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Flavonoids-rich nutrients with potent antioxidant activity prevent atherosclerosis development: the licorice example

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Abstract. *Background*: Macrophage-mediated oxidation of low-density lipoprotein (LDL) has a major role in early atherogenesis, and, thus, intervention means to inhibit this process by flavonoids-rich nutrients (pomegranate, red wine, licorice) are being extensively studied. In the present study, we questioned the ability of the isoflavan glabridin from licorice root to accumulate in macrophages and to affect cell-mediated oxidation of LDL and atherogenesis. *Results*: Macrophages accumulated glabridin, and this process was time and glabridin dose dependent. In parallel, macrophage-mediated oxidation of LDL was inhibited by up to 80%. Glabridin inhibited superoxides release from MPM by up to 60%, and in glabridin-enriched macrophages, reduced protein kinase C activity by 70% was noted, in comparison to control cells. Translocation of P-47phox, the cytosolic component of NADPH oxidase, to the plasma membrane was substantially inhibited by glabridin. Macrophages (MPM) isolated from the atherosclerotic apolipoprotein E-deficient (E^o) mice that consumed glabridin (20 μg/mouse/day) for 6 weeks demonstrated reduced capability to oxidize LDL by 80%, in comparison to placebo-treated mice. This latter phenomenon was associated with a substantial 50% reduction in the mice aortic lesion size. *Conclusions*: Glabridin accumulation in macrophages affects cell-signaling processes, which are associated with activation of the cellular NADPH oxidase system. These phenomena are responsible for the inhibition of cell-mediated oxidation of LDL and the attenuation of atherosclerosis developments by dietary glabridin.

Keywords: Antioxidants; Flavonoids; Licorice; Glabridin; Lipid peroxidation; LDL; Oxidized-LDL; Aggregated-LDL; Paraoxonase; Atherosclerosis

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1. Atherogenic modifications of low-density lipoprotein (LDL) and atherosclerosis

Most of the accumulated cholesterol in foam cells originates from plasma LDL, which is internalized into the cells via the LDL receptor. Native LDL, however, does not induce cellular cholesterol accumulation because the LDL receptor activity is downregulated by the cellular cholesterol content [1]. LDL has to undergo some modifications, such as oxidation, in order to be taken up by macrophages at enhanced rate via the macrophage scavenger receptors pathway, which, unlike the LDL receptor, are not subjected to downregulation by cellular cholesterol content [1–3]. The underlying mechanisms leading to the formation of atherosclerotic lesion under oxidative stress are complicated and represent the outcome of multiple interactive processes.

The ''oxidative modification of lipoproteins'' hypothesis of atherosclerosis proposes that LDL oxidation plays a pivotal role in early atherogenesis, and this hypothesis is supported by evidence that LDL oxidation occurs in vivo and contributes to the clinical manifestation of atherosclerosis.

2. Macrophage-mediated oxidation of LDL

The identity of the cells responsible for the oxidation of LDL along atherogenesis in the arterial wall is uncertain. Monocyte-derived macrophages are likely candidates to induce the oxidation of LDL during early atherogenesis, as they are prominent in arterial lesions, and because they generate reactive oxygen and nitrogen species. LDL oxidation by arterial wall cells was suggested to involve the activation of macrophage 15-lipoxygenase and of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase [4]. Macrophage-mediated oxidation of LDL can also result from an initial cellular lipids peroxidation. When cultured macrophages were exposed to ferrous ions, cellular lipid peroxidation took place [5]. These ''oxidized macrophages'' could easily oxidize LDL even in the absence of any added transition metal ions.

3. Paraoxonase and LDL oxidation

Human serum paraoxonase 1 (PON1) is an esterase, which is physically associated with HDL and is also distributed in tissues such as liver, kidney and intestine [6].

Human serum paraoxonase activity was shown to be inversely related to the risk of cardiovascular disease, as shown in atherosclerotic, hypercholesterolemic and diabetic patients [7]. HDL-associated PON1 has recently been shown to protect LDL, as well as the HDL particle itself, against oxidation induced by either copper ions or by free radicals generators, and this effect could be related to the hydrolysis of the specific lipoprotein's oxidized lipids such as cholesteryl linoleate hydroperoxides and some specific oxidized phospholipids. Protection of HDL from oxidation by PON1 was shown to preserve the antiatherogenic effect of HDL in reverse cholesterol transport, as shown by its beneficial effect on HDL-mediated macrophage cholesterol efflux. These effects of PON1 may be relevant to its beneficial properties against cardiovascular disease. Antioxidants were shown to preserve PON1's activity as they decrease the formation of lipid peroxides, which can, in turn, inactivates PON1.

4. Flavonoids and cardiovascular disease

Dietary consumption of flavonoids was shown to be inversely related to morbidity and mortality from coronary heart diseases. Flavonoids compose the largest and most studied group of plant phenols. Over 4000 different flavonoids have been identified to date.

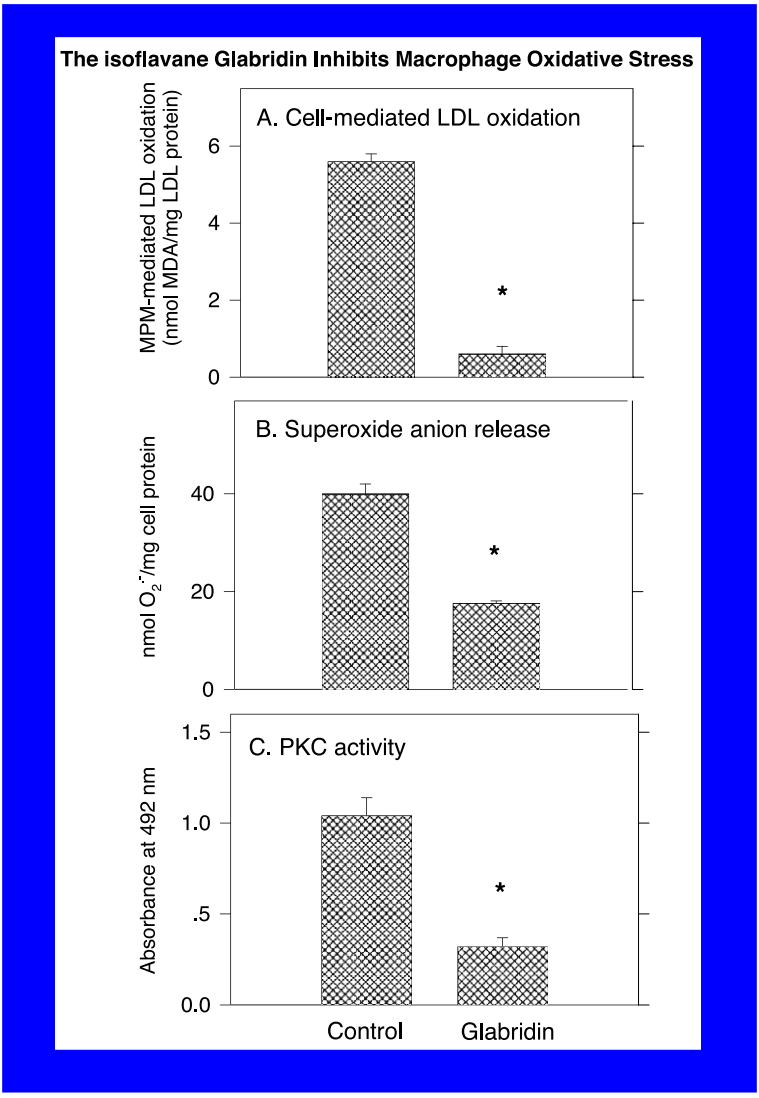

Fig. 1. Licorice-derived glabridin inhibits superoxide-induced macrophage-mediated oxidation of LDL: a role for protein kinase C.

Flavonoids are powerful antioxidants, and their activity is related to their chemical structures [8]. Plant flavonoids can act as potent inhibitors of LDL oxidation via several mechanisms including scavenging of free radicals, chelation of transition metal ions, or preservation of serum paraoxonase (PON1) activity (and as a result, hydrolysis of LDL-associated lipid peroxides).

5. Licorice, LDL oxidation and atherosclerosis: characterization of antioxidant constituents from the licorice roots

Glycyrrhiza glabra, the licorice plant, has been known as a healthy nutrient for more than 3000 years. The licorice roots have long been used as flavoring and sweetening agents.

Among licorice flavonoids, glabridin constituted the major flavonoid in the licorice root extract (500 mg/kg of root ethanolic extract). Upon LDL incubation with glabridin, the latter was shown to bind to the LDL and subsequently protect it from oxidation [9–11].

Glabridin inhibited AAPH-induced LDL oxidation in a dose-dependent manner.

Addition of glabridin (30 μM) to LDL that was incubated with AAPH or with copper ions inhibited the formation of oxidized lipids and oxysterols (7-hydroxycholesterol, 7-ketocholesterol and epoxycholesterol) by 65%, 70% and 45%, respectively. Glabridin inhibited the consumption of β-carotene and that of lycopene by 41% and 50%, respectively, after 1 h of LDL oxidation in the presence of AAPH, but failed to protect vitamin E, the major LDL-associated antioxidant, from oxidation [11]. Glabridin was also found to preserve the arylesterase activity of human serum PON1, including its ability to hydrolyze oxidized LDL cholesteryl linoleate hydroperoxides, since PON1 was found to be more potent in reducing the amount of cholesteryl linoleate hydroperoxides when added to Ox-LDL in the presence of glabridin, in comparison to its effects in the absence of glabridin. Glabridin was also shown to accumulate in macrophages in a dose- and time-dependent manner and in parallel, in glabridin-enriched cells, macrophage-mediated oxidation of LDL was inhibited by up to 80%, in comparison to control cells (Fig. 1A). These effects could be related to glabridin-mediated inhibition of superoxide anion release from macrophages in response to phorbol 12-myristate 13-acetate (PMA) (Fig. 1B), to inhibition of the translocation of P-47 (a cytosolic component of NADPH oxidase) to the plasma membrane and to a reduction in cellular protein kinase C activity [12] (Fig. 1C), which is required for P-47 phosphorylation and activation. Thus, glabridin-induced inhibition of P-47 phosphorylation may be the primary event responsible for its inhibitory effect on NADPH oxidase-induced macrophage-mediated oxidation of LDL. All of the above inhibitory effects of glabridin on the events related to cell-mediated oxidation of LDL required the hydroxyl groups on the isoflavan B ring [10].

6. The antioxidative properties of licorice consumption in humans

Evaluation of the protective effect of licorice root extract on the resistance of LDL to ex vivo oxidation was studied in normolipidemic human subjects [13], as well as in hypercholestero-lemic patients [14]. LDL, which was isolated from the plasma of ten

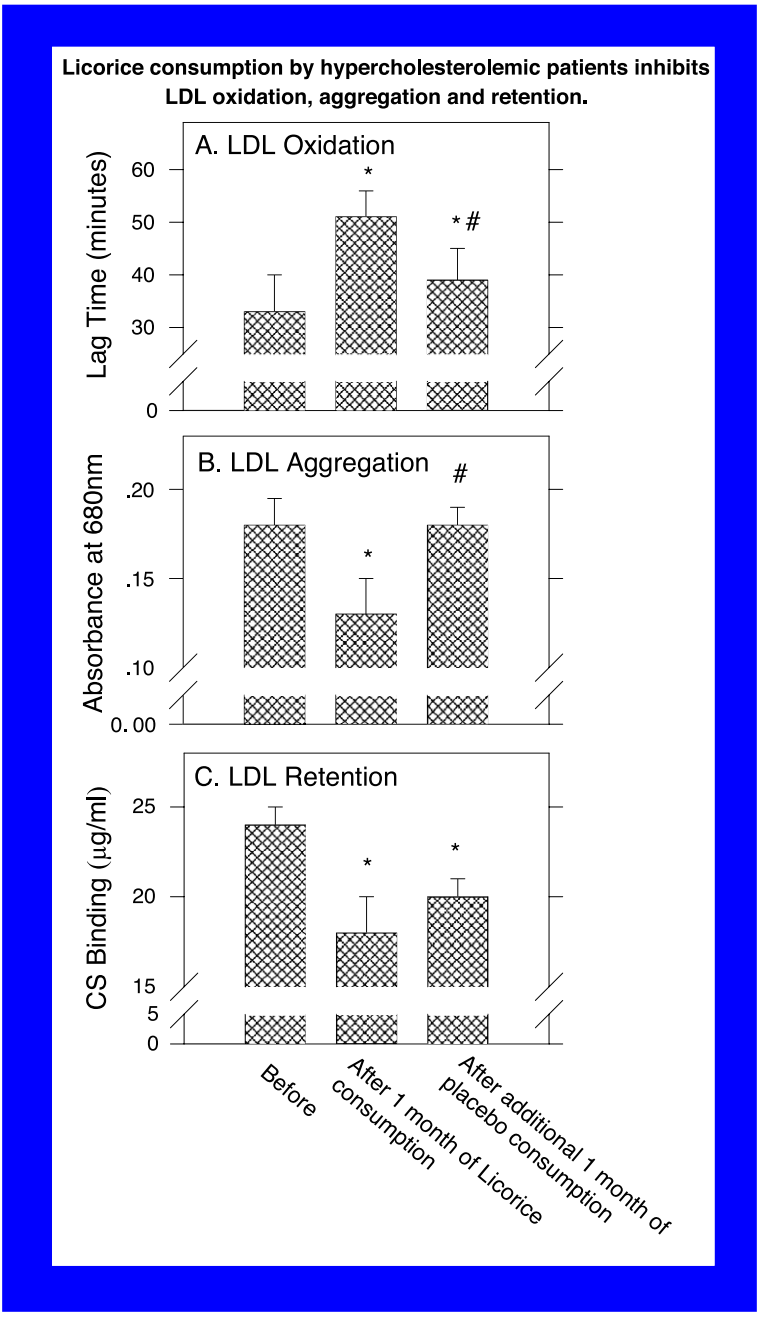

Fig. 2. Licorice consumption by hypercholesterolemic patients inhibits LDL modifications.

healthy volunteers after consumption of 100 mg of licorice root ethanolic extract per day, for a period of 2 weeks, was more resistant to copper ion-induced oxidation, as well as to AAPH-induced oxidation, by 44% and 36%, respectively, in comparison to LDL isolated prior to licorice supplementation. Supplementation of licorice root extract (0.1 g/day) to hypercholesterolemic patients for a period of 1 month was followed by an additional 1 month of placebo consumption [14]. Licorice consumption resulted in a moderate reduction in the patients' plasma susceptibility to lipid peroxidation (by 19%), and in a marked reduction in the susceptibility of the patients' plasma LDL to oxidation, as shown by a prolongation of the lag time required for the initiation of LDL oxidation by 55%, in comparison to the lag time of LDL isolated from plasma derived before licorice extract consumption (Fig. 2A). This effect was even partially sustained after an additional 1 month of placebo supplementation, since LDL derived after this period was still less susceptible to copper ion-induced lipid peroxidation, as demonstrated by an 18% increment in the lag time, in comparison to the baseline lag time (before licorice administration).

Atherogenicity of LDL is attributed not only to its oxidative modification but also to its aggregation. Upon analyzing the susceptibility to aggregation of LDL isolated from hypercholesterolemic patients that consumed licorice extract for 1 month, a significant reduction of 28% in LDL aggregation was observed (Fig. 2B). After an additional 1 month of placebo consumption, LDL aggregation rates returned towards baseline values.

Retention of LDL, which is an early step in atherogenesis, was measured by analysis of LDL binding to the proteoglycan chondroitin sulfate (CS). Following licorice consumption, LDL CS binding ability was significantly reduced by 25%, and this effect was partially sustained for the additional 1 month of placebo consumption (Fig. 2C).

Licorice extract supplementation resulted also in a 10% reduction in the patients' systolic blood pressure, which sustained for an additional month (during the placebo consumption). Thus, dietary consumption of licorice root extract by hypercholesterolemic patients may act as a moderate hypotensive nutrient and as a potent antioxidant agent, which confer its health benefit against cardiovascular disease.

7. The effect of licorice consumption by mice on LDL oxidation, macrophage foam cell formation and atherosclerosis development

Dietary supplementation of licorice (200 μg/day/mouse) to the apolipoprotein E-deficient (E^o) mice for a period of 6 weeks resulted in 80% reduction in the susceptibility of their LDL to copper ion-induced oxidation, in comparison to LDL isolated from placebo-treated mice [13]. Administration of purified glabridin to E^o mice in their drinking water was followed by analysis of its antioxidative effect against ex vivo LDL oxidation [13]. GC–MS analysis of the LDL, derived from E^o mice after consumption of glabridin, revealed that glabridin was absorbed and bound to the LDL particle. Whereas no glabridin could be detected in LDL from control mice, LDL from mice that consumed glabridin (20 μg/day/mouse) contained about 2 nmol of glabridin/mg LDL protein. LDL derived from E^o mice after consumption of 20 μg glabridin/day/mouse for a period of 6 weeks was significantly more resistant to copper ion-induced oxidation by 22% than LDL derived from placebo-treated mice. Administration of glabridin (25 μg/day/mouse) to E^o mice for a

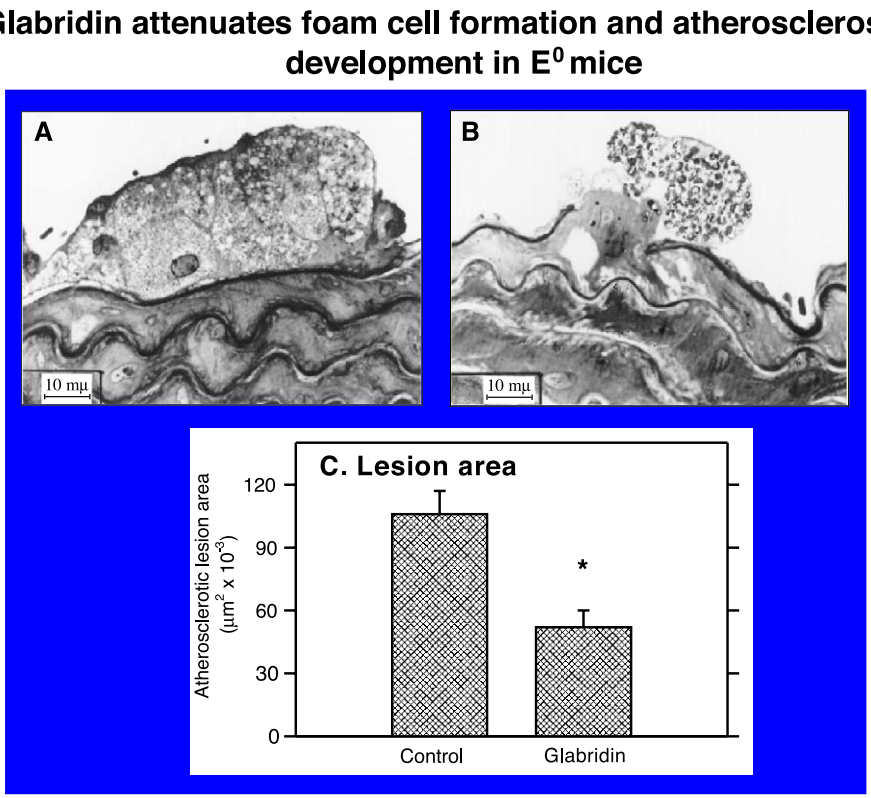

Fig. 3. Licorice-derived glabridin consumption decreased macrophage foam cell formation and attenuated atherosclerosis progression in the atherosclerotic E^o mice.

period of 3 months also reduced (by 50%) an additional atherogenic modification of LDL, i.e., its susceptibility to aggregation induced by vortexing. Most important, inhibition of atherogenic modifications of LDL (oxidation and aggregation) in E^o mice following glabridin consumption was associated with a substantial reduction in macrophage foam cell formation (Fig. 3B vs. A) and in the development of the atherosclerotic lesion area (Fig. 3C).

We thus conclude that licorice-derived glabridin can protect LDL against cell-mediated oxidation via two major pathways, including a direct interaction with the lipoprotein and/ an indirect effect through accumulation in arterial macrophages. Licorice thus represents a most potent nutrient, which can attenuate the development of atherosclerosis, secondary to its antioxidation properties against lipids peroxidation in arterial cells and in lipoproteins.

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