxytyrosol on force production were expressed as the percentage of the initial force production.

H_2O_2 scavenging

Scavenging of H_2O_2 was measured by directly measuring the hydroxytyrosol concentration after adding 1 mM H_2O_2 to a 100 µM solution of the hydroxytyrosol in a 150 mM sodium phosphate buffer (pH 7.4, 37°C). UV Spectra were recorded before and during 30 min after the addition of H_2O_2.

$OH^{\bullet}$ scavenging

Scavenging of $OH^{\bullet}$ was determined by the deoxyribose method according to Haenen *et al.* (16).

Statistics

Values are means ± SEM. Differences in the response of the muscle strips were statistically analysed by Mann Whitney U. $P<0.05$ was considered statistically significant.
Control experiments (neither H_2O_2, nor hydroxytyrosol present) were performed on muscle strips obtained from five rats. The experiments with H_2O_2 (no hydroxytyrosol present), were performed on muscle strips of eight (1 mM H_2O_2) and two (2 mM H_2O_2) rats. The experiments with hydroxytyrosol were performed on muscle strips of three rats. Muscle strips of respectively one and two rats were used when 100 and 300 µM hydroxytyrosol was incubated before the addition of H_2O_2. For the experiments in which muscle strips of two rats were used, the data are presented as mean ± half range.

RESULTS

Short-term electrical muscle stimulation

Firstly, the concentration-dependent effect of H_2O_2 on force production was investigated. The force production of the muscle strips was determined before ('short-term stimulation 1') and after ('short-term stimulation 2') the incubation with H_2O_2 and hydroxytyrosol.

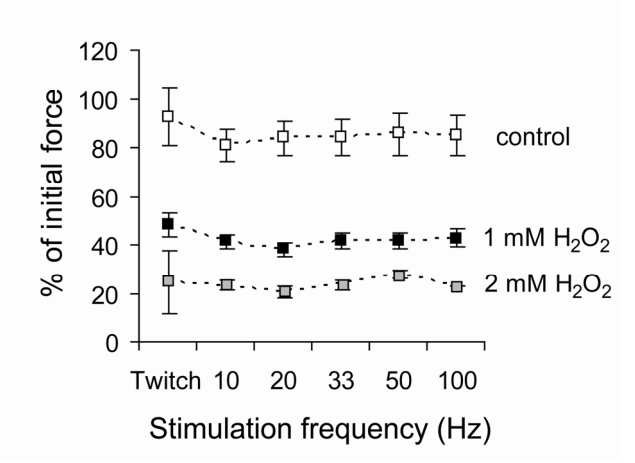

Figure 3. Effect of 1 mM (black squares) and 2 mM (grey squares) H_2O_2 on force production of diaphragm strips during short-term stimulation, compared with control (white squares). Values are the percentage of the initial force production during stimulation protocol 1.

In the control experiment without H_2O_2, force production during 'short-term stimulation 2' was approximately 80% of that of 'short-term stimulation 1', at both low and high stimulation frequencies (Figure 3, white squares).
Incubation of the diaphragm muscle strips with H_2O_2 resulted in a considerable decrease in force production at a wide range of stimulation frequencies (Figure 3). Exposure of the muscle strips to H_2O_2 resulted in a 50% (1 mM H_2O_2) to 75% (2 mM H_2O_2) decrease in force production, compared to that of control muscle strips that were not exposed to hydroxytyrosol or H_2O_2 ($P<0.05$; Figure 3 and Figure 4). Based on these results, it was decided to use a concentration of 1 mM H_2O_2 to study the protective effect of hydroxytyrosol, as this concentration gave a substantial but submaximal effect.

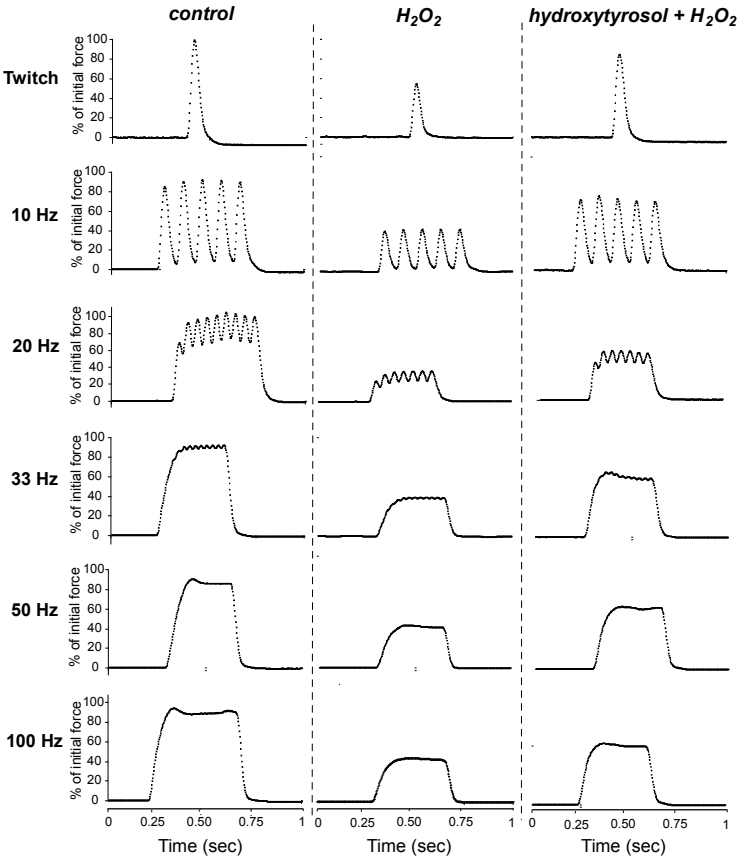

Figure 4. Effect of H_2O_2 (1 mM) on force production of diaphragm strips during short-term stimulation, compared to control. The protective effect of 50 μM hydroxytyrosol is also shown. Diaphragm strips were stimulated before and after the addition of H_2O_2 and hydroxytyrosol. Values are the percentage of the initial force production during stimulation protocol 1. Representative experiments are shown.

Hydroxytyrosol protected the muscle strips against the H_2O_2-dependent decrease in force production (Figure 4 and Figure 5). Even a relatively low concentration of 25 μM hydroxytyrosol significantly protected against 1 mM H_2O_2 ($P<0.05$) (Figure 5A). The dose-dependency of the protective effect of hydroxytyrosol against the H_2O_2-induced decline in force production during short-term 50 Hz stimulation is depicted in Figure 5B. A concentration of 300 μM hydroxytyrosol resulted in a protective effect of approximately 75% (Figure 5B).

Hydroxytyrosol alone, in a concentration of 300 μM, did not affect muscle contractile function in response to short-term electrical stimulation (data not shown).

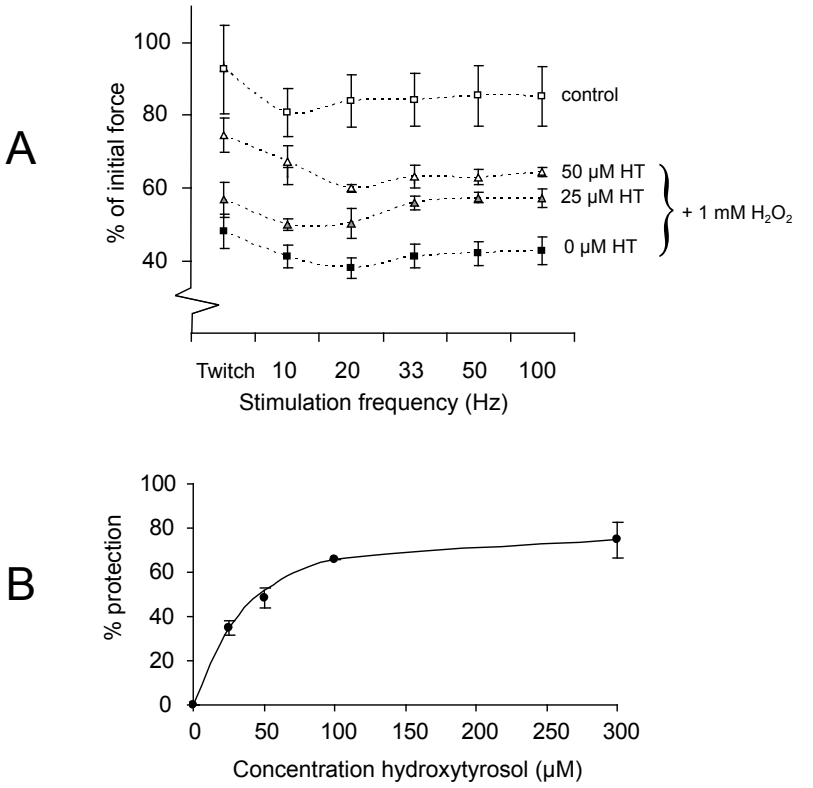

Figure 5. Protective effect of hydroxytyrosol (white and grey triangles) against the H_2O_2 (1 mM, black squares) induced decrease in force production during short-term stimulation, compared to control (white squares) (panel A). Different stimulation frequencies are shown. Values are the percentage of the initial force production during stimulation protocol 1. The concentration-dependent protection of hydroxytyrosol (HT) against the H_2O_2 (1 mM) induced decline in force production at a stimulation frequency of 50 Hz is shown in panel B.

Fatigue

During the first two min of long-term electrical muscle stimulation a rapid decline in force production was observed. Subsequently, the force production gradually decreased during the last 4 min of the fatigue protocol (Fatigue 1; Figure 6 and Figure 7). When the muscle strips were once again stimulated for 6 min (Fatigue 2), force production was approximately 20% lower compared to Fatigue 1. In these control experiments the diaphragm muscle strips were not incubated with hydroxytyrosol or H_2O_2 (Figure 6A and Figure 7).

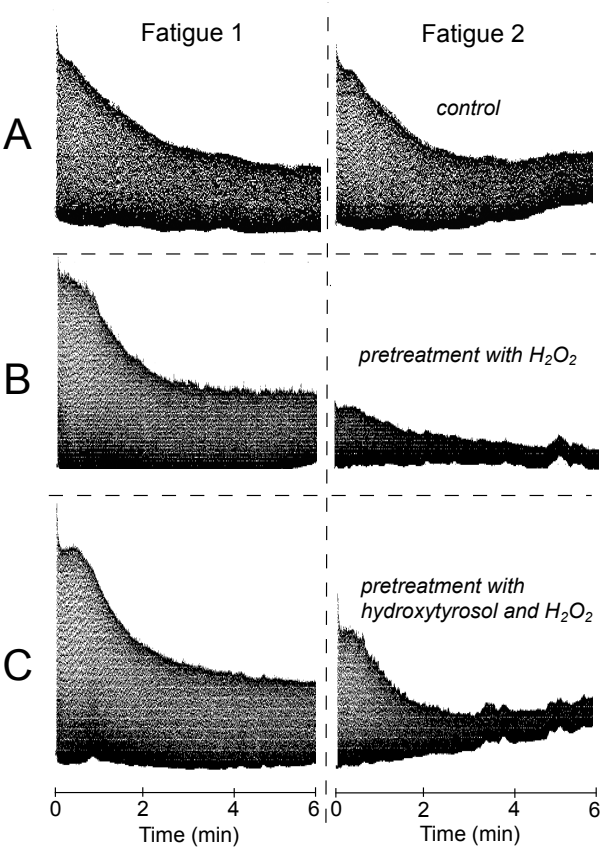

Figure 6. Effect of H_2O_2 (1 mM) on force production of diaphragm strips during long-term stimulation, compared to control. The protective effect of 50 μM hydroxytyrosol is also shown. Representative recorder traces are shown.

H_2O_2 drastically impaired the initial and long term force production during the 6-min stimulation protocol (Fatigue 2; Figure 6B and 7). Hydroxytyrosol protected the muscle strips against the H_2O_2-induced impairment in force

production, especially during the initial stage of fatigue (Fatigue 2; Figure 6C and Figure 7). Hydroxytyrosol alone, in a concentration of 300 μM, did not affect diaphragm force production during the long-term fatigue protocol (data not shown).

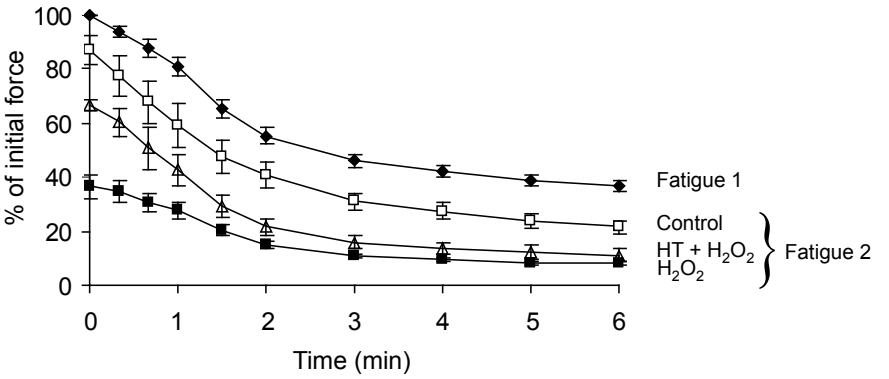

Figure 7. Effect of long-term stimulation on force production of diaphragm strips (black quadrangle). Effect of H_2O_2 (1 mM) (black squares) on force production of diaphragm strips during long-term stimulation, compared to control (white squares). The protective effect of 50 μM hydroxytyrosol (HT) is also shown (white triangles). Values are the percentage of the initial force production.

H_2O_2 and $OH^{\bullet}$ scavenging

Hydroxytyrosol did not show any H_2O_2 scavenging activity. In contrast, hydroxytyrosol was found to be a potent $OH^{\bullet}$ scavenger. The $OH^{\bullet}$ scavenging activity of hydroxytyrosol (second order rate constant, $k_s = 25.1 \cdot 10^9$ $M^{-1}s^{-1}$) was nearly seven times higher than the activity of mannitol, a well known $OH^{\bullet}$ scavenger ($k_s = 3.4 \cdot 10^9$ $M^{-1}s^{-1}$).

DISCUSSION

The production of ROS is significantly enhanced during skeletal muscle contractile activity. When the production of ROS exceeds the protective effect provided by antioxidants, oxidative damage will occur (2, 3). In skeletal muscle, this will lead to reduced force production. Therefore, increasing the antioxidant capacity of skeletal muscle cells seems to be valuable to reduce oxidative muscle damage and preserve the contractile function.

A number of studies have investigated the effect of exogenous antioxidants on skeletal muscle contractile function. For example, N-acetylcysteine (NAC) has been shown to attenuate fatigue in both rat (17) and rabbit (18) diaphragms. Moreover, NAC has been shown to delay fatigue in several human intervention studies (19-22). In contrast, incubation of rat diaphragm with NAC depressed the contractility during short-term stimulation at low frequencies. Similar to NAC, vitamin E also reduced the force production of isolated diaphragm strips during short-term stimulation at low stimulation frequencies (15). In contrast to NAC, vitamin E did not affect fatigue during long-term stimulation. However, trolox, a water-soluble vitamin E analog, attenuated the mechanical ventilation-induced impairment in contractile function of rat diaphragm (23). The antioxidant lipoic acid did not affect skeletal muscle contractile function (15). Although the effects of various antioxidants on contractile performance are not unequivocal, the common view is that antioxidants might protect skeletal muscle force production during exercise.

In the present study it was shown that hydroxytyrosol efficiently protected against the H_2O_2-induced reduction in force production especially during the initial stage of fatigue. Moreover, hydroxytyrosol also protected the muscle strips against the H_2O_2-induced reduction in force production during short-term stimulation, at a wide range of stimulation frequencies.

A relatively low concentration (25 μM) of hydroxytyrosol protected significantly against a forty-fold higher concentration of H_2O_2 (1 mM). Moreover, hydroxytyrosol displayed no H_2O_2 scavenging activity. This indicates that H_2O_2 is converted to another reactive species that subsequently impairs skeletal muscle contractile function, such as the highly reactive $OH^{\bullet}$. Because of its extreme reactivity, $OH^{\bullet}$ acts in an indiscriminate way. It is able to destruct any cellular component. This can have far-reaching consequences. For example, $OH^{\bullet}$ can induce lipid peroxidation, leading to damage of cytoskeleton and membranes and, consequently, cell dysfunction. The excellent antioxidant efficacy of hydroxytyrosol is illustrated by its potent $OH^{\bullet}$ scavenging activity, which is nearly seven times higher than the activity of mannitol, a well known $OH^{\bullet}$ scavenger.

Muscle contraction involves a chain of sequential reactions. Like any other cellular process, all of these reactions can be inhibited by radicals. One of these reactions will be relatively more susceptible to radical damage than the other reactions involved in muscle contraction. This reaction, referred to as 'the bottle neck', will become limiting in muscle contraction during oxidative stress (24). By breaking this weakest link, the chain of reactions leading to contraction is broken, thereby limiting force production and concomitant ROS production.

Reid compared this self-limiting radical production with 'a-canary-in-the-coal-mine' (24). A canary is relatively sensitive to toxic gasses. Therefore, miners used to take a canary into the mine as a biosensor. When the canary dropped

dead, this signalled the miners to immediately evacuate the mine in order to avoid the same fate. The comparison, however, falls short as the 'sensor', i.e. the weakest link in muscle contraction, is an integral part of the chain of reactions that needs to be protected. Moreover, radicals have a very limited specificity and will also react with cell components other than the sensor. With regard to the indiscriminate action of radicals, the metaphor of 'a-bull-in-a-china-store' seems to be more appropriate. The present study shows that the species that are involved are very reactive, comparable to that of $OH^{\bullet}$. Such a reactive species will destroy anything that comes in its way.

In line with 'the-canary-in-the-coal-mine' concept, Reid argued that the use of antioxidants to increase performance might backfire. Antioxidants were supposed to only reduce the sensitivity of the sensor. The trade-off of antioxidant supplementation would be better short-term performance at the expense of greater exercise-induced injury and longer recovery times (24). The sensor will relatively benefit the most from antioxidant supplementation. However, by antioxidant supplementation the other parts of the chain of reactions leading to muscle contraction are protected to the same degree as the sensor. Also after antioxidant supplementation the sensor remains the weakest link. After antioxidant supplementation, more exercise induced ROS have to be produced to inactivate the sensor, or, in other words, to break this weakest link. The high vulnerability of the weakest link relative to that of the other parts of the chain is not changed by antioxidant supplementation. This indicates that the theoretical trade-off for the use of antioxidants to increase performance is not plausible. In our opinion, by 'taming the bull', i.e. by inactivating radicals, antioxidants protect the entire 'china store'.

For an antioxidant to have beneficial health effects *in vivo* it is of course essential that it is bioavailable. Several studies in humans and rats indeed report that the uptake of hydroxytyrosol is good (25-28). Plasma concentrations of hydroxytyrosol after consumption of 25 ml of extra virgin olive oil range from 50 to 160 nM (29, 30). The consumption of supplements containing a relatively high amount of hydroxytyrosol might lead to even higher plasma hydroxytyrosol concentrations.

The concentrations of hydroxytyrosol tested were, compared to the concentrations reached *in vivo*, relatively high. However, the concentration of H_2O_2 required to induce oxidative damage in the isolated diaphragm in a relatively short time span was also relatively high, i.e. 1 mM. The level of oxidative stress in the body is lower than that generated in the organ baths. Nevertheless, hydroxytyrosol was able to provide substantial protection against the relatively high concentration of H_2O_2. This indicates that hydroxytyrosol has the potency to protect against oxidative stress in skeletal muscle *in vivo*. Several studies have demonstrated that a real-life intake of hydroxytyrosol displays a physiological relevant antioxidant effect. For example, hydroxytyrosol protects against

the oxidative modification of LDL (10). This shows that hydroxytyrosol behaves as an efficient antioxidant *in vivo*.

One of the problems in comparing the efficacy of hydroxytyrosol with that of antioxidants used in other studies is that the concentrations used of both antioxidant and oxidant and the applied study design are highly variable. Compared to the most frequent studied antioxidant in muscle concentration, i.e. NAC, hydroxytyrosol seems to have at least the same the same potency in the isolated diaphragm. Both hydroxytyrosol and NAC did reduce fatigue during long-term stimulation (17, 18). However, hydroxytyrosol, in contrast to NAC, did not reduce the force production of isolated diaphragm strips at low stimulation frequencies. It should be noted that the present study is - to our knowledge - the first study thus far on the effects of hydroxytyrosol on skeletal muscle force production and *in vivo* data on this subject are lacking.

In principle, hydroxytyrosol supplementation might have several applications. Firstly, hydroxytyrosol might mitigate exercise induced oxidative muscle damage (3, 4, 31). Furthermore, hydroxytyrosol could theoretically be used as a performance-enhancing nutraceutical in certain conditions. For example, in several respiratory diseases, including chronic obstructive pulmonary disease (COPD), diaphragm force generation is impaired. Impaired respiratory muscle function causes ventilatory failure and exercise intolerance. In the aetiology of the impaired muscle performance, oxidative stress has been implicated (32). Hydroxytyrosol supplementation might protect the diaphragm, which would drastically improve the quality of life of these patients. The present study strengthens the rationale to further investigate the benefit of antioxidants such as hydroxytyrosol in these situations.

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CHAPTER 6

The olive antioxidant hydroxytyrosol boosts the endogenous antioxidant defence and attenuates the rise in plasma lactate during resistance type exercise

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Submitted

ABSTRACT

During exercise, the production of reactive oxygen and nitrogen species is considerably enhanced. The aim of the present study was to investigate the effects of the olive antioxidant hydroxytyrosol on antioxidant capacity, oxidative damage, inflammation, and plasma lactate levels during strenuous exercise. In a cross-over design, 7 untrained healthy men were randomly assigned to supplementation with hydroxytyrosol-rich olive extract or placebo. Muscle biopsies, urine, and blood samples were collected before and after a single bout of resistance type exercise. Supplementation with hydroxytyrosol (twice 200 mg) resulted in a considerable increase in plasma total antioxidant capacity (TEAC). Moreover, the glutathione defence system in skeletal muscle tissue was up-regulated by hydroxytyrosol, as shown by an elevation in glutathione (GSH) levels and increased glutathione reductase activity after exercise. Urinary isoprostane levels were not altered by hydroxytyrosol. Inflammatory markers were neither affected by exercise, nor by hydroxytyrosol supplementation. Interestingly, hydroxytyrosol attenuated the rise in circulating plasma lactate levels during exercise.

INTRODUCTION

Exercise magnifies the production of reactive oxygen species (ROS) in skeletal muscle tissue. Potential sources for ROS production during exercise are mitochondria, plasma membrane NADPH oxidases, phospholipase A_2 dependent processes, and ischemia reperfusion (1-4). Radical production may lead to tissue injury. Moreover, ROS trigger the activation of pro-inflammatory pathways such as Nuclear Factor kappa B (NF-κB) and the invasion of the injured tissue with phagocytic cells. These pathways further promote the production of ROS in a feed forward process.
Fortunately, the body has an elaborate defence system of antioxidants that provides protection against ROS. A major part of the defence against ROS is made up by alimentary antioxidants. In spite of the intricate antioxidant network, a large number of human studies have shown that exercise induces lipid peroxidation, as demonstrated by an increase in thiobarbituric acid reactive substances (TBARS), isoprostanes and exhaled pentane (5-14). The increase in these biomarkers indicates that endogenous antioxidants do not adequately cope with increased radical production during exercise. This provides the rationale for the supplementation of antioxidants during exercise. Several studies on the effects of increased antioxidant intake on exercise-induced skeletal muscle injury and inflammation have been performed. However, these studies are ambiguous, showing inconsistent results (15, 16).

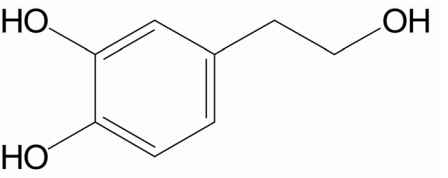

Figure 1. Molecular structure of hydroxytyrosol.

In the present study, the effect of the olive oil antioxidant hydroxytyrosol (Figure 1) during exercise was studied. Hydroxytyrosol was selected because it is a versatile antioxidant, showing strong antioxidant activity in both *in vitro* and *in vivo* studies (17-24). Furthermore, it has been recently found that hydroxytyrosol efficiently protects against oxidative stress-induced reduction in force production of skeletal muscle *in vitro* (chapter 5 of the thesis). In the present study, we investigated the effects of hydroxytyrosol supplementation on antioxidant capacity, oxidative damage, inflammation, and lactate levels in untrained, healthy volunteers during a single session of resistance type exercise.

METHODS

For the present study on the effect of hydroxytyrosol on antioxidant capacity, oxidative damage, inflammation, and lactate levels, we used the resistance exercise model as described in chapter 4 of the thesis. The placebo data of the study are described in chapter 4 of the thesis.

Subjects

Eight healthy male volunteers with no history of participating in any regular exercise program were recruited for the present study. One of the subjects was excluded from the study because he could not comply with the exercise protocol and had to stop before all the repetitions were performed. Subjects' characteristics are shown in Table 1.

Table 1. Subjects' characteristics

	Mean ± SEM
Age (yrs)	22.4 ± 2.0
Body mass (kg)	74.4 ± 3.3
Height (m)	1.82 ± 0.03
BMI ($kg \cdot m^{-2}$)	22.3 ± 0.8

Values are expressed as mean ± SEM.

All subjects were informed on the nature and possible risks of the experimental procedures before their written informed consent was obtained. This study has been approved by the Medical Ethical Review Board of the Academic Hospital, Maastricht, the Netherlands.

Pretesting

Two weeks before the experimental trial, the subjects performed a short exercise session to become familiarized with the exercise protocol and the equipment. Proper weightlifting technique was demonstrated and practiced for each of the two lower-limb exercises (leg press and leg extension). Subsequently, maximum strength was estimated using a standard multiple repetitions testing procedure (25). After warming up, the load was gradually increased, and the number of successful lifts until failure was determined. A 5-min resting period between subsequent attempts was allowed.
In a second session, at least one week after the first session and at least one week before the experimental trial, subjects' one repetition maximum (1RM) for each of the two lower-limb exercises was determined (26). After warming up, the load was set at 90% of the estimated 1RM and was increased after each successful lift until failure. Thereafter, the load was increased by 2.5-5% and

the successful lifts until failure were determined. A 5-min resting period between successive attempts was allowed. A repetition was valid if the subject was able to complete the entire lift in a controlled manner without assistance. 1RM was typically reached within three attempts.
Mean 1RM measured during pre-testing was 117 ± 6 kg on the leg extension and 195 ± 11 kg on the horizontal leg press. The starting workload applied during resistance exercise averaged 88 ± 5 kg and 146 ± 8 kg (75% of 1RM) for the leg extension and leg press, respectively.

Standardization of diet and activity prior to testing

Subjects were instructed not to consume any products rich in antioxidants, such as fruit juices, chocolate, olive oil, wine, or antioxidant supplements during three days before testing, on the test day itself, and on the day after the test day until 24 h after exercise. The low-antioxidant diet was chosen firstly to minimize a potential protective effect of high antioxidant intake and, secondly, to reduce variation in the baseline level of antioxidants, such as vitamin E and vitamin C. The volunteers consumed a carbohydrate-rich noodle dish, containing approximately 10 g of the vegetable leek, at 8.00 pm in the evening before the test day. Subjects were asked to record their food intake for three days before testing, on the test day itself, and on the day after the test day until 24 h after exercise. Food choices and quantities were recorded. By reviewing the recorded food intake, it was verified that the subjects' diet complied with the instructions. The volunteers were instructed to refrain from any sort of heavy physical exercise during the entire period, except for the resistance exercise session.

Hydroxytyrosol supplementation

The volunteers were asked to participate in the study on two separate occasions. Subjects consumed, in a randomized order, a hydroxytyrosol-rich olive extract or a placebo. The study was blinded for both the volunteers as well as the investigators. The olive extract (kindly provided by DSM, Delft, the Netherlands), providing 200 mg hydroxytyrosol per dose, was diluted in 150 ml drinking water, directly prior to ingestion. The olive extract or the placebo was supplemented twice: the evening before the test day at 8.00 pm and on the test day itself at 8.30 am (t = -30 min). The placebo had no antioxidant capacity, as determined by the trolox equivalent antioxidant capacity (TEAC) assay. The hydroxytyrosol supplement and the placebo were similar in taste and colour.

Experimental trial

The subjects arrived at the laboratory at 8.00 am, in an overnight fasted state. Then, they performed a 5-min low-intensity warm up using a Stairmaster (Jimsa Benelux BV, Rotterdam, the Netherlands). Thereafter, the resistance exercise session targeted the legs, with eight sets of ten repetitions on the horizontal leg press machine (Technogym BV, Rotterdam, the Netherlands) and eight sets of ten repetitions on the leg extension machine (Technogym). The starting workload applied during resistance exercise session was 75% of the individual 1RM for both the leg press and leg extension with 2-min rest intervals between sets. In total, the exercise regime required approximately 40 min to complete. This resistance exercise protocol has been applied previously and is routinely used as a resistance type exercise session in our institute (25, 26).
If subjects could not finish all ten repetitions at full weight, this was reduced to 65% or 55% of the individual 1RM. Only one of the evaluated volunteers was able to finish the entire protocol at 75%. The average intensity level of the exercise regime was 70%. All subjects were verbally encouraged during the test to complete the entire protocol.

Blood sampling

A Teflon catheter was inserted in an antecubital vein for venous blood sampling. Blood was collected before the intake of hydroxytyrosol or placebo (t = -30 min), just before the start of the exercise (t=0), during exercise (t=20 min), two min after cessation of exercise (t=43 min), and during subsequent recovery (t=60 min, 90 min, 120 min, 150 min, 180 min, and 24 h after exercise). Blood samples were collected in tubes containing heparin and placed on ice. Blood was aliquoted for the vitamin C and reduced glutathione (GSH) and glutathione disulphide (GSSG) analysis. To preserve the samples, 5% trichloroacetic acid (TCA) was added to the former, and 1.3% sulphosalicylic acid (SSA) was added to the latter. After centrifugation at 1000·g at 4°C for 5 min, aliquots of plasma were stored at -80°C until analysis.

Blood analyses

Plasma lactate concentrations were analysed spectrophotometrically (340 nm), measuring NADH formation using lactate dehydrogenase (LDH) (27). The increase in lactate concentration above baseline was determined and the area under the curve (AUC) of this increase in time was calculated using the trapezoidal rule (28). The half-life ($t_{1/2}$) of lactate after exercise was calculated by plotting the natural logarithm of the increase in lactate against time. This gave a

straight line, showing that the kinetics of lactate follow first order kinetics. The slope of the straight line obtained gives the elimination rate constant k.
The total antioxidant capacity (TEAC) was determined in plasma that was deproteinized with a final concentration of 5% TCA (29). The samples were incubated with the ABTS radical solution for 5 min and subsequently the absorbance at 734 nm was measured. The TEAC is expressed as μM trolox equivalents.
Uric acid was determined in plasma that was deproteinized with a final concentration of 5% TCA, using HPLC. A Hypersil BDS C-18 end-capped column, 125 x 4 mm, particle size 5 μm (Agilent, Palo Alto, CA, USA), was used, with a mobile phase of 0.1% trifluoroacetic acid (v/v) in water. UV detection was performed at 292 nm. The relative contribution of uric acid to the TEAC is calculated using the TEAC value of 1 for uric acid (29).
Hydroxytyrosol and homovanillic alcohol were determined in plasma that was deproteinized with a final concentration of 3% perchloric acid (PCA) using HPLC. The system (Agilent, Palo Alto, CA, USA) consisted of an Alltima C18 column, 150 x 3 mm, particle size 5 μm (Alltech, Deerfield, USA) with a mobile phase of 3% acetonitrile (v/v) and 0.1% trifluoric acid (v/v) in MilliQ. Diode array (280 nm) and fluorescence detection (excitation 281 nm, emission 316 nm) were used. Plasma samples were subjected to acidic hydrolysis to detect hydroxytyrosol metabolites, e.g. glucuronide or sulphate conjugates.
Vitamin C was determined in plasma by HPLC using the following method. Vitamin C was oxidized by ascorbate oxidase to dehydroascorbate. The latter reacted with o-phenylene diamine to its quinoxaline derivative. This derivative was quantified on the same system used for uric acid with a mobile phase of 0.08 M phosphate buffer (pH 7.8) and methanol, with fluorimetric detection (355-425 nm).
Vitamin E was extracted using hexane after adding vitamin E nicotinate as internal standard. HPLC analysis was performed on the same system used for uric acid with a mobile phase of methanol. UV detection was used (295 nm).
The erythrocyte non-protein thiol concentration, representing mainly GSH, was measured using 5,5'-dithiobis 2-nitrobenzoic acid (DTNB). The erythrocyte GSSG concentration was measured using 2-vinylpyridine pre-treatment using the recycling method (30).

NF-κB activity in peripheral blood mononuclear cells (PBMCs)

PBMCs were isolated from the blood samples (collected into leucosep tubes at t=0, 43 min, 90 min, and 24 h after exercise) using Lymphoprep (Bio-Connect, Huissen, the Netherlands). Nuclear extracts were immediately prepared and stored at -80°C until analysis. The protein concentration was determined according to the method of Bradford (BioRad, Veenendaal, the Netherlands).

NF-κB activity was measured using the TransAM NF-κB p50 Activation Assay (Active Motif, Rixensart, Belgium) and expressed as µg Jurkat NF-κB equivalents per µg nuclear protein.

Blood based cytokine production

At t=0, 43 min, 90 min, and 24 h after exercise, the blood based TNFα, IL-10 and IL-6 production was determined after stimulating whole blood for 24 h with 0.1 ng/ml lipopolysaccharide (LPS). TNFα, IL-10 and IL-6 concentrations were quantified using Pelikine Compact human Enzyme linked immuno sorbent assay (ELISA) kits (CBL/Sanquin, The Netherlands).

Urine collection

Morning urine was collected on the test day and on the morning of the day after the test day. Aliquots of urine were stored at -80°C until analysis of $F_{2\alpha}$-isoprostanes and creatinine.

Urine analysis

$F_{2\alpha}$-isoprostanes (8-iso $PGF_{2\alpha}$) in urine were analysed by an enzyme immunoassay (Cayman Chemical, Ann Arbor, MI, USA) and were related to creatinine values.

Muscle biopsies

Muscle biopsy samples were taken 30 min before the start of the exercise, i.e. prior to hydroxytyrosol or placebo ingestion, and 30 min after exercise, to determine the acute effects of exercise. Muscle biopsies were obtained from the middle region of the m. vastus lateralis (15 cm above the patella) and approximately 3 cm below entry through the fascia using the percutaneous needle biopsy technique. The second muscle biopsy was taken from the contra-lateral leg. Muscle samples were freed from any visible nonmuscle material and rapidly frozen in liquid nitrogen. Muscle biopsies were stored at -80°C until analysis.

Preparation of muscle homogenates

Frozen muscle biopsies were weighed and subsequently ground with a liquid nitrogen cooled mortar. The powder was resuspended in demineralised water to a final concentration of 250 mg tissue/ml. SSA (final concentration of 1.3%) was added to a small part of the homogenates, for the analysis of GSH and GSSG. The homogenates were directly frozen in liquid nitrogen and were

subsequently stored at -80°C until analysis. The homogenates were centrifuged at 14,000·g at 4°C for 3 min. The muscle biopsy supernatant was used for analysis.

Muscle biopsy analyses

Glutathione S-transferase (GST) activity, glutathione reductase activity, uric acid and protein levels in muscle biopsies were determined according to the methods of Julicher *et al*. (30). GST activity was determined by the enzymatic reaction of chlorodinitrobenzene with GSH. The increase in the formation of the coloured product of the reaction was measured at 340 nm for 2 min. Glutathione reductase activity was measured by determining the consumption of nicotinamide adenine dinucleotide phosphate (NADPH) in the enzymatic reduction of GSSG to GSH. The decrease in absorption at 340 nm was measured during 2 min. Skeletal muscle GSH and GSSG concentrations were measured according to the method for the determination of erythrocyte GSH and GSSG concentrations, as described above. Uric acid concentrations were determined in muscle biopsies of four subjects, because of the limited amount of muscle tissue of three subjects.

Statistics

All data are expressed as means ± SEM. Analysis of variance (ANOVA) was applied to determine differences between hydroxytyrosol and placebo supplementation in plasma antioxidant concentrations and inflammatory markers. Sign tests were used to compare the effects of hydroxytyrosol and placebo supplementation on AUC and $t_{1/2}$ of lactate. Pre-versus post-exercise values were calculated for skeletal muscle antioxidants and urinary isoprostanes. The Wilcoxon signed-rank test was used to compare the effect of hydroxytyrosol with placebo. Statistical significance was set at $P<0.10$.

RESULTS

Plasma antioxidant capacity

At 8.00 pm, the evening before the start of the exercise, 200 mg hydroxytyrosol or placebo was given. The following morning, before the second intake of hydroxytyrosol or placebo, no differences between the hydroxytyrosol or placebo group in any of the parameters studied was observed, including the TEAC. Moreover, neither hydroxytyrosol nor hydroxytyrosol metabolites could be detected in plasma of the volunteers that had received hydroxytyrosol.

After the second intake of placebo or hydroxytyrosol, plasma samples were taken at relatively short time intervals. It was found that 200 mg hydroxytyrosol resulted in a considerable increase in plasma TEAC (Figure 2A) ($P<0.05$). Thirty minutes after hydroxytyrosol intake, the TEAC of plasma increased with 17%. No significant changes in TEAC were observed after intake of the placebo (Figure 2A).

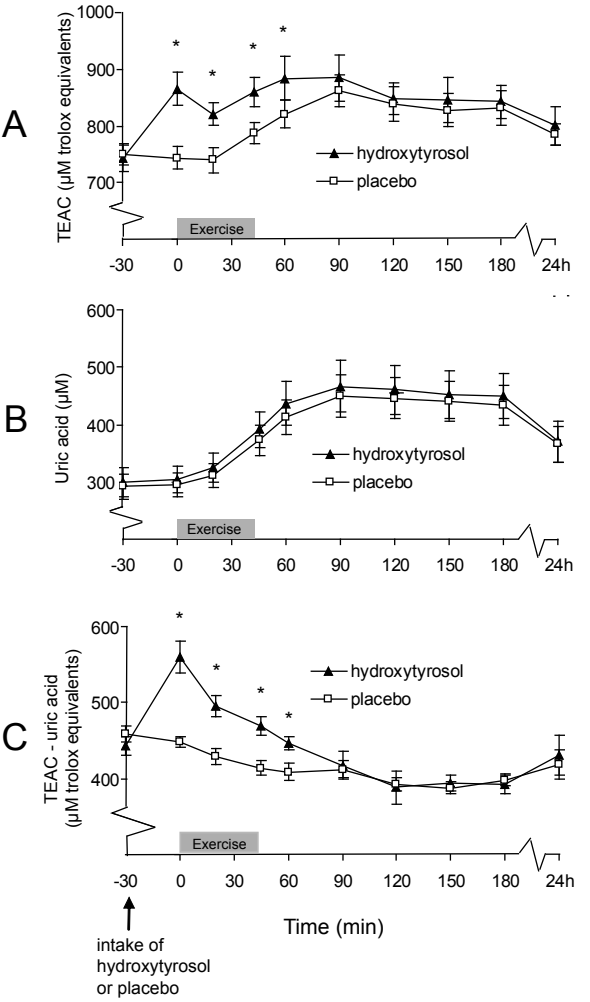

Figure 2. The effect of hydroxytyrosol on plasma TEAC values (panel A), uric acid concentrations (panel B) and TEAC values, corrected for uric acid (panel C) during resistance exercise (duration is indicated by the grey bar) and subsequent recovery. Data provided are means ± SEM.
* Significantly different from placebo ($P<0.05$).

Exercise also considerably increased the TEAC. At t=0 (i.e. 30 min after intake of hydroxytyrosol), t=20 min, and directly after exercise (t=43 min and t=60 min), hydroxytyrosol supplementation resulted in higher TEAC values, compared to placebo ($P<0.05$). During further recovery, TEAC values after hydroxytyrosol and placebo supplementation became similar (Figure 2A). The increase in uric acid concentrations during exercise was similar after hydroxytyrosol and placebo supplementation (Figure 2B). Fifty minutes after exercise, the uric acid concentration reached a maximum value after which it slightly decreased.

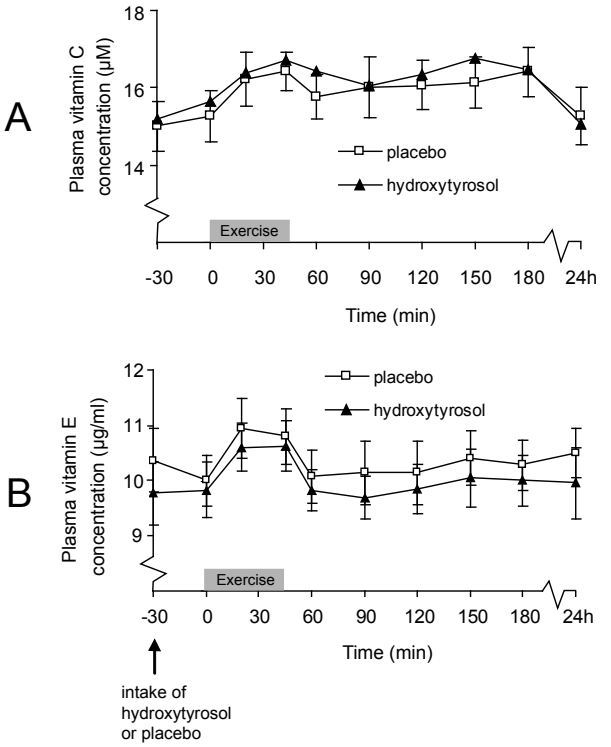

Figure 3. Plasma vitamin C (panel A) and vitamin E (panel B) concentrations during resistance exercise (duration is indicated by the grey bar) and subsequent recovery, after hydroxytyrosol and placebo supplementation. Data provided are means ± SEM.

The antioxidant uric acid significantly contributes to the TEAC. This contribution of uric acid to the TEAC can be calculated. Subtracting the contribution of uric acid from the TEAC gives the 'uric acid-subtracted TEAC' value. The 'uric acid-subtracted TEAC' decreased during exercise. It was found that hydroxytyrosol supplementation resulted in an increased 'uric acid-subtracted TEAC',

compared to placebo, at the start of the exercise (t=0), during exercise (t=20 min) and directly after exercise (t=43 min and 60 min) ($P<0.05$; Figure 2C). During further recovery, 'uric acid-subtracted TEAC' values after hydroxytyrosol and placebo supplementation were similar (Figure 2C).
Hydroxytyrosol is known to be extensively metabolized e.g. by conjugation to glucuronic acid, sulphate or glutathione (31). Accordingly, the plasma levels of free hydroxytyrosol were below the lower limit of quantification (0.2 μM; HPLC). Total conjugated hydroxytyrosol was determined in plasma samples subjected to acidic hydrolysis. Peak concentration of total conjugated hydroxytyrosol was approximately 5 μM. Hydroxytyrosol is also metabolized to homovanillic alcohol (31). The maximal plasma concentration of homovanillic alcohol was approximately 1 μM.
Plasma vitamin C and vitamin E concentrations temporarily elevated during exercise. Hydroxytyrosol supplementation didn't affect plasma vitamin E and vitamin C levels during exercise and recovery (Figure 3A and B).
Erythrocyte GSH levels also temporarily increased during exercise. Hydroxytyrosol didn't affect the erythrocyte GSH levels during exercise and recovery. The erythrocyte GSSG concentration remained during exercise at baseline value, after both hydroxytyrosol and placebo supplementation.

Antioxidant activity in skeletal muscle

The increase in GSH concentrations in skeletal muscle, 30 min after exercise, was 5.5 ± 4.3 nmol/mg protein. The supplementation of hydroxytyrosol increased skeletal muscle GSH levels after exercise. After hydroxytyrosol supplementation, the increase in GSH levels was 14.4 ± 7.6 nmol/mg protein (Figure 4A) ($P<0.10$). Neither exercise, nor hydroxytyrosol did affect GSSG concentrations in skeletal muscle (Figure 4C).
Glutathione reductase activity in skeletal muscle increased after exercise on average with 4.9 ± 2.2 mU/mg protein (Figure 4B). The increase in glutathione reductase activity induced by exercise was higher after hydroxytyrosol supplementation, i.e. an increment of 7.8 ± 2.9 mU/mg protein was found (Figure 4B) ($P<0.10$). The increase in skeletal muscle GST activity by exercise was similar after hydroxytyrosol and placebo supplementation (Figure 4D). The increase in uric acid concentrations in skeletal muscle after exercise was also comparable after placebo or hydroxytyrosol supplementation (Figure 4E).

8-iso $PGF_{2\alpha}$ levels in urine

The increase in the concentration of 8-iso $PGF_{2\alpha}$ in urine during exercise was similar after hydroxytyrosol and placebo supplementation. In case of placebo, urinary 8-iso $PGF_{2\alpha}$ increased from 0.117 ± 0.021 nmol/mmol creatinine pre-

exercise to 0.164 ± 0.030 nmol/mmol creatinine post-exercise (Figure 5). After hydroxytyrosol supplementation, urinary 8-iso $PGF_{2\alpha}$ increased from 0.107 ± 0.014 nmol/mmol creatinine pre-exercise to 0.165 ± 0.028 nmol/mmol creatinine post-exercise (Figure 4F).

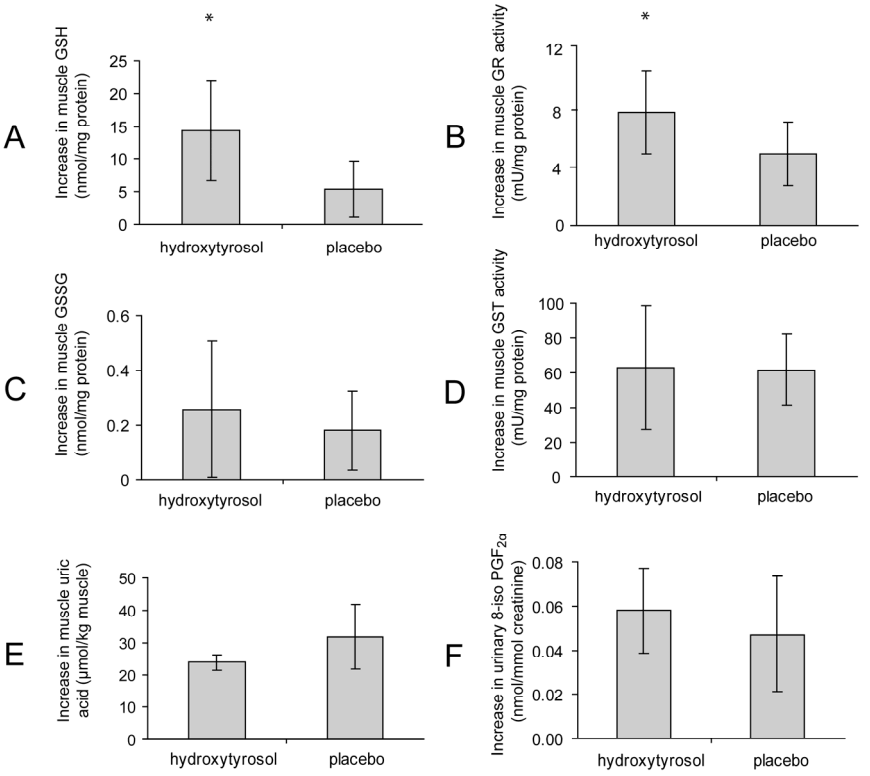

Figure 4. The effect of hydroxytyrosol on exercise-induced increases in skeletal muscle GSH (panel A), glutathione reductase (GR) activity (panel B), GSSG (panel C), GST activity (panel D), uric acid (panel E) and urinary isoprostanes (panel F). Data provided are means ± SEM.
* Significantly different from placebo (P<0.10).

Inflammatory markers

Exercise did not induce changes in NF-κB activity in PBMCs. The supplementation of hydroxytyrosol also didn't affect the NF-κB activity in PBMCs. Furthermore, the *ex vivo* LPS-induced production of TNFα, IL-10 and IL-6 was neither affected by exercise, nor by hydroxytyrosol supplementation (data not shown).

Lactate

Hydroxytyrosol supplementation tended to decrease the plasma peak concentration of lactate directly after exercise (t=43 min) (10.2 ± 1.2 vs 11.0 ± 1.0 mM; Figure 5A). The AUC, reflecting the production of lactate, and the $t_{1/2}$ of lactate were calculated for each volunteer. This reveals that there are large inter-individual differences in both AUC and $t_{1/2}$. The AUC values ranged from 296 to 783 and the $t_{1/2}$ of lactate ranged from 12 to 27 min. Hydroxytyrosol decreased the AUC of lactate from 560 ± 65 to 522 ± 67 (Figure 5B) (P=0.063).

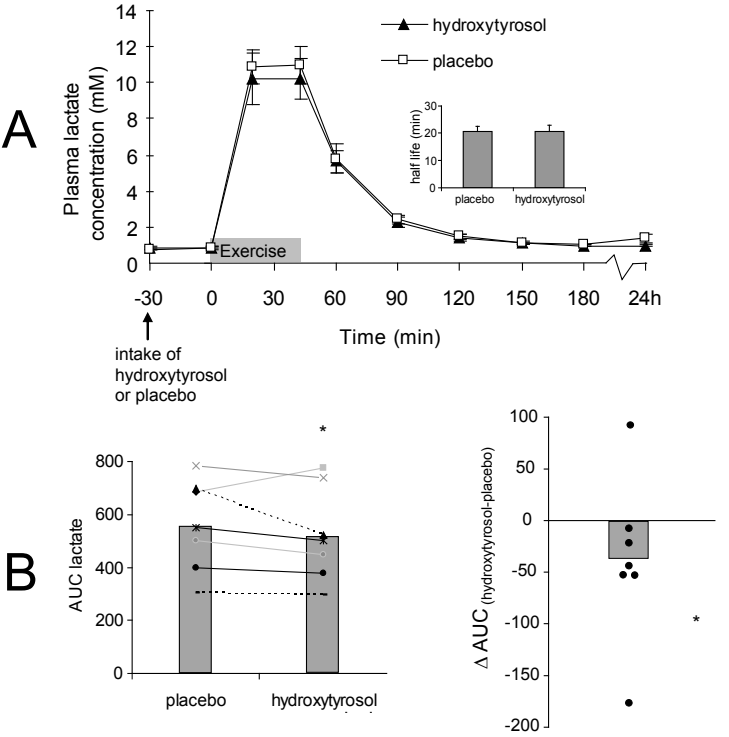

Figure 5. The effect of hydroxytyrosol on lactate levels during resistance exercise (duration is indicated by the grey bar) and subsequent recovery (panel A). The $t_{1/2}$ of lactate after hydroxytyrosol or placebo supplementation is shown in the insert of panel A. The effect of hydroxytyrosol on the AUC of lactate is shown in panel B. Data provided are means ± SEM (panel A) or individual values plus mean values (grey bars) (panel B).
$\Delta AUC = AUC_{hydroxytyrosol} - AUC_{placebo}$
* Significantly different from placebo (P<0.10).

Hydroxytyrosol did not affect the clearance, i.e. the elimination of lactate out of the blood compartment as the $t_{1/2}$ of lactate was identical after hydroxytyrosol and placebo supplementation ($t_{1/2}$ = 20.6 ± 2.2 and 20.5 ± 2.0 min respectively; insert Figure 5A). The average $t_{1/2}$ of lactate of 21 min corresponds to values previously reported (32).

DISCUSSION

The interest in antioxidant supplements in the field of exercise has largely increased. The rationale to use antioxidants during exercise is to empower the endogenous antioxidant defence in order to cope with the increased radical flux induced by exercise. This may prevent muscle damage, which is expected to enhance performance and shorten the recovery period. Up to date, the effect of antioxidant supplementation during exercise is inconclusive.

Muscle endogenous antioxidant defence systems are up-regulated in response to exercise (13, 33). ROS produced during exercise may act as signals to increase the production of antioxidant enzymes. NF-κB and mitogen-activated protein kinase (MAPK) are two major oxidative stress-sensitive signal transduction pathways that have been shown to activate the gene expression of a number of antioxidant enzymes (34, 35). In literature, it has been speculated that antioxidant supplementation might block this adaptive response to exercise (33).

In the present study the effect of the olive antioxidant hydroxytyrosol during exercise was studied. Hydroxytyrosol was chosen because of its excellent antioxidant characteristics. It is a potent scavenger of reactive oxygen and nitrogen species (17). Furthermore, hydroxytyrosol has been shown to provide protection against oxidative damage to all kinds of cells *in vitro* (18, 20-22). Hydroxytyrosol also displays antioxidant effects *in vivo*. For example, it provides protection against the oxidation of low density lipoprotein (LDL), one of the key steps in the initiation of atherosclerosis (36-39).

The effect of hydroxytyrosol on skeletal muscle has not been studied in detail so far. Most of the research on hydroxytyrosol is focused on its beneficial cardiovascular properties. Recently, we have shown that hydroxytyrosol efficiently protects against oxidative stress-induced reduction in force production of rat skeletal muscle *in vitro* (chapter 5 of the thesis). The aim of the present study was to investigate the *in vivo* effects of hydroxytyrosol in untrained, healthy volunteers during a single session of strenuous resistance type exercise.

The present study shows that the supplementation of hydroxytyrosol boosts the antioxidant defence system, as can be deduced from the large increase in TEAC of plasma. The TEAC increased by more than 100 μM trolox equivalents, while

only approximately 5 μM of hydroxytyrosol metabolites could be detected in plasma, which contribute for maximally 10 μM trolox equivalents to the TEAC. Apparently, the antioxidant activity of hydroxytyrosol itself and its known metabolites can explain only a small part of the increase in TEAC after hydroxytyrosol supplementation. The molecular mechanism responsible for the high increase in TEAC remains to be elucidated. It is possible that hydroxytyrosol is converted to other, unknown metabolites with high antioxidant activity. It should be noted that antioxidants do not act in isolation; they synergize to form an intricate network of antioxidants. It is possible that hydroxytyrosol regenerates other antioxidants that are present in the antioxidant network.

Importantly, hydroxytyrosol did not hamper the exercise-induced adaptive antioxidant response. The exercise-induced increase in erythrocyte GSH and plasma vitamin E, vitamin C, and uric acid concentrations was not affected by hydroxytyrosol supplementation. Moreover, the glutathione defence system in skeletal muscle was up-regulated by hydroxytyrosol, as shown by an elevation in GSH levels after exercise. Furthermore, the exercise induced increase in glutathione reductase activity was even higher after hydroxytyrosol supplementation, compared to placebo. This indicates that hydroxytyrosol rather boosts than blocks the exercise induced adaptive response.

Hydroxytyrosol supplementation didn't attenuate the rise in the concentration of 8-iso $PGF_{2\alpha}$ in urine 24h after a single session of resistance exercise. Based on this result, hydroxytyrosol doesn't seem to be effective in protecting against ROS generated by exercise.

Several studies on the use of antioxidants to attenuate exercise-induced muscle injury and oxidative stress during exercise have been performed. Most of the research in this area is focused on common dietary antioxidants such as vitamin E and vitamin C. With regard to endurance exercise, the effect of antioxidant supplements is not straightforward, as studies in this area show inconsistent results (16). Regarding a bout of resistance exercise, some reports suggest a potential beneficial role of antioxidant supplementation, whereas others indicate no benefit (15).

In general, the efficacy of an antioxidant to protect against exercise-induced oxidative stress depends on several methodological aspects, such as (I) the specific population studied, (II) the type of exercise, (III) the type of antioxidant, dosage, and timing of administration of the antioxidant, (IV) the biomarkers used, and (V) the sampling time.

Most studies reporting a benefit of antioxidant supplementation in attenuating muscle injury and oxidative stress following resistance exercise have included sedentary, non-resistance trained subjects. In trained subjects, endogenous antioxidant defences may be up-regulated, and, therefore, these individuals may not benefit greatly from exogenous antioxidant intake in order to reduce muscle injury (34). We selected untrained subjects for the present study, based

on our speculation that oxidative damage would be greater in a non-trained group, thereby increasing the window of opportunity to detect an effect of hydroxytyrosol supplementation on oxidative damage and antioxidant capacity. Studies on the use of antioxidants during exercise have used several types of exercise, ranging from long-term endurance exercise to short-term resistance exercise. Aerobic endurance exercise will induce a different flux of radicals than anaerobic resistance exercise does (35). In designing the study, we speculated that the effect of an acute and high flux of radicals would be more sensitive to antioxidant supplementation than that of a more prolonged and moderate flux of radicals. In order to maximize the sensitivity of the study, a single session of strenuous resistance exercise was applied. It has been suggested that the effect of antioxidants depends on the degree of muscle damage (33). During moderate exercise, antioxidants might hamper the beneficial adaptive response. When strenuous exercise is applied, such as in the present study, antioxidants are expected to prevent oxidative stress-induced muscle injury (33).

The efficacy of the antioxidant studied also depends on the type of antioxidant, dose, and timing of the administration of the antioxidant. In the present study, hydroxytyrosol increased the TEAC of plasma with more than 100 μM trolox equivalents, clearly demonstrating the high antioxidant potency of hydroxytyrosol in a dose of just 200 mg. For comparison, administration of 2 g of the flavonoid quercetin increased the TEAC merely by 15 μM trolox equivalents (unpublished results). The increase in TEAC of plasma lasted only approximately 90 minutes after intake of hydroxytyrosol. Supplementing hydroxytyrosol not only 30 minutes before, but also directly after exercise would have prolonged the increase in TEAC, which might have been more effective, in retrospect.

Furthermore, the observed efficacy of an antioxidant will also depend on the biomarkers used. Plasma protein carbonyl content is often used as a biomarker for exercise induced oxidative damage (6, 7, 9). We also intended to include this biomarker. However, in our hands, the routinely applied procedure for determination of protein carbonyl content lacked sufficient specificity, due to interference by pyruvate (data not shown).

The observed effect of an antioxidant is also dependent on sampling time. In the present study the sampling time was limited to 24h post exercise. Up to 24h post exercise, inflammatory markers were neither affected by exercise, nor by hydroxytyrosol supplementation. It is possible that in a more long term study design, effects of exercise or hydroxytyrosol would have been detected. The anti-inflammatory effect of hydroxytyrosol has been shown both in preclinical and clinical studies (40-42).

It was expected that the large increase in TEAC of plasma and the upregulation of the glutathione defence system in muscle due to hydroxytyrosol would have

reduced oxidative stress. In spite of the inability to attenuate the exercise-induced rise in urinary isoprostanes, hydroxytyrosol did reduce the production of lactate during exercise. Sastre *et al.* have reported a positive correlation between oxidative stress, namely the GSSG to GSH ratio, and the lactate to pyruvate ratio during exhaustive physical exercise (43). Recently, it has been shown that long-term co-supplementation (90 days) with vitamin E, β-carotene and vitamin C reduced the maximal blood lactate concentration during exercise. The present study shows that also short-term supplementation with hydroxytyrosol is able to reduce the production of lactate during strenuous exercise. Although the mechanism is still unclear, it is obvious that antioxidants are able to affect lactate levels during exercise. Additional research is needed to clarify this finding.

The present study does not support the alleged antagonistic action of antioxidants on muscle cell adaptation. On the contrary, hydroxytyrosol rather strengthens than blocks the endogenous antioxidant defence during a single session of strenuous resistance type exercise in untrained men. In spite of the inability of hydroxytyrosol to attenuate the exercise-induced rise in urinary isoprostanes, hydroxytyrosol considerably increases the TEAC of plasma and attenuates the rise in plasma lactate during exercise.

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CHAPTER 7

General discussion

GENERAL DISCUSSION

Over the past decades, the interest in antioxidants has largely increased. Antioxidants are considered to support health. Consequently, the market for supplements containing antioxidants with health promoting claims is growing. The general purpose of the intake of these 'exogenous' antioxidants is to empower the endogenous antioxidant system to combat ROS-mediated damage.
Phenolic antioxidants present in olive oil have gained a lot of interest. Current evidence indicates that olive oil phenolic compounds provide beneficial health effects, e.g. on the cardiovascular system. Hydroxytyrosol is one of the main phenolic compounds present in virgin olive oil and is endowed with a wide range of antioxidant effects.
During the production of olive oil, large quantities of olive oil mill wastewater (OMWW) are produced. This wastewater constitutes a serious environmental problem, as it has severe negative impact on soil and water quality. OMWW has a higher content of phenolic compounds than olive oil itself. The recovery of phenolic compounds from OMWW is very valuable, not only from an environmental point of view, but also because these compounds might be useful in the pharmaceutical, food and cosmetic industry. Supplements with health promoting claims are currently being developed from OMWW. The content of phenolic compounds present in some of these extracts is 300-500 times higher than that in olive oil itself.

This thesis focuses on the health benefits of hydroxytyrosol from its mechanism of action to its use as nutraceutical. The present thesis touches upon two specific aspects of hydroxytyrosol. Firstly, the health benefit of hydroxytyrosol on the cardiovascular system was further explored (**chapter 2 and 3**). Secondly, the protective effect of hydroxytyrosol against exercise induced oxidative stress in skeletal muscle was studied **(chapter 4 - 6)**.

Effect of hydroxytyrosol on the cardiovascular system

The molecular mechanism of the beneficial cardiovascular effects of hydroxytyrosol has not been fully elucidated yet. In **chapter 2**, the antioxidant profile of hydroxytyrosol was studied in detail. The intrinsic antioxidant activity forms the fundament for the physiological impact of hydroxytyrosol. Regarding the antioxidant profile of hydroxytyrosol some controversies exist. For example, the reports on superoxide radical ($O_2^{\bullet-}$) scavenging are ambiguous. In chapter 2 it was shown that hydroxytyrosol is a potent scavenger of hydroxyl radicals ($OH^{\bullet}$), peroxynitrite (ONOOH), and $O_2^{\bullet-}$, but not of hypochlorous acid (HOCl) and hydrogen peroxide (H_2O_2).

Also regarding the protective effect of hydroxytyrosol against low density lipoprotein (LDL) oxidation some controversies exist. The oxidation of LDL is a key factor in the initiation of atherosclerosis. Hydroxytyrosol efficiently protects against LDL oxidation *in vitro* and *in vivo*. However, no protective effect of hydroxytyrosol is usually demonstrated *ex vivo* against the oxidation of LDL isolated from humans after hydroxytyrosol consumption.

Chapter 2 shows that this controversy is due to the isolation of LDL, which greatly reduces the protective effect of hydroxytyrosol against LDL oxidation. It was established that hydroxytyrosol is an amphiphilic compound, present in the aqueous as well as the lipophilic environment in comparable concentrations. During the LDL isolation procedure, most of the hydroxytyrosol is lost due to its amphiphilic nature. This indicates that the reduction in the protective effect of hydroxytyrosol might be due to the loss of hydroxytyrosol from LDL during the LDL isolation procedure. Therefore, in *ex vivo* experiments the effect of hydroxytyrosol is generally underestimated because of the LDL isolation procedure. Several studies have been published that are consistent in showing a protective effect of hydroxytyrosol against LDL oxidation *in vivo*.

In addition to oxidative modifications of LDL, oxidative damage to endothelial cells may also affect cardiovascular health. Under physiological conditions, endothelial $NO^{\bullet}$ is of pivotal importance in the regulation of the vascular tone. Oxidative damage to the delicate $NO^{\bullet}$ system in endothelial cells might disturb the vascular tone. A diminished $NO^{\bullet}$ mediated vasorelaxation leads to a rise in blood pressure, which is a well-established risk factor for the development or progression of atherosclerosis.

In **chapter 3,** the effect of oxidative stress on the $NO^{\bullet}$-mediated vasorelaxation of rat aorta was studied. Cumene hydroperoxide (CHP) was used to mimic oxidative stress induced by lipid hydroperoxides, which involves the formation of reactive species such as $OH^{\bullet}$. CHP impaired the $NO^{\bullet}$ mediated relaxation of rat aorta by the acetylcholine receptor agonist carbachol. This was due to a reduction in $NO^{\bullet}$ production. Chapter 3 shows that hydroxytyrosol efficiently protected the aorta against the CHP induced impairment of the $NO^{\bullet}$ mediated relaxation. The protective effect of hydroxytyrosol might be explained by its potent scavenging activity, which can be attributed to its catechol moiety. The results in chapter 2 and 3 provide a molecular basis for the contribution of hydroxytyrosol to the cardiovascular health benefits of olive oil consumption.

Effect of hydroxytyrosol on skeletal muscle

The second focus of the thesis is on the potential of hydroxytyrosol to protect against oxidative stress in skeletal muscle. During exercise, the production of reactive oxygen and nitrogen species is significantly enhanced. Unbalanced production of reactive species will lead to oxidative damage to biomolecules,

such as proteins and lipids. Fortunately, the body has an elaborate defence system of antioxidants that provides protection against these reactive species.
In **chapter 4**, it was investigated whether the endogenous antioxidant defence system is able to cope with the increased radical flux induced by exercise. It was expected that antioxidant levels would be reduced by increased consumption of antioxidants. Surprisingly, a single session of resistance type exercise was found to increase the concentration of several antioxidants in untrained healthy males. Plasma total antioxidant capacity (TEAC) significantly increased after exercise, which appeared to be predominantly due to elevated uric acid concentrations. Plasma vitamin C and vitamin E and erythrocyte glutathione (GSH) levels were also elevated during exercise. Also in skeletal muscle, uric acid levels were found to increase after exercise. Moreover, the activity of the antioxidant enzymes glutathione S-transferase (GST) and glutathione reductase was found to be significantly elevated by exercise.
ROS produced during exercise may act as signals to increase the production of antioxidant enzymes. Nuclear Factor kappa B (NF-κB) and mitogen-activated protein kinase (MAPK) are two major oxidative stress-sensitive signal transduction pathways that have been shown to activate the gene expression of a number of antioxidant enzymes. In spite of the exercise-induced adaptive increase in antioxidant capacity of blood and skeletal muscle, a single session of resistance exercise induced oxidative damage to lipids, as shown by an increase in urinary $F_{2\alpha}$-isoprostanes.
The fact that the endogenous antioxidant defence is not able to sufficiently cope with the increased radical flux induced by exercise provides a rationale for the use of antioxidant supplements during exercise. However, in literature, it has been speculated that antioxidant supplementation might block the adaptive antioxidant response to exercise. In **chapter 5 and 6**, it was investigated whether hydroxytyrosol would be a suitable candidate to protect against ROS generated during exercise.
Firstly, the antioxidant potential of hydroxytyrosol was investigated in isolated rat muscle strips (**chapter 5)**. H_2O_2 was used to induce oxidative stress in rat skeletal muscle. The *in vitro* rat diaphragm preparation was applied, which is a well-established model to study skeletal muscle function *in vitro*. It was observed that hydroxytyrosol efficiently protects against the oxidative stress-induced impairment of force production during both short-term and fatiguing long-term electrical muscle stimulation.
In **chapter 6**, the *in vivo* protective effect of hydroxytyrosol against oxidative stress induced by a single session of resistance exercise was investigated in a double-blinded intervention study in untrained healthy volunteers. Supplementation with 200 mg hydroxytyrosol resulted in a considerable increase in plasma total antioxidant capacity (TEAC). Hydroxytyrosol did not hamper the exercise-induced adaptive antioxidant response. The exercise-induced increase

in erythrocyte glutathione (GSH) and plasma vitamin E, vitamin C, and uric acid concentrations was not affected by hydroxytyrosol supplementation. Moreover, the glutathione defence system in skeletal muscle was up-regulated by hydroxytyrosol, as shown by an elevation in GSH levels after exercise. Furthermore, the exercise-induced increase in glutathione reductase activity was even higher after hydroxytyrosol supplementation, compared to placebo. This indicates that hydroxytyrosol rather boosts than blocks the exercise induced adaptive response. Therefore, during exhaustive exercise, when the generation of ROS overwhelms the endogenous defensive mechanisms, hydroxytyrosol might be supplemented to strengthen the antioxidant capacity.
Hydroxytyrosol did not attenuate the exercise-induced rise in urinary isoprostanes, indicating that hydroxytyrosol did not protect against oxidative stress. Hydroxytyrosol did, however, reduce the production of lactate during exercise. Irrespective of the mechanism involved, attenuation of lactate production during exercise by hydroxytyrosol is expected to enhance performance.

CHAPTER 8

Summary and perspective

SUMMARY

Hydroxytyrosol is an excellent antioxidant. The fundament for the physiological impact of hydroxytyrosol is its high intrinsic antioxidant activity. Hydroxytyrosol is a potent scavenger of several reactive species, i.e. superoxide radical ($O_2^{\bullet-}$), hydroxyl radical ($OH^{\bullet}$) and peroxynitrite (ONOOH). The amphiphilic character of hydroxytyrosol facilitates its uptake in the intestines. Moreover, because of its amphiphilic nature, hydroxytyrosol is expected to provide protection in both lipid and aqueous cellular compartments.

Hydroxytyrosol has beneficial effects on cardiovascular health. It protects against the oxidative stress mediated impairment of blood vessel relaxation, thereby preserving one of the key mechanisms in regulating blood pressure. Moreover, the oxidation of low density lipoprotein (LDL) is efficiently prevented by hydroxytyrosol. The oxidation of LDL is regarded as one of the key steps of the initiation of atherosclerosis.

The applicability of hydroxytyrosol is not merely restricted to cardiovascular health. The present thesis shows that hydroxytyrosol efficiently protects against the oxidative stress mediated reduction in force production of isolated skeletal muscle. Furthermore, the supplementation of hydroxytyrosol in healthy men considerably boosts the antioxidant capacity of blood and muscle and attenuates the rise in plasma lactate during exercise.

PERSPECTIVE

The application field of hydroxytyrosol is broad. Hydroxytyrosol is of interest in all kinds of situations involving oxidative stress. Based on the results described in this thesis, it would be interesting to further study the beneficial cardiovascular effects of hydroxytyrosol. Especially for people at risk (such as having a high blood pressure or high LDL to HDL ratio) hydroxytyrosol might be beneficial. With regard to the application of hydroxytyrosol, one should also be cautious by taking the law of Paracelsus into account. This law states that every compound, so also hydroxytyrosol, will be toxic provided that the dose is high enough. This applies especially to people not at risk taking supplements high in hydroxytyrosol over a long period of time. Therefore, it is important to establish not only the efficacy, but also the safety of long-term use of hydroxytyrosol. It should be noted that in the experiments described in this thesis, no signs of toxicity due to hydroxytyrosol administration were observed.
Another promising option is to further elaborate on the effect of hydroxytyrosol during exercise. The increase in TEAC of plasma lasted only approximately 90 minutes after intake of hydroxytyrosol. Supplementing hydroxytyrosol not only 30 minutes before, but also directly after exercise would have prolonged the increase in TEAC, which might have been more effective in protecting against oxidative damage. Here also specific target populations can be pointed out that might benefit most from hydroxytyrosol supplementation during exercise, for example people suffering from chronic obstructive pulmonary disease (COPD) or type 2 diabetes. Besides an impaired antioxidant defence, these patients have a relatively high production of radicals even under basal conditions. A negative health effect of a high radical flux induced by exercise is therefore expected to be more pronounced in these individuals. Interestingly, exercise is recommended in the treatment of these patients. Hydroxytyrosol supplementation during exercise might therefore especially be relevant for these patients, as it strengthens the antioxidant defence against ROS generated during exercise.

SAMENVATTING

Hydroxytyrosol is een uitmuntende antioxidant. Het fundament voor de fysiologische impact van hydroxytyrosol wordt gevormd door de hoge intrinsieke antioxidant activiteit. Dit proefschrift laat zien dat hydroxytyrosol een effectieve scavenger is van verschillende reactieve deeltjes, zoals superoxide radicalen ($O_2^{\bullet-}$), hydroxyl radicalen ($OH^{\bullet}$) en peroxynitriet (ONOOH). Het amphiphilisch karakter van hydroxytyrosol bevordert de opname van hydroxytyrosol in de darm. Bovendien wordt verwacht dat, vanwege het amphiphilisch karakter, hydroxytyrosol in staat is om als antioxidant bescherming te bieden in zowel hydrofobe als hydrofiele compartimenten van de cel.
Hydroxytyrosol heeft positieve cardiovasculaire gezondheidseffecten. Hydroxytyrosol is in staat om bescherming te bieden tegen de oxidatieve stress gemedieerde reductie in bloedvat relaxatie. Hierdoor blijft een essentieel mechanisme in de regulatie van de bloeddruk behouden. Daarnaast wordt de oxidatie van LDL effectief tegengegaan door hydroxytyrosol. De oxidatie van LDL wordt gezien als een van de belangrijkste stappen in de initiatie van atherosclerosis.
De toepasbaarheid van hydroxytyrosol is niet alleen beperkt tot cardiovasculaire gezondheid. Dit proefschrift toont aan dat hydroxytyrosol effectieve bescherming biedt tegen de oxidatieve stress gemedieerde reductie in krachtontwikkeling van geïsoleerd skeletspierweefsel. Daarnaast versterkt het supplementeren van hydroxytyrosol tijdens fysieke inspanning de antioxidant capaciteit van bloed en spieren van gezonde vrijwilligers. Bovendien verlaagt hydroxytyrosol de productie van melkzuur tijdens fysieke inspanning.

PERSPECTIEF

Het toepassingsgebied van hydroxytyrosol is breed. Het supplementeren van hydroxytyrosol kan aantrekkelijk zijn in allerlei situaties waar oxidatieve stress een rol speelt. Het is interessant om de positieve cardiovasculaire gezondheidseffecten van hydroxytyrosol verder te onderzoeken. Met name voor risicogroepen (zoals mensen met een hoge bloeddruk of een hoge LDL/HDL ratio) zou hydroxytyrosol positieve gezondheidseffecten kunnen hebben. Men moet echter wel altijd de wet van Paracelsus in het achterhoofd houden. Deze wet zegt dat elke stof, dus ook hydroxytyrosol, toxisch is mits de dosis hoog genoeg is. Dit geldt in het bijzonder voor mensen zonder risicofactoren, die hydroxytyrosol supplementen in hoge dosering gebruiken gedurende lange tijd. Het is daarom van belang dat niet alleen de effectiviteit, maar ook de veiligheid van langdurig gebruik van hydroxytyrosol nader onderzocht wordt. In het onderzoek dat werd uitgevoerd in het kader van dit proefschrift werden geen tekenen van toxiciteit van hydroxytyrosol waargenomen.
Een andere veelbelovende mogelijkheid voor verder onderzoek is om het effect van hydroxytyrosol tijdens fysieke inspanning verder te ontrafelen. De toename in de totale antioxidant capaciteit van het bloed was waarneembaar tot ongeveer 90 minuten na inname van hydroxytyrosol. Deze relatief korte periode zou verlengd kunnen worden door meerdere malen hydroxytyrosol in te nemen. Hierdoor zou hydroxytyrosol mogelijk effectiever bescherming kunnen bieden tegen oxidatieve schade veroorzaakt door fysieke inspanning.
Er zijn situaties waarbij op voorhand verwacht kan worden dat hydroxytyrosol een positief gezondheidseffect heeft. Hierbij kan worden gedacht aan hydroxytyrosol supplementatie tijdens fysieke inspanning van patiënten met chronisch obstructief longlijden (COPD) of diabetes type 2 patiënten. Deze patiënten hebben, naast een verminderde antioxidant capaciteit, een hoge productie van radicalen onder normale omstandigheden. De door fysieke inspanning verhoogde productie van radicalen zou bij deze patiënten kunnen leiden tot meer geprononceerde negatieve gezondheidseffecten, in vergelijking met gezonde mensen. Het is opvallend dat fysieke inspanning juist aanbevolen wordt in de behandeling van deze patiënten. Hydroxytyrosol supplementatie tijdens fysieke inspanning zou daarom in het bijzonder relevant kunnen zijn voor deze patiënten, omdat dit de endogene antioxidant bescherming tegen de door fysieke inspanning gevormde radicalen kan versterken.

APPENDIX

Dankwoord

About the author

Publications

DANKWOORD

Promoveren doe je niet alleen. Graag wil ik iedereen bedanken die mij geholpen heeft om deze mijlpaal te bereiken.
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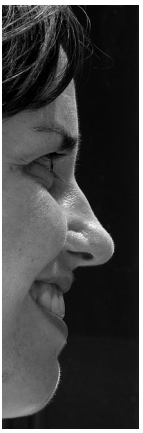
ABOUT THE AUTHOR



Saskia J. Rietjens was born on July 12th 1980 in Roermond. Her secondary school period took place at 'Bisschoppelijk College Broekhin' in Roermond, where she received her gymnasium certificate in 1998.

From 1998-2003 she studied Biomedical Health Sciences at Radboud University Nijmegen with a specialization in Toxicology. Experience in clinical research was obtained during an internship at the department of Internal Medicine of University Medical Centre St Radboud. Additional research experience was obtained during an internship at the Nephrology Research Laboratory, Nijmegen Centre for Molecular Life Sciences, University Medical Centre St Radboud.

In September 2002, she moved to Maastricht to specialize in Nutritional Toxicology. Her MSc thesis entitled 'Oxidative stress: damage and protection' was based on research conducted at the department of Pharmacology and Toxicology of Maastricht University. After obtaining her MSc in 2003, she continued her work as a PhD-student at the same department, under the supervision of Dr. Guido Haenen and Prof. dr. Aalt Bast, resulting in the present thesis. During this period, she also completed the Postgraduate Education in Toxicology, which will result in the registration as Toxicologist.

From September 2007 onward, she works as researcher and consultant in Clinical Toxicology at the National Poisons Information Centre of the National Institute for Public Health and the Environment (RIVM), Bilthoven.

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