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Protection of Mitochondrial Functions against Oxidative Stresses by Isoflavans from *Glycyrrhiza glabra*

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Abstract

Isoflavan derivatives, glabridin (**1**), hispaglabridin A (**2**), hispaglabridin B (**3**), 4′-*O*-methylglabridin (**4**) and 3′-hydroxy-4′-*O*-methylglabridin (**5**), isolated from *Glycyrrhiza glabra*, were investigated for their ability to protect liver mitochondria against oxidative stresses.

Mitochondrial lipid peroxidation linked to respiratory electron transport and that induced non-enzymatically were inhibited by these isoflavans. Hispaglabridin A (**2**) strongly inhibited both peroxidations and 3′-hydroxy-4′-*O*-methylglabridin (**5**) was the most effective at preventing NADH-dependent peroxidation. 3′-Hydroxy-4′-*O*-methylglabridin (**5**) protected mitochondrial respiratory enzyme activities against NADPH-dependent peroxidation injury. Dihydroxyfumarate-induced mitochondrial peroxidation was also prevented by this isoflavan.

Isoflavans from *G. glabra* were shown to be effective in protecting mitochondrial function against oxidative stresses.

Biological membranes are sensitive to lipid peroxidation induced by reactive oxygen species. The oxidation of unsaturated fatty acids in biological membranes may cause impairment of membrane function, decrease in the membrane fluidity, inactivation of membrane-bound receptors and enzymes, increase of non-specific permeability to ions and disruption of membrane structure. Inner membranes of mitochondria are particularly at risk from lipid peroxidation, because mitochondria utilize oxygen at a high rate and inner membranes have a large content of polyunsaturated fatty acids together with peroxidation catalysts such as iron and copper (Hingh et al 1995). Mitochondrial damage due to lipid peroxidation causes serious derangements, such as ischaemia–reperfusion injury and neurodegenerative diseases, as well as being associated with ageing (Sohal & Weindruchi 1996).

Liquorice (root and rhizome of *Glycyrrhiza* spp.) has been used for centuries as a medicine because of its wide-ranging therapeutic properties (Fenwick et al 1990). Liquorice is known to contain the sweet principle glycyrrhizin, a biologically active triterpene glycoside. It also contains various flavonoids, isoflavonoids, chalcones and coumarins (Fenwick et al 1990). In our continuous search for chemical constituents in liquorice, a number of phenolic materials have been isolated from *Glycyrrhiza inflata* (Kajiyama et al 1992), *G. uralensis* (Demizu et al 1988) and *G. pallidifora* (Kajiyama et al 1993). Recently, some flavonoids in liquorice have been evaluated as antioxidants and shown to be effective as food additives (Gordon & An 1995). Our biochemical study has also revealed that retrochalcones in *G. inflata* have antiperoxidative and superoxide-scavenging activities in biological membranes (Haraguchi et al 1998). The search for bioactive components in liquorice has led to the isolation of isoflavan derivatives, glabridin (**1**), hispaglabridin A (**2**), hispaglabridin B (**3**), 4′-*O*-methylglabridin (**4**), 3′-hydroxy-4′-*O*-methylglabridin (**5**), from *G. glabra* (Kinoshita et al 1996), which have been found to exhibit potent antioxidative activity. This paper describes the anti-

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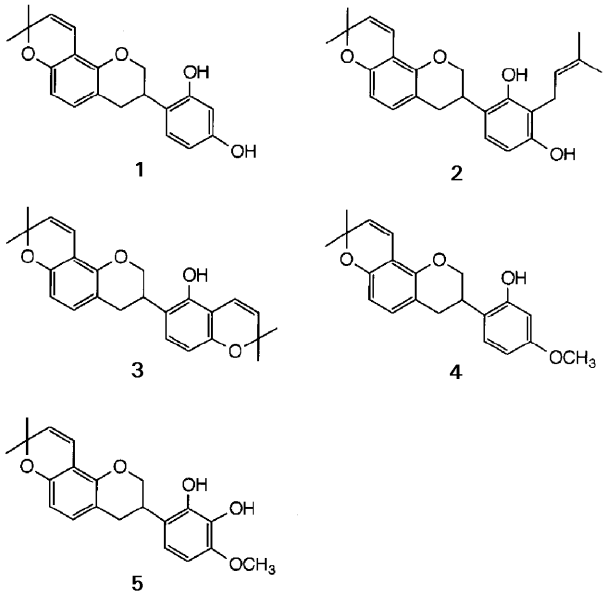

Figure 1. Structure of antioxidative isoflavans from *Glycyrrhiza glabra*. Glabridin (**1**), hispaglabridin A (**2**), hispaglabridin B (**3**), 4′-*O*-methylglabridin (**4**) and 3′-hydroxy-4′-*O*-methylglabridin (**5**).

oxidative characteristics and protective activity of these isoflavans against oxidative stresses in mitochondria. The structures of the isoflavans in *G. glabra* mentioned here are listed in Figure 1.

Materials and Methods

Chemicals

Glabridin (**1**), hispaglabridin A (**2**), hispaglabridin B (**3**), 4′-*O*-methylglabridin (**4**) and 3′-hydroxy-4′-*O*-methylglabridin (**5**) were isolated from the root of *G. glabra*. These isoflavans were completely purified, and NMR (^{1}H and ^{13}C) and MS spectral data supported their purity (Kinoshita et al 1996). Butylated hydroxytoluene, ADP, thiobarbituric acid and dihydroxyfumarate were purchased from Sigma Chemical Co. (St Louis, MO). NADH and NADPH were obtained from Oriental Yeast Co. (Tokyo, Japan).

Preparation of mitochondria

Male Wistar rats (100–150 g) were etherized and then dissected, the livers were quickly removed and dropped into ice-cold 3 mM Tris–HCl buffer (pH 7·4) containing 0·25 M sucrose and 0·1 mM EDTA. Mitochondria were obtained as a pellet after centrifugation at 15 000 *g*, and then resuspended in 100 mM HEPES buffer (pH 7·2). Submitochondrial particles were prepared by sonication of the mitochondrial suspension for 1 min at 4°C using a Model 450 Sonifier (Branson Ultrasonics Corporation, USA) (Haraguchi et al 1996a). Protein concentrations of the suspensions were determined by the method of Lowry et al (1951).

Measurement of lipid peroxidation

Rat liver submitochondrial particles (equivalent 0·3 mg protein) were incubated at 37°C in 1 mL of reaction mixture containing 50 mM HEPES–NaOH (pH 7·0), 2 mM ADP, 0·1 mM $FeCl_3$, 10 μM rotenone and 0·1 mM NADH (Haraguchi et al 1996a). The reaction was initiated by the addition of NADH. After 5 min, 2 mL of TCA–thiobarbituric acid–HCl reagent (15% w/v trichloroacetic acid; 0·375% thiobarbituric acid; 0·25 N HCl) and 90 μL of 2% butylated hydroxytoluene were added to the reaction mixture. The solution was heated for 15 min in a boiling water bath. After cooling, the flocculent precipitate was removed by centrifugation at 1000 *g* for 10 min. The absorbance of thiobarbituric acid reactive substances in the supernatant was determined at 535 nm (Buege & Aust 1978).

Ascorbate-induced mitochondrial lipid peroxidation was measured in a solution consisting of 50 mM HEPES buffer (pH 7·4), 20 mM KCl, 10 μM $FeSO_4$, 0·2 mM ascorbate and the mitochondrial suspension (Nagababu & Lakshmaiah 1992) at 37°C for 20 min. The formation of thiobarbituric acid reactive substances was determined by the same method as described above.

Mitochondrial peroxidation and assay for enzyme activity

NADPH-dependent peroxidation of rat liver submitochondrial particles were acheived in a medium containing 0·1 M mannitol, 5 mM potassium phosphate (pH 7·4), 10 mM Tris–HCl (pH 7·4), 0·1 mM EDTA, 1 mM ADP and 0·3 mM $FeCl_3$ at 25°C (Nishida et al 1987). The reaction was started by the addition of 0·5 mM NADPH. At intervals during incubation, 1 mL of the mitochondrial suspension was taken out from the mixture and NADH-cytochrome *c* reductase and succinate-cytochrome *c* reductase activities were measured.

The reductase activity was assayed by measuring the increase in the absorbance at 550 nm resulting from the reduction of cytochrome *c*. The reaction mixtures contained 50 mM potassium phosphate buffer (pH 7·4), 5 mM NaN_3, 2·1 mg of oxidized cytochrome *c*, and either 200 μM NADH or 20 mM sodium succinate in a total volume of 3 mL (Haraguchi et al 1997).

Dihydroxyfumarate-induced mitochondrial peroxidation was carried out in a solution consisting of 50 mM phosphate buffer (pH 7·4), 0·1 mM $FeCl_3$, 1 mM ADP and 0·3 mM dihydroxyfumarate at 30°C (Yagi & Haraguchi 1997). At intervals during incubation, mitochondrial suspensions were taken out from the mixture and respiratory enzyme activities were measured as described above.

Results

In mitochondrial membranes, an electron transport chain is involved in enzymatic lipid peroxidation. It has been reported that NAD(P)H supports enzymatically-induced lipid peroxidation in submitochondrial particles in the presence of an iron chelate (Glinn et al 1991). Isoflavans isolated from *G. glabra* showed potent inhibiton against mitochondrial lipid peroxidation induced by Fe^{3+}-ADP/NADH. Glabridin (**1**), hispaglabridin B (**3**) and 4′-*O*-methylglabridin (**4**) exhibited almost complete inhibiton at 30 μM. Hispaglabridin A (**2**) showed more potent antioxidative activity; 8 μM of this compound completely inhibited mitochondrial lipid peroxidation. 3′-Hydroxy-4′-*O*-methylglabridin (**5**) was the most effective antioxidant; complete inhibition of mitochondrial NADH-dependent lipid peroxidation was obtained at 1 μM. Their IC50 values (concentration required to inhibit lipid peroxidation by 50%) are shown in Table 1. Butylated hydroxytoluene, a popular antioxidant, required 10 μM for complete inhibition of mitochondrial lipid peroxidation.

Table 1 also shows the effect of isoflavans isolated from *G. glabra* on ascorbate-induced lipid peroxidation (Slater 1984); all were potent inhibitors of this type of non-enzymatically stimulated peroxidation in mitochondria. Hispaglabridin A (**2**) was the most effective inhibitor of ascorbate-induced lipid peroxidation (complete inhibition was obtained at 2·6 μM). Butylated hydroxytoluene required 4·5 μM for complete inhibition.

Table 1. Inhibition of rat-liver mitochondrial lipid peroxidation by isoflavans isolated from *Glycyrrhiza glabra*.

Compound	IC50 (μM)*	
	NADH-dependent	Ascorbate-induced
1	23·4	23·1
2	4·9	1·7
3	19·8	20·8
4	22·4	20·8
5	0·1	22·9

*Inhibitory activity is expressed as the mean of 50% inhibitory concentration of triplicate determinations, obtained by interpolation of concentration–inhibition curves.

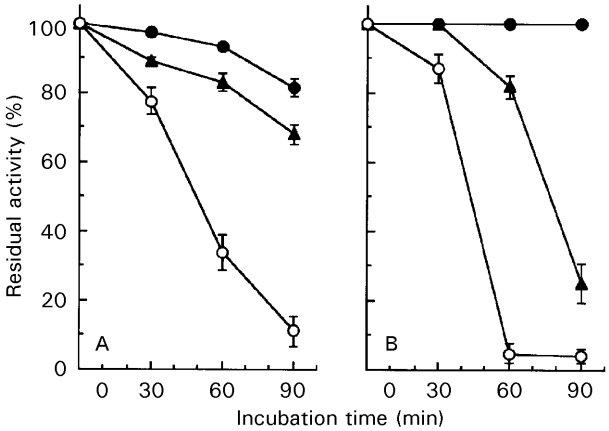

Figure 2. Effect of 3′-hydroxy-4′-*O*-methylglabridin (**5**) on NADPH-dependent oxidative injury in rat-liver mitochondrial respiratory chain. A. NADH-cytochrome *c* reductase activity. B. Succinate-cytochrome *c* reductase activity. Each plot is the mean of triplicate determinations, with the standard deviation indicated by a vertical bar. (●) 1 μM; (▲) 0·3 μM, (○) control.

Various oxidative stresses affect mitochondrial enzyme activities (Veitch et al 1992). NADH-cytochrome *c* reductase and succinate-cytochrome *c* reductase are the most sensitive sites to mitochondrial peroxidative injury. NADPH-dependent lipid peroxidation in submitochondrial particles results in a remarkable loss of these enzyme activities (Narabayashi et al 1982). When rat liver mitochondria were incubated with Fe^{3+}-ADP/NADPH, membrane lipids were peroxidized and NADH- and succinate-cytochrome *c* reductase activities decreased; almost complete loss of activities were observed for 90 and 60 min incubation, respectively. As shown in Figure 2, 3′-hydroxy-4′-*O*-methylglabridin (**5**) protected both enzyme activities against NADPH-dependent peroxidation.

The mitochondrial respiratory chain generates superoxide anions (O_2^-), and subsequently, hydrogen peroxide (H_2O_2) at the level of complex I and at the ubiquinone-cytochrome *b* segment (Boveris 1984). Lipid peroxides produced by hydroxy radicals (OH·) derived from H_2O_2 and O_2^- affect mitochondrial function (Forman & Boveris 1982). The autoxidation of dihydroxyfumarate generates O_2^- and H_2O_2. Once formed, O_2^- leads to the generation of OH· through non-enzymatic dismutation, which is catalysed by Fe^{3+}-ADP (Kukreja et al 1988). When mitochondrial suspensions were incubated with dihydroxyfumarate, respiratory enzyme activities decreased. The addition of Fe^{3+}-ADP to the incubation mixture accelerated the loss of enzyme activities. As shown in Figure 3, 3′-hydroxy-4′-*O*-methylglabridin (**5**)

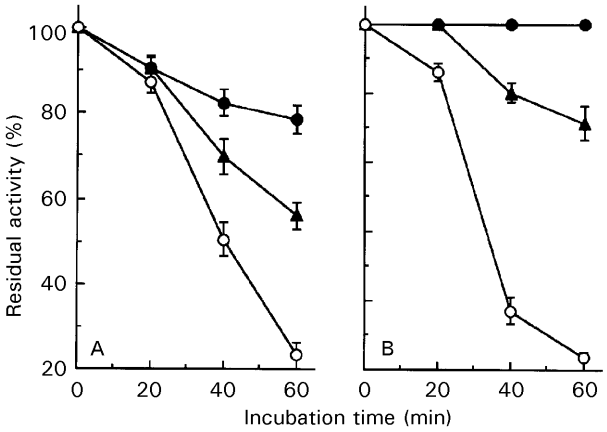

Figure 3. Effect of 3′-hydroxy-4′-*O*-methylglabridin (**5**) on dihydroxyfumarate-induced oxidative injury in mitochondrial respiratory chain. A. NADH-cytochrome *c* reductase activity. B. Succinate-cytochrome *c* reductase activity. Each plot is the mean of triplicate determinations, with the standard deviation indicated by a vertical bar, (●) 1 μM; (▲) 0·3 μM; (○) control.

protected the enzyme activities of NADH- and succinate-cytochrome *c* reductase against dihydroxyfumarate-induced peroxidation.

Discussion

Mitochondrial lipid peroxidation induced by Fe^{3+}-ADP/NADH was inhibited by isoflavans isolated from *G. glabra*. Antioxidative compounds effective in preventing NAD(P)H-dependent lipid peroxidation may inhibit the enzymic reduction of Fe^{3+}-ADP. However, lipid peroxidation started by Fe^{3+}-ascorbate avoids this problem (Halliwell 1995). Isoflavans isolated from *G. glabra* were also effective at preventing this non-enzymic lipid peroxidation in mitochondria. There was little difference in the efficiency of the antioxidative activity of 3′-hydroxy-4′-*O*-methylglabridin (**5**) between peroxidation systems employed. Whatever the mode of induction of lipid peroxidation, there can be differences in the localization of radical formation centres in the membrane. Hence, the efficiency of peroxidation inhibitors can also differ (Kagan et al 1990). Mitochondria are considered to be the most important site where peroxidative processes can arise, causing damage to electron-transfer activities (Guarnieri et al 1985). 3′-Hydroxy-4′-*O*-methylglabridin (**5**) protected respiratory-enzyme activities in the mitochondrial electron transport system against oxidative stresses.

It is considered that the catechol portion of the B-ring (C-3′ and C-4′) contributes to the antiperoxidative effects of flavonoids (Ratty & Das 1988; Cholbi et al 1991). A common hydroxy function exists at the C-2′ position of antioxidative isoflavans isolated from *G. glabra*. In both NADH-dependent and ascorbate-induced lipid peroxidation, hispaglabridin A (**2**) was a potent inhibitor. Contrasting with glabridin (**1**), hispaglabridin A (**2**) has a prenyl moiety between two hydroxyls, which would provide this isoflavan with an affinity for a hydrophobic environment. The most potent inhibitor of NADH-dependent lipid peroxidation, 3′-hydroxy-4′-*O*-methylglabridin (**5**), has an electron-donating methoxy group in the ortho-position of a phenolic hydroxy function, which would contribute to the stability of phenoxy radicals generated in the antiperoxidation process (Haraguchi et al 1996b).

The alcohol extract of liquorice which is used commercially in cosmetics and as a food additive, contains 10–14% of isoflavans mentioned in this article. Absorption and distribution of these compounds after oral administration have not been examined. The concentration of these isoflavans from *G. glabra* in plasma and their protective activity on mitochondrial function in-vivo are under investigation, and structure–activity relationship studies using various radical initiators are underway.

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