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The Inhibitory Effect of Tannins on Lipid Peroxidation of Rat Heart Mitochondria

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Abstract

We induced lipid peroxidation in rat heart mitochondria with ferrous sulphate ($FeSO_4$) and compared the inhibitory effect of various tannins on the peroxidation.

Oxygen consumption and malondialdehyde (MDA) formation were used to quantitate the amount of lipid peroxidation, and the free radical scavenger activity of tannins was measured with a diphenyl-*p*-picryl hydrazyl (DPPH) method.

Of 25 tannins and related compounds tested, catechin benzylthioether and procyanidin B-2 benzylthioether were the most potent in inhibiting lipid peroxidation, with inhibitory effects stronger than that of trolox, a water soluble analogue of vitamin E. The concentrations (IC50) required for catechin benzylthioether and procyanidin B-2 benzylthioether to inhibit oxygen consumption to 50% of control values were 0·85 and 2·0 μM, respectively, while their IC50 values from the inhibition of MDA formation were 0·9 and 1·70 μM, respectively. The IC50 values for catechin and procyanidin B-2 to inhibit oxygen consumption were 34·0 and 11·0 μM. Both compounds were less potent than their benzylthioether derivatives. However, the ability of catechin and procyanidin B-2 to scavenge DPPH were similar to that of their benzylthioether derivatives.

We conclude that conjugation with a benzylthioether group enhances the inhibitory effect of tannins on lipid peroxidation, and that the mechanism is not an increase in its scavenger activity.

Tannins are polyphenols widely present in plants (Haslam et al 1989). Since phenolic substances are known to have antioxidant properties, tannins could also be developed for the prevention of lipid peroxidation, biological damage caused by free radicals formed under oxidative stress (Antonius 1988). The heart is a target organ for this injury by oxygen free radicals (Ambrosio 1991; Ferrari et al 1991). The redox reaction is most active in mitochondria and cardiac mitochondria are constantly susceptible to oxidative stress (Hruszkewycz 1988; Wiswedel et al 1989). Several studies have demonstrated that lipid peroxidation in cardiac mitochondria may play an important role in the pathogenesis of cardiac dysfunction (Fukuchi et al 1991). It has also been reported that high concentration of antioxidants in adipose tissue is associated with a reduced risk of myocardial infarction (Kardinaal et al 1993).

With the recent advances in purification technology, a large number of tannins have been isolated and identified, including gallotannins (Haddock et al 1982; Nishizawa et al 1982; Nonaka & Nishioka 1983; Lee at al 1989), ellagitannins (Okuda at al 1989), galloyl quinates (Nishimura et al 1984), flavan-3-ol tannins (Hashimoto et al 1987) and benzylthioethers (Hsu et al 1985). Few studies have been undertaken to compare the relative potencies of these tannins, including their effect on lipid peroxidation. We now compare a total of 25 tannins and related compounds and examine their structure-activity relationships.

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Materials and Methods

Seven gallotannins (gallic acid, 1,6-di-*O*-galloyl-β-D-glucose, 1,2,6-tri-*O*-galloyl-D-glucose, 1,3,6-tri-*O*-galloyl-D-glucose, 3,4,6-tri-*O*-galloyl-D-glucose, 1,2,3,6-tetra-*O*-galloyl-β-D-glucose, 1,2,3,4,6-penta-*O*-galloyl-β-D-glucose), six ellagitannins (ellagic acid, 2,3-HHDP-D-glucose, corilagin, pedunculagin, geraniin, chebulagic acid), three galloyl quinates (4-*O*-galloyl quinic acid, 3,4-di-*O*-galloyl quinic acid, 3,5-di-*O*-galloyl quinic acid), seven flavan-3-ols (catechin, epicatechin-3-*O*-gallate, epigallocatechin-3-*O*-gallate, gallocatechin-3-*O*-gallate, procyanidin B-1, procyanidin B-2, epiafzelechin-(4β-8)-epicatechin) and two benzylthioethers (catechin-benzylthioether, procyanidin B-2 benzylthioether) were tested. The naturally occurring tannins were purified in our laboratory. Benzylthioether derivatives were semi-synthesized by acid-catalysed degradation of the parent compounds with benzylmercaptan (Hsu et al 1985). The structures of these tannins were confirmed from physical data and NMR spectra. The structures of catechin, procyanidin B-2 and their benzylthioether derivatives are shown in Fig. 1.

Mitochondria were prepared from the heart of male rats as previously described (Vercesi et al 1978). Sprague-Dawley rats, 200–300 g, were decapitated and their hearts were quickly excised, opened and thoroughly washed with ice-cold 0·25 M sucrose. The hearts were finely minced with a pair of scissors into 10 vol sucrose-EGTA-HEPES (SEH) buffer which contained 0·25 M sucrose, 0·5 mM EGTA and 3 mM HEPES (pH 7·2). The suspension of minced heart was treated with nagarse at a concentration of 0·1 mg mL^{-1}. After 15 min incubation at 0°C with occasional stirring, the supernatant

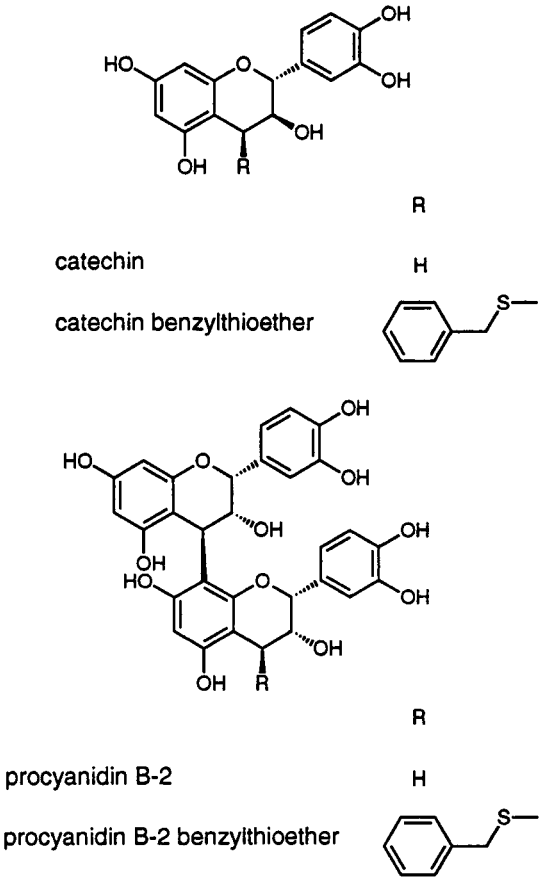

FIG. 1. Structure of catechin, procyanidin B-2 and their benzylthioether derivatives.

was discarded and the remaining tissue washed twice with 0·25 M sucrose. The dispersed heart tissue was then homogenized with a glass pestle and diluted with sucrose solution to 10 mL g^{-1}. The homogenates were centrifuged at 800 *g* for 5 min in the JA 20 rotor of a Beckman J2/21 refrigerated high-speed centrifuge. The supernatant was decanted and centrifuged at 1800 *g* for 5 min. After recentrifugation of the supernatant at 6000 *g* for 10 min, the pellet was resuspended in a KCl-Tris solution containing 175 mM KCl and 20 mM Tris-HCl (pH 7·4) and centrifuged again for 10 min at 12 000 *g*. The mitochondria-rich, red-brown lower layer was suspended in KCl-Tris solution (2 g heart tissue mL^{-1}) stored at 0°C for 3 days before it was used. Freshly prepared mitochondria, like liver and myocardial homogenate (Deng et al 1990) was resistant to lipid peroxidation, so that a cold-storage period was required. Protein concentration of the mitochondrial suspension was determined (Lowry et al 1951).

Lipid peroxidation in rat heart mitochondria was measured according to the method described by Sassa et al (1990). Peroxidation was started by addition of ADP and $FeSO_4$ to the mitochondrial suspension. Final concentrations of ADP and $FeSO_4$ were 1 and 0·1 mM, respectively. The amount of oxygen consumed during the incubation period was monitored with a Clark-type oxygen electrode in a Gilson 5/6 oxygraph (Gilson Medical Electronics, USA). The total volume of the assay medium in the reaction chamber was 1·6 mL. At the end of incubation, 0·3 mL mitochondrial suspension was mixed with 0·1 mL 15·2% trichloroacetic acid. Malondialdehyde was assayed with a thiobarbituric acid technique and the level was used to represent the amount of lipid peroxides formed during incubation (Dousset et al 1990). For estimation of conjugated diene, 0·3 mL mitochondrial suspension was mixed with 0·7 mL ethanol and then centrifuged at 3000 *g* for 10 min. The ultraviolet absorption of supernatant at 234 nm was measured with ethanol as a blank (Esterbauer et al 1989).

For studying the antioxidant effect, tannin was first dissolved in KCl-Tris buffer and then pipetted into the reaction chamber before the incubation of mitochondria and ADP/Fe^{2+}. Oxygen consumption, malondialdehyde and conjugated diene formation in rat heart mitochondria in the presence of tannin were compared with those in their absence. The concentration of added tannin needed to inhibit lipid peroxidation to 50% of control (IC50) was plotted from the concentration-response curve. The effect of Trolox, a water-soluble analogue of vitamin E (Wu et al 1991) was also tested.

The radical-scavenging activity of tannins was determined from the reduction in the optical absorbance at 517 nm due to scavenging of the stable free radical of diphenyl-*p*-picrylhydrazyl (DPPH, Sigma, USA). According to the method of Blois (1958), 10 mL 100 mM acetate buffer, pH 5·5, 10 mL ethanol and 5 mL 500 μM ethanolic solution of DPPH were mixed, 5 mL 2·5–200 μM test compounds were added, and the change in optical density was monitored.

Results

Table 1 shows the IC50 values for the tested tannins and for Trolox. Catechin benzylthioether and procyanidin B-2 benzylthioether were the most potent in both tests. Trolox was less potent than these four tannins. Concentration-response curves for catechin benzylthioether, procyanidin B-2 benzylthioether, procyanidin B-2, catechin and Trolox for the inhibition of oxygen consumption and malondialdehyde formation in rat heart mitochondria are shown in Figs 2 and 3.

The IC50 values for procyanidin B-2 benzylthioether, catechin benzylthioether, procyanidin B-2, catechin and Trolox for the inhibition of conjugated diene formation in rat heart mitochondria were 3·0, 6·2, 17·0, 30·0 and 46·0 μM, respectively. The concentration-response curves for this effect are shown in Fig. 4.

Fig. 5 shows the concentration-response curves for procyanidin B-2 benzylthioether, catechin benzylthioether, catechin, procyanidin B-2, and Trolox to decolourize DPPH after incubation for 90 min. The IC50 values for these compounds were 14, 16, 17·5, 35 and 38 μM, respectively. Compared with their effect on oxygen consumption and malondialdehyde formation, the scavenging effect of catechin benzylthioether and procyanidin B-2 benzylthioether were much closer to that of catechin, procyanidin B-2 and Trolox.

Discussion

This study showed that tannins protected cardiac mitochondria against lipid peroxidation. Most of the test compounds

Table 1. Concentration (IC50 μM) for tannins and Trolox to inhibit oxygen consumption and malondialdehyde formation in rat heart mitochondria to 50% of control. Data are mean of three samples.

Chemical	Molecular weight	O_2 consumption	Malondialdehyde formation
Gallotannins			
Gallic acid	170	22·0	26·0
1,6-Di-*O*-galloyl-β-D-glucose	484	50·0	45·0
1,2,6-Tri-*O*-galloyl-D-glucose	636	35·0	30·5
1,3,6-Tri-*O*-galloyl-D-glucose	636	21·0	24·5
3,4,6-Tri-*O*-galloyl-D-glucose	636	42·0	38·0
1,2,3,6-Tetra-*O*-galloyl-β-D-glucose	788	19·0	23·3
1,2,3,4,6-Penta-*O*-galloyl-β-D-glucose	940	18·0	23·3
Ellagitannins			
Ellagic acid	302	120·0	115·5
2,3-HHDP-D-glucose	482	68·5	65·0
Corilagin	643	12·5	19·5
Pedunculagin	784	7·8	12·0
Geraniin	952	20·0	25·0
Chebulagic acid	954	10·0	16·0
Galloyl quinates			
4-*O*-Galloyl quinic acid	362	31·5	28·0
3,4-Di-*O*-galloyl quinic acid	514	52·0	55·0
3,5-Di-*O*-galloyl quinic acid	514	17·5	23·5
Flavan-3-ols			
Catechin	290	34·0	35·0
Epicatechin-3-*O*-gallate	442	8·4	5.4
Epigallocatechin-3-*O*-gallate	458	12·2	9·4
Gallocatechin-3-*O*-gallate	458	14·0	8·6
Procyanidin B-1	576	16·0	15·5
Procyanidin B-2	576	11·0	9·0
Epiafzelechin-(4β-8)-epicatechin	562	6·8	10·5
Benzylthioethers			
Catechin benzylthioether	412	0·8	0·9
Procyanidin B-2 benzylthioether	652	2·0	1·7
Trolox	250	60·0	55·0

were more potent than Trolox, a water-soluble analogue of vitamin E, in inhibiting $FeSO_4$-induced oxygen consumption and malondialdehyde formation. Since catechin benzylthioether, and procyanidin B-2 benzylthioether were most potent in these effects, we concluded that conjugation with a benzylthioether group enhances the inhibitory effect of flavan-3-ol tannins on lipid peroxidation.

Lipid peroxidation is a complex process. In addition to the effect of oxygen consumption and malondialdehyde formation, we measured the inhibitory effect for catechin benzylthioether, procyanidin B-2 benzylthioether, procyanidin and catechin on conjugated diene formation. Conjugated diene is an intermediate product of lipid peroxidation while malondialdehyde is the end product. These four tannins were also more potent than α-tocopherol in inhibiting diene formation. From the IC50 values, it could be estimated that the potencies for catechin benzylthioether to inhibit oxygen consumption and malondialdehyde formation was 60 times that of α-tocopherol. For the effect on conjugated diene formation, its potency was 7·4 times that of α-tocopherol.

It has been reported that conjugation of a galloyl group in the 3-*O*-position increases antioxidant as well as the free

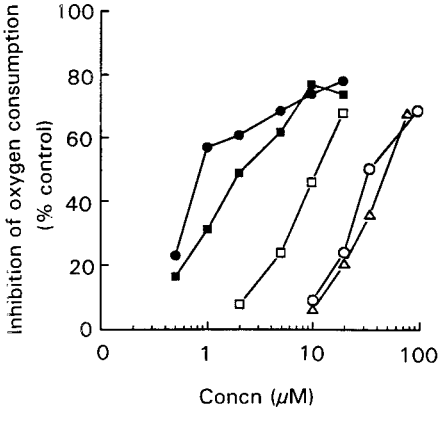

FIG. 2. Dose-response curves for catechin benzylthioether (●), procyanidin B-2 benzylthioether (■), procyanidin B-2 (□), catechin (○) and Trolox (△) to inhibit oxygen consumption due to lipid peroxidation in rat heart mitochondria. Data points were mean values obtained from three different samples.

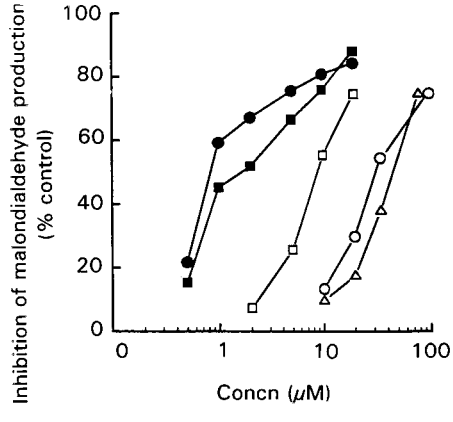

FIG. 3. Dose-response curves for catechin benzylthioether (●), procyanidin B-2 benzylthioether (■), procyanidin B-2 (□), catechin (○) and Trolox (△) to inhibit malondialdehyde formation due to lipid peroxidation in rat heart mitochondria. Data points were mean values obtained from three different samples.

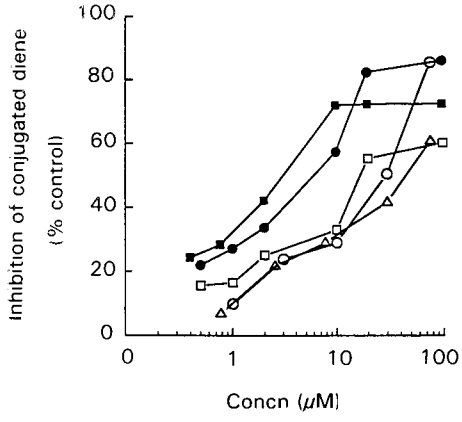

FIG. 4. Dose-response curves for catechin benzylthioether (●), procyanidin B-2 benzylthioether (■), procyanidin B-2 (□), catechin (○) and Trolox (△) to inhibit diene formation due to lipid peroxidation in rat heart mitochondria. Data points were mean values obtained from three different samples.

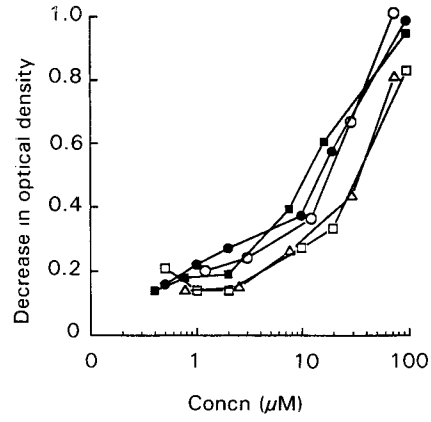

FIG. 5. Dose-response curves for catechin benzylthioether (●), procyanidin B-2 benzylthioether (■), procyanidin B-2 (□), catechin (○) and Trolox (△) to scavenge DPPH expressed as decrease in optical density at 517 nm. Data points were mean values obtained from three different samples.

radical scavenger activities of flavan-3-ol tannins (Uchida et al 1990). We found that the IC50 value for epicatechin-3-*O*-gallate, the most potent flavan-3-ol tannin, to inhibit oxygen consumption and malondialdehyde formation was 8·4 and 5·4 μM, respectively. Because the IC50 values for catechin benzylthioether and procyanidine B-2 benzylthioether to inhibit oxygen consumption was 0·85 and 2·0 μM, while their IC50 values to inhibit malondialdehyde formation were 0·9 and 1·70 μM, respectively, it is clear that these benzylthioethers were more potent than flavan-3-ol tannins. However, we recently found that magnolol and honokiol isolated from *Magnolia officinalis* were 1000 times more potent than vitamin E in inhibiting lipid peroxidation on rat heart mitochondria (Lo et al 1994). The IC50 values for magnolol and honokiol to inhibit oxygen consumption was 0·08 and 0·1 μM, while their IC50 values to inhibit malondialdehyde formation were 0·11 and 0·1 μM, respectively. The potencies of benzylthioethers were less than those of magnolol and honokiol.

To understand the mechanism for the antioxidant effect of tannins, we monitored the decrease in optical absorbance at 517 nm following the trapping of the unpaired electron of DPPH. A positive DPPH test suggested that catechin benzylthioether, procyanidin B-2 benzylthioether, procyanidin and catechin were free-radical scavengers. Unlike their effects on oxygen consumption, conjugated diene and malondialdehyde formation, the free radical scavenging activities of catechin benzylthioether and procyanidin B-2 benzylthioether shown with the DPPH test were similar to that of procyanidin and catechin. Disparity between the effects of antioxidants on oxygen consumption, malondialdehyde formation and the DPPH assay is most likely due to the fact that the DPPH test is a chemical reaction in which mitochondria are not used. Orientation of the phenolic hydroxyl group of antioxidants in mitochondrial membrane and the differential affinity of antioxidants to various types of lipid peroxyl radicals formed in the mitochondrial membrane may all influence their relative potencies. Our study showed that conjugation of tannins with a benzylthioether group does not enhance the free radical scavenger activity of tannins; however, it may increase the inhibitory effect on lipid peroxidation.

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