Flavonoids can replace α-tocopherol as an antioxidant

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Abstract Endogenous antioxidants such as the lipid-soluble vitamin E protect the cell membranes from oxidative damage. Glutathione seems to be able to regenerate α-tocopherol via a so-called free radical reductase. The transient protection by reduced glutathione (GSH) against lipid peroxidation in control liver microsomes is not observed in microsomes deficient in α-tocopherol. Introduction of antioxidant flavonoids, such as 7-monohydroxyethylrutoside, fisetin or naringenin, into the deficient microsomes restored the GSH-dependent protection, suggesting that flavonoids can take over the role of α-tocopherol as a chain-breaking antioxidant in liver microsomal membranes.

Key words: Lipid peroxidation; α-Tocopherol; Glutathione; Flavonoid; Antioxidant; Free radical reductase

1. Introduction

Tissue damage due to an increased radical production has been reported to play a role in a large number of (patho)-physiological processes such as cancer, ageing, inflammation and ischemia–reperfusion. An important target of free radicals appears to be the biomembrane. The radicals which are not captured by the defence mechanism are able to induce lipid peroxidation (LPO) in membranes. As a consequence of LPO, membrane functions are inactivated: pores are formed in the membrane [1], enzymes are inactivated and the membrane fluidity is altered.

Endogenous antioxidants such as the lipid-soluble chain-breaking antioxidant vitamin E (tocopherol) protect membranes from this oxidative damage [2,3]. Vitamin E is a generic term used to collectively describe a range of tocopherol and tocotrienol molecules. In vivo, α-tocopherol is the most abundant and therefore the most important vitamer. It terminates the chain reaction of LPO by scavenging lipid peroxyl radicals ($LOO^{\bullet}$). In this reaction, α-tocopherol itself becomes a radical, but it is much less reactive than $LOO^{\bullet}$ [2].

Reduced glutathione (GSH) has also been shown to protect against peroxidation of lipids in rat liver [4]. GSH seems to be able to regenerate α-tocopherol from the tocopheroxyl radical via a so-called free radical reductase, mainly present in the liver [4–7]. This recycling process keeps the levels of α-tocopherol high enough to protect the membranes against LPO.

Recently, it has been shown that the anesthetic agent propofol (2,6-diisopropopylphenol), which like α-tocopherol contains an aromatic hydroxyl (OH) group, was able to replace α-tocopherol as an antioxidant [8]. Flavonoids which are a class of naturally occurring compounds are known to be excellent antioxidants. They usually contain one or more aromatic hydroxyl groups and this moiety is, comparable to α-tocopherol, responsible for the antioxidant activity of the flavonoid. Based on this structural similarity, we hypothesized that flavonoids might act similarly to α-tocopherol and could mimic its antioxidant activity in the membrane. Therefore, we investigated if also the flavonoids could replace α-tocopherol. The hepatic microsomal free radical reductase system is very well suited to test this hypothesis.

2. Materials and methods

2.1. Materials

7-Monohydroxyethylrutoside (monoHER) was kindly provided by Novartis Consumer Health, Nyon, Switzerland. Naringenin (95%) was obtained from Sigma Chemicals (St. Louis, MO, USA) and fisetin was purchased from Aldrich (Milwaukee, WI, USA). Vitamin E and $FeSO_4$ were obtained from Merck (Darmstadt, Germany). All other chemicals were of the highest grade available.

2.2. Preparation of the microsomes

Male Wistar rats (200–220 g) obtained from Harlan Olac CPB (Horst, The Netherlands) were killed by decapitation. Livers were removed and homogenized in ice-cold phosphate buffer (50 mM phosphate+0.1 mM EDTA, pH 7.4, 1:2 w/v). The homogenate was centrifuged at $10\,000\times g$ (20 min at 4°C). Subsequently, the supernatant was centrifuged at $100\,000\times g$ for 60 min (4°C). The pellet was resuspended in phosphate buffer and centrifuged again at $100\,000\times g$ (60 min, 4°C). Finally, the microsomal pellet was resuspended in phosphate buffer (microsomes originating from 2 g liver/ml buffer) and 1 ml aliquots were stored at −80°C. Protein concentrations were determined using the Bio-Rad Protein Assay (Bio-Rad Laboratories GmbH, München, Germany) with a bovine serum albumin calibration curve.

2.3. Extraction of α-tocopherol

The stored microsomes were thawed and mixed with two volumes of ice-cold acetone and shaken vigorously at 4°C. After 5 min, the microsomes were centrifuged for 15 min at $1500\times g$ (4°C). The supernatant was removed and the extraction was repeated two times. After the third centrifugation step, the acetone was evaporated on ice under an atmosphere of dry nitrogen. The pellet obtained after evaporation was resuspended in 50 mM Tris–HCl buffer (pH 7.4, 37°C) and washed by centrifugation (45 min, $115\,000\times g$).

2.4. Preincubation with vitamin E or flavonoids

Stock solutions of the flavonoids were freshly prepared in nitrogen-purged DMSO and nanopure water (1:1) just before use, as some of the flavonoids tended to oxidize very quickly. The α-tocopherol-deficient microsomes were preincubated with 4.3 μM vitamin E or with flavonoids using concentrations of approximately three times their

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IC_{50} values of LPO inhibition, i.e. 30.6 μM monoHER, 1.5 μM fisetin or 2.7 mM naringenin. The mixture was shaken vigorously at 4°C for 5 min. Subsequently, the microsomes were washed once by centrifugation.

2.5. LPO assay

Microsomes (originating from 0.5 g liver/ml) were incubated at 37°C, with shaking, and oxygen was freely admitted in Tris–HCl (50 mM, pH 7.4). All compounds were added on ice, after which the incubates were transferred to a water bath (37°C), where 200 μM ascorbate was added. The reaction was started by adding 10 μM freshly prepared $FeSO_4$ in nitrogen-purged nanopure water. The maximum DMSO concentration in the final incubation mixture was 2.5%, which was found to have no influence on the assay. LPO was assayed by measuring thiobarbituric acid (TBA) reactive substances according to Haenen et al. [6]. At $t = 0$, 5, 10, 15, 30, 45 and 60 min, a 0.3 ml aliquot of the incubation mixture was mixed with 2 ml of an ice-cold TBA–trichloroacetic acid–HCl–butylhydroxytoluene (BHT) solution to stop the reaction. After heating (15 min, 80°C) and centrifugation (15 min), the absorbance at 535–600 nm was determined. The TBA–trichloroacetic acid–HCl–BHT solution was prepared by dissolving 41.6 mg TBA/10 ml trichloroacetic acid (16.8% w/v in 0.125 N HCl). 1 ml BHT (1.5 mg/ml ethanol) was added to 10 ml TBA–trichloroacetic acid–HCl. The data are expressed as mean ± S.E.M. ($n = 4$–5).

The IC_{50} was determined with heat-inactivated microsomes by measuring the percentage of LPO inhibition at several concentrations and calculating the concentration at which 50% inhibition was obtained. The IC_{50} values were given as the mean of 3–5 independent experiments.

2.6. Determination of α-tocopherol

To determine α-tocopherol, it was extracted from the membrane by heptane according to the method of Burton et al. [9]. α-Tocopherol was quantified using high performance liquid chromatography (HPLC) (C18 nucleosil column, Chrompack, The Netherlands; eluted with 98% methanol, flow rate 0.6 ml/min) with fluorescence detection (λ_{ex} 295 nm, λ_{em} 340 nm).

2.7. Determination of flavonoids

To determine the flavonoids monoHER, naringenin and fisetin, they were extracted from the membrane using two volumes (v/v) of acidic MeOH (20% (v/v) buffer containing 25 mM NaH_2PO_4, 25 mM acetic acid and 90 μM EDTA (pH 3.33)+80% (v/v) methanol). After centrifugation (3 min, 1°C), the supernatant was mixed with an equal amount of 25 mM phosphate buffer (pH 3.33) containing 90 μM EDTA. After a second centrifugation step (3 min, 1°C), flavonoids were quantified by a standard addition procedure using HPLC (YMC ODS-AQ, 3 μm, 150×4.6 mm, Bester, Amstelveen, The Netherlands; eluted with 51 volumes 10 mM NaH_2PO_4+10 mM acetic acid+36 μM EDTA and 49 volumes MeOH, flow rate 0.7 ml/min) with electrochemical detection (Decade with glassy carbon working electrode set at 0.7, 0.7 and 1.1 V vs. Ag/AgCl reference electrode for monoHER, fisetin and naringenin, respectively).

3. Results

The IC_{50} values determined with heat-inactivated microsomes for naringenin, monoHER and fisetin were 918 [10], 12.7 and 0.2 μM, respectively. GSH (1 mM) clearly protected against LPO in control liver microsomes. After heating to 100°C for at least 2 min to inactivate all enzymatic activity, the GSH protection was completely abolished. After extraction with acetone, the α-tocopherol level dropped from 0.197 ± 0.005 nmol α-tocopherol/mg protein to a concentration of 0.022 ± 0.014 nmol/mg protein. In these α-tocopherol-extracted microsomes, GSH (1 mM) could not protect against LPO anymore, but after preincubation with α-tocopherol (4.3 μM), the protective effect of GSH was restored (Fig. 1A). The resulting membrane concentration was 4.62 ± 0.45 nmol/mg protein (Table 1). When the α-tocopherol-deficient microsomes were preincubated with flavonoids using concentrations of approximately three times their IC_{50} values of LPO inhibition, i.e. monoHER (30.6 μM), fisetin (1.5 μM) or naringenin (2.7 mM), these flavonoids mimicked the effect of vitamin E reintroduction (Fig. 1). The flavonoids themselves protected already against LPO to a certain extent, but all three flavonoids showed a larger protection against LPO in combination with GSH. Membrane concentrations of monoHER and naringenin were 3.8- and 325-fold higher than those obtained for

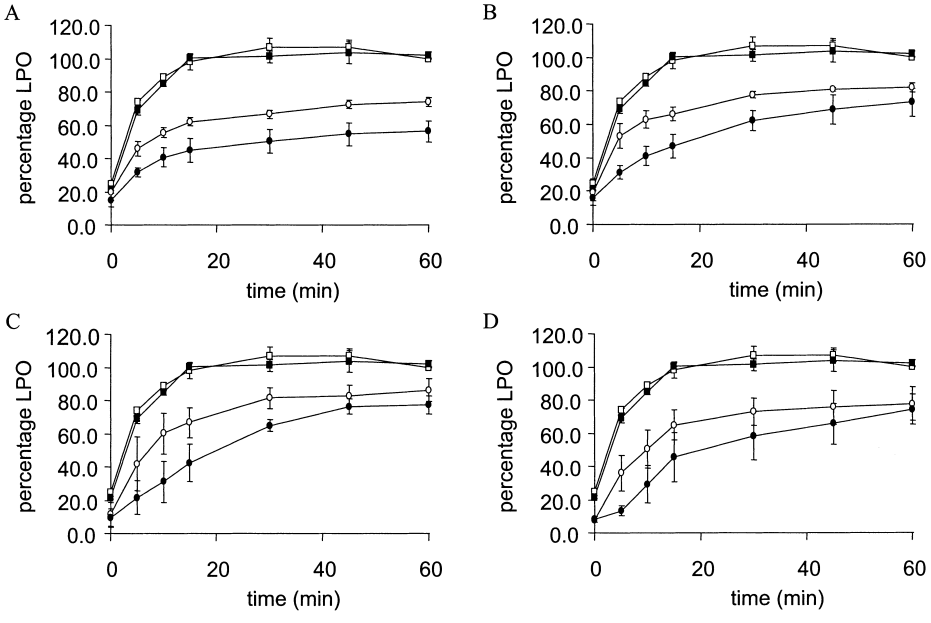

Fig. 1. Representative time-course of LPO in α-tocopherol-deficient microsomes in the presence (■) or absence (□) of GSH and (A) after preincubation with vitamin E (4.3 μM) in the presence (●) or absence (○) of GSH, (B) after preincubation with monoHER (30.6 μM) in the presence (●) or absence (○) of GSH, (C) after the reintroduction of fisetin (1.5 μM) in the presence (●) or absence (○) of GSH and (D) after the introduction of naringenin (2.7 mM) in the presence (●) or absence (○) of GSH.

Table 1
Membrane concentrations of vitamin E, monoHER, naringenin and fisetin after preincubation with 4.3 μM, 30.6 μM, 2.7 mM and 1.5 μM, respectively

Compound	Membrane concentration (nmol/mg protein)
α-Tocopherol	4.62 ± 0.45
MonoHER	17.62 ± 1.65
Naringenin	1505 ± 141
Fisetin	0.83 ± 0.42

vitamin E. Concentrations of fisetin were however 5.6-fold lower (Table 1).

4. Discussion

The protective effect of GSH against Fe^{2+}/ascorbate-induced LPO in liver microsomes depends on the presence of α-tocopherol. We showed that in α-tocopherol-deficient microsomes, GSH did not protect against LPO which is in good agreement with earlier findings [11]. Furthermore, after the addition of α-tocopherol to these depleted microsomes, the GSH-dependent protection was restored. The GSH-dependent protection was completely lost in microsomes that were heated (100°C). This suggests that the GSH-dependent protection depends on a heat labile factor and it is therefore assumed that this factor is the previously described free radical reductase [4–7].

The flavonoids chemically resemble the chain-breaking antioxidant α-tocopherol, because they generally contain one or more aromatic hydroxyl groups. These hydroxyl groups have been shown to be important for the antioxidant activity of the flavonoids [10,12–14] in the same way as for α-tocopherol [2]. We have shown that the introduction of the flavonoids monoHER, fisetin and naringenin into α-tocopherol-deficient microsomes restores the GSH-dependent protection. This suggests that flavonoids act similarly to and can take over the role of α-tocopherol in the membranes. These results also suggest that the free radical reductase is not specific for α-tocopherol, but also shows affinity for other antioxidants such as the flavonoids. This coincides with the effects found for propofol [8].

Although membrane concentrations of monoHER and naringenin are higher than the α-tocopherol concentration in control microsomes (0.197 ± 0.005 nmol α-tocopherol/mg protein), the concentration of fisetin is in the same order of magnitude. These data suggest that fisetin could take over the antioxidant role of vitamin E with a similar potency. Naringenin is much less efficient and very high plasma concentrations would be needed to give an effect. Although monoHER is somewhat less efficient than vitamin E, plasma levels equal to the preincubation concentration of 30.6 μM, which would be needed for the observed effect, are easily obtained. After administration of 500 mg/kg monoHER intraperitoneal to mice, the peak plasma concentration reaches levels of approximately 100 μM (personal communication, M.A.I. Abou El Hassan, Department of Medical Oncology, University Hospital Vrije Universiteit). These concentrations clearly exceed the concentration that is necessary for monoHER to take over the role of α-tocopherol.

The concentrations of the flavonoids were added in the same ratio as their IC_{50} values in LPO determined with heat-inactivated control microsomes (0.2, 12.7 and 918 μM for fisetin, monoHER and naringenin, respectively). Assuming that the GSH protection depends on the concentration of the antioxidant would mean that fisetin, with a membrane concentration of 0.83 nmol/mg protein, was the most potent compound and that naringenin (1505 nmol/mg protein) was the least potent. This potency correlates with the oxidation potential (Ep/2) of the compounds. Ep/2 values are 120 mV for fisetin and 190 mV and 600 mV for monoHER and naringenin, respectively [10]. However, although the Ep/2 value for α-tocopherol is 859 mV [15], a membrane concentration of 4.62 nmol α-tocopherol/mg protein was sufficient to restore the GSH-dependent protection, whereas for naringenin 1505 nmol/mg protein was needed. This suggests that other parameters such as binding to the free radical reductase or membrane localization [16] probably also play a role. Taken together, a high Ep/2 value and a lower affinity for the enzyme or less favorable membrane localization might explain the low potency of naringenin in restoring the GSH-dependent protection.

It is generally accepted that antioxidants co-operate and that free electrons are transferred from one antioxidant to another [17–20]. Flavonoids are excellent antioxidants and therefore it is important to know their place in the antioxidant network. In the present study, we have shown that there is an interaction between flavonoids and glutathione. Moreover, flavonoids can mimic the antioxidant activity of α-tocopherol in the membrane. These properties might explain the beneficial effect of flavonoids in for instance cardiovascular diseases [21].

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