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Oxidative damage shifts from lipid peroxidation to thiol arylation by catechol-containing antioxidants

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Abstract

Background
Catechol-containing antioxidants are able to protect against lipid peroxidation by nonenzymatic scavenging of free radicals with their catechol moiety. However, antioxidants become oxidized during their actual scavenging activity and oxidation products such as semiquinone radicals and quinones are formed. These products may display toxic effects. Therefore, the aim of the present study is to evaluate the effect of catechol-containing antioxidants as well as of their oxidation products on free radical damage. 4-Methylcatechol, a catechol-containing antioxidant that gives stable oxidation products, has been used as model compound.

Methods
The formation of the oxidation products of 4-methylcatechol has been evaluated using electron spin resonance (ESR) measurements. The influence of both the antioxidant and its oxidation products on free radical damage has been investigated in liver microsomes by means of various parameters including lipid peroxidation, thiol content and calcium sequestration.

Results
The oxidation products of 4-methylcatechol inactivated both the glutathione (GSH)-dependent protection against lipid peroxidation and the calcium sequestration in liver microsomes.

Conclusion
The found effects of the oxidation products of 4-methylcatechol are probable due to their arylation of free thiol groups of the enzymes responsible for the GSH-dependent protection and calcium sequestration, i.e. the free radical reductase and calcium ATP-ase. Therefore, it is concluded that a catechol-containing antioxidant might shift radical damage from lipid peroxidation to sulfhydryl arylation.

Introduction

Aerobic life-forms are associated with the generation of reactive oxygen species (ROS). ROS can cause oxidative damage to biological macromolecules like DNA, lipids and proteins (1). An important target of free radicals are the poly-unsaturated fatty acids in biomembranes. Their peroxidation, known as lipid peroxidation, may impair membrane functions, increase permeability, reduce the membrane fluidity, inhibit the signal transduction over the membrane and inactivate membrane-bound enzymes and receptors (2). One of the primary targets in toxicity induced by lipid peroxidation is calcium ATP-ase. Microsomal calcium sequestration by calcium ATP-ase is dependent on critically sulfhydryl groups of the enzyme that are affected by lipid peroxidation (3).

Fortunately, cells contain an elaborate defense system to neutralize ROS. Endogenous antioxidants interact in an elaborate network. For example, several groups have stated that glutathione (GSH) protects against microsomal lipid peroxidation in an interplay with vitamin E. During the protection against ROS, vitamin E radicals are formed. It is proposed that this radical is converted back to vitamin E by a free radical reductase at the expense of GSH (4-8).

In some cases, however, an imbalanced radical production, oxidative stress, arises. It is generally accepted that oxidative stress is implicated in several pathophysiological processes such as cardiovascular heart diseases, diabetes and chronic obstructive pulmonary disease (COPD) (9-11).

To prevent oxidative stress-induced pathologies, antioxidant therapy can be used. An important antioxidant pharmacophore is the catechol moiety present in various antioxidants such as catecholamines and numerous flavonoids like quercetin (12). They are able to scavenge highly reactive species such as peroxynitrite and the hydroxyl radical (13,14). It should be noted that during this activity the antioxidant is converted into oxidized products, i.e. semiquinone radicals and quinones. These products may also be toxic (15-17).

The aim of this study is to evaluate the effect of catechol containing antioxidants on free radical damage. To study the effect of the oxidation products of catechol containing antioxidants, we used 4-methylcatechol which gives a stable oxidation product, 4-methyl-ortho-benzoquinone. This strategy has been used previously (18,19). The effect of 4-methylcatechol as well as the effect of its quinone was examined.

Materials and methods

Chemicals

Reduced GSH, Nitro Blue Tetrazolium (NBT) and chromatographically purified xanthine oxidase (grade III) were from Sigma (St Louis, MO). 4-Methylcatechol was obtained from Fluka (Buchs, Switserland). 4-Methyl-ortho-benzoquinone was synthesized according to Willstätter and Pfannenstiel by the Ag_2O-mediated oxidation of 4-methylcatechol (20).

Methods

Male Wistar rats (Harlan Olec CPB, Zeist, The Netherlands) of 200-250 g were used. Liver microsomes (1mg/ml) were prepared as described previously (4). Lipid peroxidation was measured with the thiobarbituric acid assay (4) and expressed as the absorbance at 535 versus 600 nm (Δ $A_{535-600}$). Oxidative stress was induced by (a) 50 μM xanthine and 55 mU xanthine oxidase or (b) 1 mM H_2O_2 and 40 nM horseradish peroxidase. The microsomes were suspended in a 150 mM Tris-HCl buffer, pH 7.4, containing 150 mM NaCl. In the experiment where the calcium sequestration was measured another buffer was used (vide infra).

Pretreatment of the microsomes with 4-methylcatechol and xanthine-xanthine oxidase consisted of an incubation of 10 min at 37 °C with 100 μM catechol, 50 μM xanthine and 55 mU xanthine-oxidase. The incubation was terminated by the addition of an equal volume of ice-cold buffer. The microsomes were immediately centrifuged (40 min, 115,000 x g at 4°C). The pellet was resuspended in the buffer and, in order to induce lipid peroxidation, incubated with 10 μM Fe^{2+} and 0.2 mM ascorbate.

Thiol content of the pretreated microsomes was measured using 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB). After preincubation, the microsomes were immediately centrifuged (5 min, 115,000 x g at 4°C) and the pellet was resus-pended in the buffer. After incubation with 0.6 mM DTNB for 5 minutes, the reduction of DTNB was measured spectrophotometrically at 412 nm.

Electron spin resonance (ESR) spectra were recorded on a Brucker EMX. It was indicated when $MgCl_2$, which stabilizes the semi-*ortho*-quinone, was added. Instrumental settings were: modulation amplitude 0.1 G; time constant 30 msec; scan time 30 min; amplifier power 25 mW; receiver gain $2 \cdot 10^5$.

Calcium sequestration was determined by incubating microsomes (1 mg/ml) in 115 mM KCl, 10 mM NaCl, 1 mM KH_2PO_4, 20 mM Hepes, 4 mM $MgCl_2$, pH 7.2 at 37°C in the presence of an ATP regenerating system (10 mM creatine phosphate and 10 U/ml creatine kinase). Calcium sequestration was started by the addition of 2 mM ATP. Changes in the free calcium

concentration were monitored by measuring Quin2 fluorescence (50 μM Quin2) on a Perkin Elmer MPF-2A fluorescence spectrophotometer. An excitation wavelength of 339 nm (10 nm slit) and an emission wavelength of 500 nm (10 nm slit) were used. The concentration of free calcium was within the physiological range (200-350 nM). Calcium sequestered was quantified by releasing the sequestrated calcium with ionomycin (2 μM). The calcium concentration was quantified by adding known quantities of calcium into the cuvette.

Protein content was assayed according to Bradford using bovine serum albumin as standard (21).

All experiments were performed at least in triplicate. Unless otherwise mentioned, a typical example is shown.

Results

Oxidative stress induced by xanthine-xanthine oxidase (Figure 2.1A) or H_2O_2–horseradish peroxidase (Figure 2.1B) resulted in the peroxidation of rat liver microsomes.

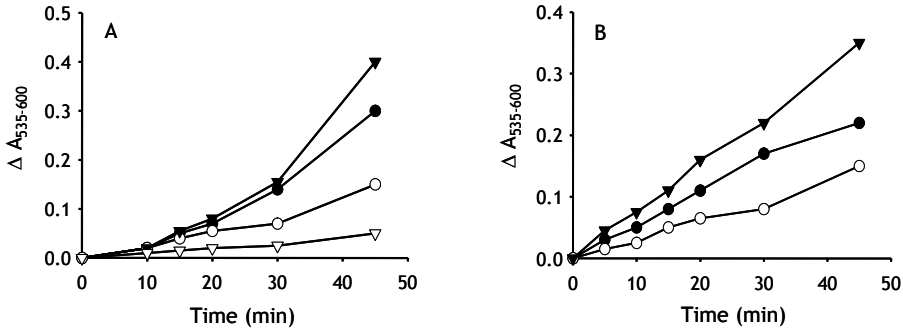

Figure 2.1 Protection against microsomal lipid peroxidation by 4-methylcatechol. Lipid peroxidation (expressed as $\Delta A_{535\text{-}600}$) was induced by xanthine-xanthine oxidase (panel A) or by H_2O_2-horseradish peroxidase (panel B). The concentrations of 4-methylcatechol were: 0 μM (closed triangle) 2 μM (closed circle), 4 μM (open circle) or 10 μM (open triangle).

This also resulted in a 20% reduction of the thiol content of the microsomes (Table 2.1). Addition of 4-methylcatechol gave a concentration-dependent protection against lipid peroxidation, irrespective of the method used to induce lipid peroxidation. Both the rate and the final extent of lipid peroxidation were reduced by adding 4-methylcatechol.

Table 2.1 Lipid peroxidation and protein thiol content of liver microsomes after the antioxidant actions of 4-methylcatechol.

H_2O_2 and horse radish peroxidase			Xanthine and xanthine oxidase		
Conc 4-MC (μM)	*Lipid peroxidation (%)*	*Protein thiols (%)*	*Conc 4-MC (μM)*	*Lipid peroxidation (%)*	*Protein thiols (%)*
0	100 ± 6	81 ± 2	0	100 ± 9	82 ± 2
100	0	50 ± 1	100	0	49 ± 1

Pre-treatment of the microsomes with H_2O_2-horseradish peroxidase or xanthine-xanthine oxidase comprised 10 minutes. Lipid peroxidation is expressed relative to a control incubation without H_2O_2-horseradish peroxidase or xanthine-xanthine oxidase in control membranes ± SD (n=3). One hundred micromolars of 4-methylcatechol (4-MC) itself did not induce lipid peroxidation or reduce the protein thiol content.

The protection by 4-methylcatechol was non-enzymatic, since heating the microsomes had no effect on the inhibition of lipid peroxidation by 4-methylcatechol (data not shown). In contrast to the complete protection by 100 μM 4-methylcatechol against xanthine-xanthine oxidase or H_2O_2-horseradish peroxidase induced lipid peroxidation, 4-methylcatechol increased the amount of thiols that was consumed from 20% to 50%. 4-Methylcatechol itself had, at a concentration of 100 μM, no effect on the thiol content of the microsomes (Table 2.1).

During the protection by 4-methylcatechol against H_2O_2-horseradish peroxidase (Figure 2.2) or xanthine-xanthine oxidase (not shown) induced oxidative stress, 4-methylcatechol becomes oxidized and 4-methyl-benzoquinone is formed. Using ESR a semi-*ortho*-quinone radical and a secondary radical could be detected in the incubation with H_2O_2-horseradish peroxidase (Figure 2.3).

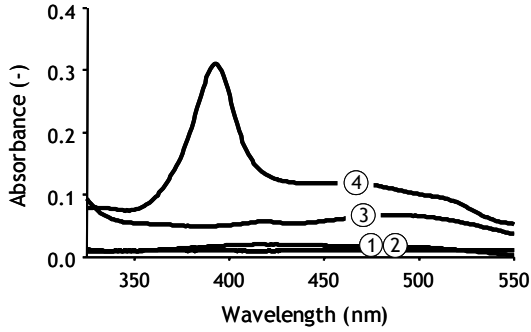

Figure 2.2 UV-spectrum of 100 μM 4-methylcatechol (4-MC; trace 1), of 1 mM H_2O_2 and 40 nM horseradish peroxidase (trace 2) and of 100 μM 4-MC with 1 mM H_2O_2 immediately (trace 3) or 10 minutes after (trace 4) the addition of 40 nM horseradish peroxidase. During this reaction 4-MC becomes oxidized and 4-methyl-quinone is formed.

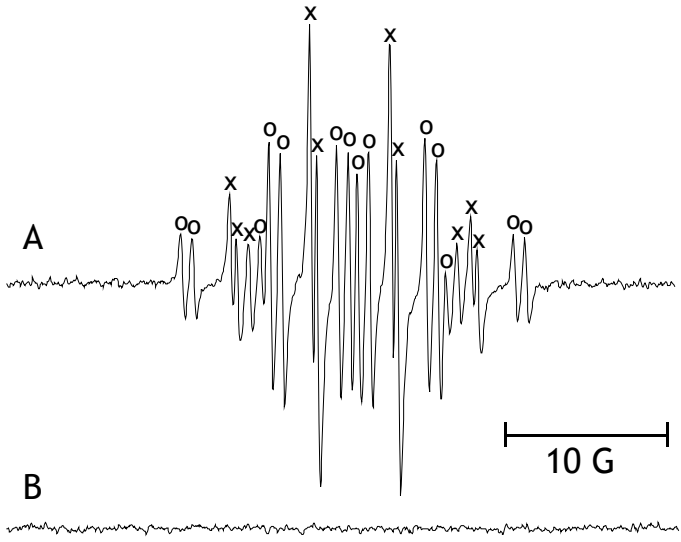

Figure 2.3 ESR spectra of radicals generated from 10 mM 4-methylcatechol by 40 nM horseradish peroxidase and 1 mM H_2O_2 with (panel A) and without 50 mM $MgCl_2$ (panel B). After stabilization of the semi-*ortho*-quinone with $MgCl_2$, a combined spectrum of the semiquinone radical (o) and a secondary radical (x) is obtained. 4-Methylcatechol and H_2O_2/horse radish peroxidase controls with and without $MgCl_2$ gave no detectable signals.

The effect of the products of 4-methylcatechol formed during its antioxidant activity on the endogenous protection by the GSH-dependent free radical reductase against microsomal lipid peroxidation was determined. Therefore microsomes subjected to the combination of 100 μM 4-methylcatechol and xanthine-xanthine oxidase were isolated. No lipid peroxidation was observed in these membranes. After 20 minutes the membranes were washed, in order to remove the xanthine-oxidase and the 4-methylcatechol.

Subsequently, in these pretreated membranes the GSH-dependent protection against lipid peroxidation was assessed. To this end, the pretreated liver microsomes were peroxidized by addition of the combination of iron and ascorbate. In control membranes, addition of 1 mM GSH induced a lag time in the occurrence of lipid peroxidation (Figure 2.4A). In microsomes, pretreated with the combination of xanthine-xanthine oxidase and 4-methylcatechol, the GSH-induced lag time was absent (Figure 2.4B).

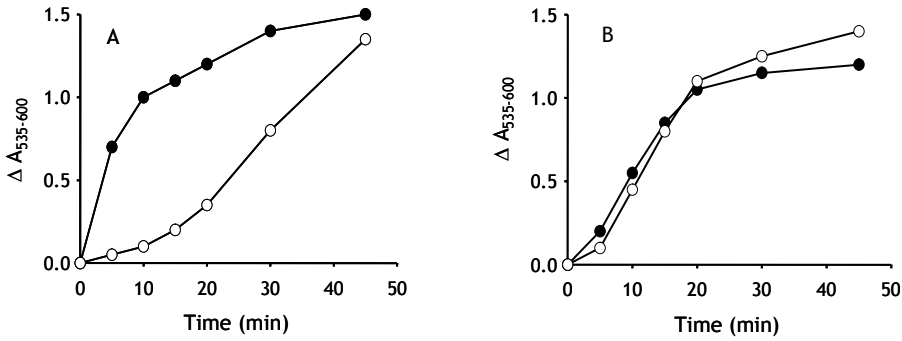

Figure 2.4 Lipid peroxidation in control microsomes (panel A) and microsomes pretreated with 100 μM 4-methylcatechol in combination with xanthine-xanthine oxidase (panel B). Lipid peroxidation (expressed as $\Delta A_{535\text{-}600}$) was induced by the combination of 10 μM Fe^{2+} and 0.2 mM ascorbate. Further additions were: none (closed circle) or 1 mM GSH (open circle).

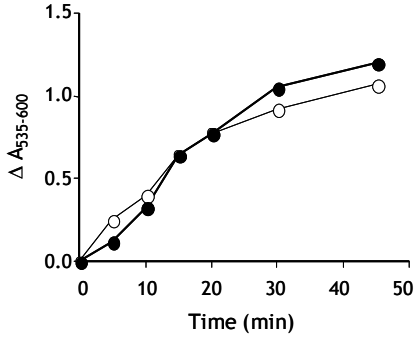

Figure 2.5 Lipid peroxidation in microsomes pretreated with 100 μM 4-methyl-*ortho*-benzoquinone Lipid peroxidation (expressed as $\Delta A_{535\text{-}600}$) was induced by the combination of 10 μM Fe^{2+} and 0.2 mM ascorbate. Further additions were: none (closed circle) or 1 mM GSH (open circle).

To investigate whether thiol arylation by 4-methyl-*ortho*-benzoquinone is responsible for the observed inactivation, the effect of addition of GSH was assessed. It was found that the addition of GSH prevented the reduction of the GSH-dependent protection by 4-methyl-*ortho*-benzoquinone (data not shown). This is probably due to the fact that the non-protein thiol GSH, instead of the sulfhydryl group on the free radical reductase, is arylated. Furthermore, preincubation of the microsomes with 4-methyl-*ortho*-benzoquinone resulted in a concentration dependent reduction of the thiol content of the microsomes (Table 2.2).

Table 2.2 Calcium sequestration and protein thiol content of liver microsomes after N-ethylmaleimide or 4-methyl-ortho-benzoquinone treatment.

H_2O_2 and horse radish peroxidase			Xanthine and xanthine oxidase		
Conc (μM)	*Ca^{2+} sequestration (%)*	*Protein thiols (%)*	*Conc (μM)*	*Ca^{2+} sequestration (%)*	*Protein thiols (%)*
0	100 ± 5	100 ± 2	0	100 ± 5	100 ± 2
100	53 ± 2	59 ± 2	100	50 ± 3	49 ± 1
250	23 ± 1	33 ± 1	250	6 ± 3	31 ± 2
1000	0	4 ± 1	1000	n.d.	2 ± 1

Both calcium sequestration and thiol content of the proteins are expressed relative to the sequestration and the thiol content in control membranes ± SD (n=3). One hundred micromolars of 4-methylcatechol itself did not inactivate calcium sequestration or reduce the protein thiol content.

Of the other enzymes located on the microsomal membrane, the calcium-ATP-ase was also investigated. The enzyme contains an essential and vulnerable sulfhydryl group, since the synthetic sulfhydryl-alkylating agent N-ethylmaleimide impaired the capacity of the enzyme to catalyse the sequestration of calcium (Table 2.2). It was found that 4-methyl-*ortho*-benzoquinone was equally potent as N-ethylmaleimide in inactivating this enzyme and reducing the thiol content of the microsomes (Table 2.2).

Discussion

4-Methylcatechol is effective in preventing microsomal lipid peroxidation, confirming that the catechol moiety is a potent antioxidant pharmacophore (12,22). This antioxidant pharmacophore is present in numerous flavonoids and other polyphenols. It has previously been described that catechol-containing antioxidants are very potent scavengers of e.g. peroxynitrite (13).

In the protection against lipid peroxidation by the catechols, the catechols are oxidized. Products like semiquinone radicals and quinones are produced during the actual antioxidant activity, i.e. the reaction with radicals. It has been well described that these compounds are potentially toxic, especially due to their ability to arylate protein thiols (16,17,23,24). Indeed, a reference quinone, i.e. 4-methyl-*ortho*-benzoquinone, is able to react with the protein thiols as efficient as the synthetic sulfhydryl-alkylating agent N-ethylmaleimide. This observation suggests that the oxidation

products of catechol-containing antioxidants shift the damage provoked by oxidative stress from lipid peroxidation to sulfhydryl arylation.

One of the consequences of this sulfhydryl arylation is that it may indirectly stimulate lipid peroxidation by inactivating the endogenous defences against lipid peroxidation, i.e. the GSH-dependent free radical reductase. This means that, despite the direct protection offered by catechol-containing antioxidants, lipid peroxidation is stimulated indirectly by the reaction products of these antioxidants formed during this protection.

One of the primary toxic effects of lipid peroxidation is inhibition of calcium ATP-ase. Catechol containing antioxidants inhibit lipid peroxidation, but the reactive products formed during this protection also inhibit calcium ATP-ase. So, despite the apparent protection afforded by catechol-containing antioxidants against lipid peroxidation, the toxic effect on a final target, i.e. calcium ATP-ase, is identical.

Sulfhydryl reactivity of the metabolites of catechol-containing compounds has been observed previously (25,26). For example, the toxicity of the catechol-containing compound α-methyldopa is suggested to be due to the scavenging of superoxide radicals generated by cytochrome-P450, a reaction leading to the production of a reactive semiquinone and quinone (26). Another example of sulfhydryl reactivity is the microsomal glutathione S-transferase (GST) that can be activated by covalent binding of its free thiolgroup (25). By this mechanism 4-methyl-*ortho*-benzoquinone and reactive metabolites of a catecholamine activate the microsomal GST (19).

In conclusion, free radical scavengers offer protection against oxidative stress since they can react with damaging reactive species. Despite this protection, the toxicity of reactive oxidation products generated during the actual antioxidant activity might be substantial, e.g. weakening of the endogenous antioxidant defence and impairment of vital enzymes. This should be considered in the application of antioxidants.

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