The olive oil antioxidant hydroxytyrosol efficiently protects against the oxidative stress-induced impairment of the NO• response of isolated rat aorta

S. J. Rietjens1, A. Bast,1 J. de Vente,2 and G. R. M. M. Haenen1

Departments of 1Pharmacology and Toxicology and 2Psychiatry and Neuropsychology, Faculty of Medicine, Maastricht University, Maastricht, The Netherlands

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The olive oil antioxidant hydroxytyrosol efficiently protects against the oxidative stress-induced impairment of the NO• response of isolated rat aorta. Am J Physiol Heart Circ Physiol 292: H1931–H1936, 2007. First published December 15, 2006; doi:10.1152/ajpheart.00755.2006.—The Mediterranean diet, which is abundant in antioxidants, is associated with a relatively low incidence of coronary heart disease. 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•)-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•). CHP (300 μM) impaired the NO•-mediated relaxation of rat aorta by the acetylcholine agonist carbachol (P < 0.05). This was due to a reduction in NO• production. A diminished NO•-mediated relaxation disturbs the vascular tone and leads to a rise in blood pressure, which is a well-established risk factor for coronary heart disease. Hydroxytyrosol (10 μM) efficiently protected the aorta against the CHP-induced impairment of the NO•-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• scavenger, which can be attributed to its catechol moiety. Because of its amphiphilic characteristics, 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.

minor quantities in olive oil and is mainly found in the olive itself (1) (Fig. 1).

The aim of the present study is to investigate the effects of oxidative stress on the nitric oxide radical (NO•)-mediated vasorelaxation of isolated rat thoracic aorta. Under physiological conditions, NO• is of pivotal importance in the regulation of vascular tone. Endothelium-derived NO• stimulates soluble guanylyl cyclase 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•) by homolytic cleavage of LOOH induces oxidative stress. OH• 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, the protective effect of one of its main metabolites, homovanillic alcohol (Fig. 1), is also studied. To elucidate the molecular mechanism of the protection against CHP provided by the antioxidants, the potential to scavenge OH• is determined.

Materials and Methods

Chemicals. Hydroxytyrosol was obtained from Cayman Chemical (Ann Arbor, MI); oleuropein 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 from Sigma-Aldrich (St. Louis, MO); and H2O2, ascorbate, 2-thiobarbituric acid, and trichloroacetic acid from Merck (Darmstadt, Germany). Anti-formaldehyde-fixed cGMP serum was raised in sheep (15), and 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 95% O2-5% CO2. Each aortic ring was connected to an isometric transducer, and the tension was adjusted to 5 mN. The composition of the Krebs buffer was (mM) 117.5 NaCl, 5.6 KCl, 1.18 MgSO4, 2.5 CaCl2, 1.28 NaHPO4, 25 NaHCO3, and 5.5 glucose. During the experiment, the buffer was changed every 15 min. At the beginning of the experiment, the aortic rings were washed for 60 min.

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To determine the NO•-mediated relaxation, the aortic rings were first precontracted using the \(\alpha\)-adrenergic agonist PE. A dose-response curve of PE from \(10^{-8}\) to \(3 \times 10^{-5}\) M was constructed. Subsequently, the NO•-mediated relaxation using the muncarcin 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 aortic 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 (\(\approx 20\) 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 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 (pD2) and the maximal effects (Emax) of PE and CCh was calculated. The higher the pD2, the more potent PE for 30 min. After two 15-min washes with Krebs buffer, the aortic rings were fixed for 2 h with an ice-cold fixativesolution of 4% freshly prepared depolymerized 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 OCT compound.

A cryostat was used to cut 8-µm sections, which were thawed onto chrome-alum/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 (1:4,000) diluted in TBS containing 0.3% Triton X-100 (TBS-T). The specificity of the cGMP antibody has been described elsewhere (15).

After 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, sections were incubated with Alexa Fluor-donkey anti-sheep IgG conjugate (1:100 dilution) 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 nonspecific 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 ×40 magnification using a cooled charge-coupled device F-view camera on an Olympus AX70 microscope. Exposure time was held constant at 2 s, except when nitroprusside was used, where exposure time was 0.5 s to prevent overexposure. Two 8-µm sections from each aortic ring were analyzed.

**OH•-scavenging activity.** OH•-scavenging activity was determined by the deoxyribose method according to Halliwell et al. (7). This method is based on the competition between the antioxidants and the detector molecule 2-deoxyribose for OH• to derive the second-order rate constant for the interaction of the antioxidant with OH•. The degradation of 2-deoxyribose after reaction with OH• 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 H2O2 (2.8 mM), 2-deoxyribose (2.8 mM), ascorbate (100 µM), and ferric chloride (20 µM). In another experiment, CHP (2.0 mM), instead of H2O2, was used as a source for radicals. The protective effect of hydroxytyrosol was determined.

The second-order rate constant of the scavenger (k) with OH• is calculated using a rate constant of 3.1 \(\times 10^{-9}\) M1 s−1 for deoxyribose (kobs) (7). The absorbance at 532 nm (A) depends on the concentration of the scavenger ([S]) and the absorbance without the scavenger (A0). For calculation of k, the following formula was used:

\[
\frac{1}{A} = \frac{1}{A_0} + \frac{1}{k_{obs} k_D [S]}
\]

where [DR] is the concentration of 2-deoxyribose (2.8 mM). The kobs value of the scavenger is obtained from the slope of the linear plot of the reciprocal value of A vs. [S]. The OH•-scavenging activity of the olive oil compounds was compared with that of the well-known OH• 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
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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 ± SE. Differences in the response of the aorta between the several conditions were statistically analyzed by Student’s t-test. $P < 0.05$ was considered 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 as means ± half range.

RESULTS

Organ bath experiments. During incubation of the aorta with 300 $\mu$M CHP for 30 min, no contraction was observed. Incubation of the aorta with 300 $\mu$M CHP resulted in only a small decrease in $E_{\text{max}}$ of the PE response (Fig. 2A; $P < 0.05$). $pD_2$ of the PE response was not affected after incubation with CHP (Fig. 2A). A similar minor effect on the PE response was also observed after incubation with 100 and 1,000 $\mu$M CHP (data not shown).

The response of the aortic rings to CCh was only slightly diminished after incubation with 100 $\mu$M CHP, whereas it completely disappeared after incubation with 1,000 $\mu$M CHP (data not shown). Incubation with 300 $\mu$M CHP significantly decreased the CCh response ($P < 0.05$), $E_{\text{max}}$ decreased approximately 50%, and $pD_2$ decreased by 0.71 (Fig. 2B). Thus oxidative stress, caused by incubation of the aorta with 300 $\mu$M CHP, drastically impaired the NO\textsuperscript{-}-mediated relaxation by CCh, whereas the PE response was hardly diminished.

Hydroxytyrosol, oleuropein, tyrosol, and homovanillic alcohol (at 30 $\mu$M) 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 $\mu$M) did not protect against the CHP-induced reduction in the NO\textsuperscript{-}-mediated relaxation by CCh (Fig. 3).

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

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 (Fig. 5B) was similar to the cGMP immunostaining of untreated aorta (Fig. 5A). In CHP-treated aortic tissue incubated with sodium nitroprusside, cGMP accumulates in smooth muscle

Fig. 2. Effect of 300 $\mu$M cumene hydroperoxide (CHP) on phenylephrine (PE) and carbachol (CCh) responses. Aorta was contracted with PE (from $10^{-8}$ to $3 \times 10^{-6}$ 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 or CCh. Effects of CHP (○) are expressed as percentage of control (●) PE and CCh curves (no addition is set at 100%). Insets: effects of CHP on maximal effect ($E_{\text{max}}$) and negative logarithm of molar concentration that produces half-maximal effect ($pD_2$) of PE and CCh responses. *$P < 0.05$.

Fig. 3. Protective effect of hydroxytyrosol, tyrosol, oleuropein, and homovanillic alcohol against CHP (300 $\mu$M)-induced impairment of NO\textsuperscript{-}-mediated aortic relaxation. Values represent protection ($[E_{\text{max}}$ (shaded bars) and $pD_2$ (open bars)] of CCh response. Final concentration of antioxidants was 30 $\mu$M.

Fig. 4. Dose-dependent protection of hydroxytyrosol against CHP (300 $\mu$M)-induced impairment of NO\textsuperscript{-}-mediated relaxation. Values represent protection ($[E_{\text{max}}$ (●) and $pD_2$ (○)] of CCh response. Control (without CHP and hydroxytyrosol) is set at 100%. *$P < 0.05$.
To elucidate the mechanism of protection against CHP, the ability of the compounds from olive oil to scavenge OH• was determined. Oleuropein and hydroxytyrosol were very potent OH• scavengers. The OH•-scavenging activity was nearly seven times higher than the activity of mannitol, a well-known OH• scavenger (Fig. 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 ($k_s = 13.2 \times 10^9 \text{M}^{-1}\cdot\text{s}^{-1}$) is comparable to the protection observed when H$_2$O$_2$ 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 (Fig. 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.

Lipophilicity. Lipophilicity is a key factor in many biological effects of compounds. The lipophilicity of hydroxytyrosol was measured by determination of the octanol-water partitioning coefficient ($P_{oww}$ value), 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•-mediated relaxation of rat aorta was determined. During oxidative stress, polyunsaturated fatty acids, 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•, 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•-mediated relaxation by CCh. $pD_2$ decreased 0.7 after incubation with CHP, which means that the acetylcholine receptor-mediated response decreased 80%. $E_{max}$ decreased ~50% after incubation with CHP, which implies that the smooth muscle itself is also affected. Endothelium-derived NO• is an important regulator of vascular tone. Damage to the NO•-regulating mechanism is involved in hypertension and the development or progression of atherosclerosis (11, 13). CHP hardly decreased the PE response, which means that the $\alpha_1$-adrenoceptor-mediated contraction is not affected.

Hydroxytyrosol very efficiently protected the aorta of the rat against the CHP-induced impairment in the NO•-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 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 guanylyl cyclase activity in smooth muscle cells. This indicates that CHP most likely impairs the release of NO• from the endothelium.

We demonstrated that preincubation with hydroxytyrosol before the addition of CHP results in substantially more cGMP levles and compounds sharing identical moieties. Scavenging activities are expressed as the second-order rate constant of the scavenger (kₙ) with OH•.

Catechol, each ortho OH group has a high electron-donating effect. The observed second-order rate constant of hydroxytyrosol (kₙ) 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 at exactly the site of OH• generation. This phenomenon has been called site-specific scavenging. 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 (Pₐw ~ 1), which means that its concentration in cytosol and membranes is practically the same. Pₐw of 1.1 indicates that hydroxytyrosol will readily cross membranes. Its 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.

Amphiphilic compounds, such as hydroxytyrosol, are generally well absorbed. Several studies indeed report that uptake of hydroxytyrosol is good (18, 19, 21). The consumption of olive oil is high in the Mediterranean area 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 hydroxytyrosol concentration of 50 nM (22) to 160 nM (12).

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 (6). 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 (16, 20). For example, it has been shown that hydroxytyrosol is able to protect against the oxidation of low-density lipoprotein (17), 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 (2). 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 the endothelial function is supported by a recent study of Ruano et al. (14), in which they showed that the intake of high-phenolic olive oil, compared with low-phenolic olive oil, improved endothelial function in patients with hypercholesterolemia.

In conclusion, hydroxytyrosol is very efficient in protecting the aorta against the oxidative stress-induced impairment in the NO$^-$-mediated relaxation. NO$^-$ is of pivotal importance in the regulation of the vascular tone. The high potency of hydroxytyrosol is able to protect against the oxidation of low-density lipoprotein (16, 20). For example, it has been shown that phenolic compounds present in olive oil might also contribute to the blood pressure-lowering effect. The beneficial effect of phenolic compounds on the endothelial function is supported by a recent study of Ruano et al. (14), in which they showed that the intake of high-phenolic olive oil, compared with low-phenolic olive oil, improved endothelial function in patients with hypercholesterolemia.

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