Macrophage Enrichment with the Isoflavan Glabridin Inhibits NADPH Oxidase-induced Cell-mediated Oxidation of Low Density Lipoprotein

A POSSIBLE ROLE FOR PROTEIN KINASE C*†

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Macrophage-mediated oxidation of low density lipoprotein (LDL) is considered to be of major importance in early atherogenesis; therefore, intervention means to inhibit this process are being extensively studied. In the present study, we questioned the ability of the isoflavon glabridin (from licorice) to accumulate in macrophages and to affect cell-mediated oxidation of LDL. We first performed in vitro studies, using mouse peritoneal macrophages (MPMs) and the J-774 A.1 macrophage-like cell line. Both cells accumulated up to 1.5 μg of glabridin/mg of cell protein after 2 h of incubation, and this process was time- and glabridin dose-dependent. In parallel, in glabridin-enriched cells, macrophage-mediated oxidation of LDL was inhibited by up to 80% in comparison with control cells. Glabridin inhibited superoxide release from MPMs in response to phorbol 12-myristate 13-acetate, or to LDL when added together with copper ions, by up to 60%. Translocation of P-47, a cytosolic component of NADPH oxidase to the plasma membrane was substantially inhibited. In glabridin-enriched macrophages, protein kinase C activity reduced by ~70%. All of the above effects of glabridin required the presence of the two hydroxy groups on the flavonoid’s B phenol ring. In order to assess the physiological significance of these results, we next performed in vivo studies, using the atherosclerotic apolipoprotein E-deficient (Eo) mice. MPMs harvested from glabridin-treated Eo mice (20 μg/mouse/day for a period of 6 weeks) demonstrated reduced capability to oxidize LDL by 80% in comparison with placebo-treated mice. This latter phenomenon was associated with a reduction in the lesion oxysterols and a 50% reduction in the aortic lesion size.

We thus conclude that glabridin accumulation in macrophages is associated with reduced cell-mediated oxidation of LDL and decreased activation of the NADPH oxidase system. These phenomena could be responsible for the attenuation of atherosclerosis in Eo mice, induced by glabridin.

The LDL1 oxidation hypothesis of atherosclerosis is supported by evidence for the accumulation of oxidized LDL in the atherosclerotic lesion, by increased LDL oxidizability in patients with increased risk for atherosclerosis, and by the antiatherogenicity of several potent antioxidants against LDL oxidation (1–4). Extensive investigation is being made to identify natural food products that can offer antioxidant defense against LDL oxidation. Flavonoids are polyphenolic compounds naturally present in fruits and vegetables and are an integral part of the human diet (5). Consumption of flavonoids in the diet was shown to be inversely associated with morbidity and mortality from coronary heart disease (6, 7). Flavonoids are powerful antioxidants and their antioxidative capacity is related to their chemical structure (8, 9). We have recently shown that dietary supplementation of healthy human volunteers with flavonoid-rich nutrients such as olive oil, red wine, or licorice root, resulted in a reduction in LDL oxidizability (10–12). Furthermore, consumption of red wine or its flavonol quercetin or of licorice extract by atherosclerotic apolipoprotein E-deficient (Eo) mice resulted in a significant reduction in the development of atherosclerotic lesions, along with a reduction in their LDL oxidation (12, 13). We have also demonstrated that the isoflavon glabridin binds to the LDL particle and substantially inhibits its oxidation (12, 14–16). The hydroxy groups on the glabridin B ring were found to be most important for its antioxidative properties (16). In vivo, antioxidants can bind to, and accumulate in, cells, including arterial wall macrophages. Macrophage-mediated oxidation of LDL is considered to be of major importance during early atherogenesis, and the formation of oxidized LDL can then induce cellular cholesterol accumulation and foam cell formation (17). Macrophage-mediated oxidation of LDL is affected by the balance between cellular oxygenases (18, 19) and antioxidants, such as the glutathione system (20). We have shown activation of the macrophage NADPH oxidase following binding of LDL to its receptor under oxidative stress (21), leading to LDL oxidation. Recently, this enzyme was shown to be also present in the arterial wall adventitia (22). Several studies have shown that flavonoids attenuate cell-mediated oxidation of LDL when

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1 The abbreviations used are: LDL, low density lipoprotein; H-7, 1-(5-isoquinolinesulfonyl)-2-methylpiperezine dihydrochloride; MPM, mouse peritoneal macrophage; PBS, phosphate-buffered saline; HPLC, high performance liquid chromatography; PMA, phorbol 12-myristate 13-acetate; PKC, protein kinase C.
added to the extracellular medium (23–26). Under these conditions, it is not clear whether the antioxidants exert their effect within the lipoprotein particle, in the medium, or at the cellular level. Only few studies examined the capability of macrophages enriched with vitamin E to oxidize LDL (27, 28). In one study, it has been reported that loading J-774 A.1 macrophages with high concentrations of vitamin E can protect LDL against macrophage-mediated oxidation (27). In another study, when MPMs or human monocytes were incubated with vitamin E at a more physiological concentration, no effect on cell-mediated oxidation of LDL was obtained (28). Flavonoids not only can act as antioxidants, but they can also inhibit some cellular enzymes (29–35). Since some of these enzymes are involved in macrophage-mediated oxidation of LDL, the present study analyzed the effect (and mechanisms) of macrophage enrichment with the isoflavon glabridin on the capability of these cells to oxidize LDL.

**EXPERIMENTAL PROCEDURES**

**Materials**

EGTA, ATP, phenylmethanesulfonyl fluoride, leupeptin, cytochrome c from horse heart, phorbol 12-myristate 13-acetate (PMA), FAD, β-NADPH, superoxide dismutase from bovine erythrocytes, SDS, 1-(5-isouquinolinesulfonyl)-2-methylpiperazine dihydrochloride (H-7), calphostin C, staurosporine, and N-(2-guanidinoethyl)-5-isouquinolinesulfonamide hydrochloride were all purchased from Sigma.

**Cells and Cell Fractionation**

The J-774 A.1 murine macrophage-like cell line was purchased from the American Type Culture Collection (ATCC, Rockville, MD). The macrophages were plated at 1 x 10⁶ cells/35-mm dish in Dulbecco's modified Eagle's medium supplemented with 5% heat-inactivated fetal calf serum. The cells were fed every 3 days (36). Mouse peritoneal macrophages (MPMs) were harvested from the peritoneal fluid 4 days after intraperitoneal injection of 3 ml of thioglycolate in saline (24 g/mouse) (37).

The cells (10–20 x 10⁶/mouse) were washed and centrifuged three times with phosphate-buffered saline (PBS) at 1000 x g for 10 min. Then the cells were resuspended to 10⁶/ml in Dulbecco's modified Eagle's medium containing 10% horse serum (heat-inactivated at 56 °C) of the compounds at 230 nm, using acetonitrile/water as eluents (16). The amounts of glabridin and 2′, 4′-dimethylglabridin were dissolved in 100% ethanol to stock solutions of 1.063 g/ml and dialyzed against 150 mM NaCl, 1 mM EDTA (pH 7.4) at 4 °C. LDL was prepared by discontinuous density gradient ultracentrifugation as described previously (29). The lipoprotein was washed at d = 1.063 g/ml and dialyzed against 150 mM NaCl, 1 mM EDTA (pH 7.4) at 4 °C. LDL was then sterilized by filtration (0.45 μm), kept at 3–6 mg of protein/ml under nitrogen in the dark at 4 °C and used within 2 weeks. Prior to the oxidation studies, LDL was dialyzed against PBS, EDTA-

**Lipoproteins**

Plasma LDL was derived from fasted normalized volunteers. LDL was prepared by discontinuous density gradient ultracentrifugation as described previously (30). The lipoprotein was washed at d = 1.063 g/ml and dialyzed against 150 mM NaCl, 1 mM EDTA (pH 7.4) at 4 °C. LDL was then sterilized by filtration (0.45 μm), kept at 3–6 mg of protein/ml under nitrogen in the dark at 4 °C and used within 2 weeks. Prior to the oxidation studies, LDL was dialyzed against PBS, EDTA-

**LDL Oxidation by Macrophages**

Cells (1 x 10⁶/35-mm dish) were incubated with LDL (100 μg of protein/ml) in Ham's F-10 medium in the presence of 2 μM CuSO₄. LDL was also incubated under similar conditions in the absence of cells. The extent of LDL oxidation was measured directly in the medium (after centrifugation at 1000 x g for 10 min), by the thiobarbituric acid-reactive substances assay, using malondialdehyde for the standard curve preparation (40). Macrophage-mediated oxidation of LDL was calculated by subtraction of the oxidation rate in the absence of cells from that obtained in the presence of the macrophages.

**Glabridin and 2′, 4′-Dimethylglabridin:**

**Preparation and Cellular Content**

**Preparation—Glabridin was isolated from the acetone extract of the roots of Glycyrrhiza glabra (the licorice plant) and purified on silica gel column chromatography (14). Glabridin (100 mg; 0.31 mmol) was dissolved in acetone (4 ml) in a round bottom flask, and methyl iodide (240 μl, 2.64 mmol) and K₂CO₃ (384 mg, 2.76 mmol) were added. The reaction mixture was heated to 50 °C with stirring and, after 8 h, was filtered, and the solvent was evaporated. The residue was chromatographed on a silica gel column, using CH₃Cl/hexane (4.1, v/v) and then CH₃Cl alone as eluents, to afford 2′, 4′-dimethylglabridin (70.6 mg, 65% yield), which was identified as described before (16). No attempt was made to optimize the yield. The purity of the isolated and synthesized compounds used in this study was above 98%, as determined by high performance liquid chromatography (HPLC) using RP-18 column (Merek, Darmstadt, Germany; 25-cm length, 0.4-cm diameter, 5-μm particle size). The analysis was performed by detecting the absorbance of the compounds at 230 nm, using acetonitrile/water as eluents (16).

**Superoxide Generation by Macrophages**

The production of the superoxide anion (O₂⁻) by mouse peritoneal macrophages was measured as the superoxide dismutase-inhibitable reduction of acetyl ferriicytochrome C by the microtiter plate technique as described previously (42). Cells (2 x 10⁶/well) were suspended in 100 μl of Hank's balanced salts solution containing acetyl ferriocytochrome C (150 μM). Superoxide production by the cells was stimulated by the addition of PMA (50 ng/ml) or LDL (100 μg of protein/ml) in the presence of 2 μM CuSO₄. The reduction of acetyl ferriocytochrome C was followed by the change in absorbance at 550 nm between 2- and 5-min intervals on a Thermomax Microplate Reader (Molecular Devices, Menlo Park, CA). The maximal rates of superoxide generation were determined and expressed as μM of superoxide produced per 10⁶ cells/10 min using the extinction coefficient of E₅₅₀ = 21 mmole/liter⁻¹ cm⁻¹.

**Cell Free Superoxide Generation Assay—Superoxide generation was measured as described previously (43). Membranes and cytosol were prepared from control MPM or from MPM that were incubated for 2 h with 20 μg of glabridin. Membranes were solubilized in a buffer containing sodium deoxycholate (1.16%), glycerol (50%), NaCl (1 mM), CaCl₂ (1.2 mM), and sodium dodecyl sulfate (20 mM), pH 7.0. The final concentration of solubilized membranes was adjusted to 4 x 10⁶ cell equivalents/ml. The reaction solution contained the following: acetyl ferriocytochrome C (150 μM), MgCl₂ (4 mM), FAD (10 μM), EGTA (1 mM), NADPH (200 μM), and...
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SOD (100 μM) in 75 mM potassium phosphate, pH 7.0. Control wells also contained superoxide dismutase (2.5 mg). The reaction was monitored at 22 °C. Light absorbance at 550 nm was determined at 2-min intervals.

**Translocation of the NADPH Oxidase P-47 Cytosolic Component to the Plasma Membrane**

Immunoblot detection of cytosolic NADPH oxidase components was performed as described (42). Samples were solubilized in 2× sample buffer (12% SDS, 8 M urea, 250 mM Tris base, 8 mM EDTA, 0.2 mM leupeptin, 2 mM phenylmethylsulfonyl fluoride, pH 6.9). The amount of protein in each sample was quantified by the Pierce BCA protein assay with bovine serum albumin as a standard. Cytosols or membranes were analyzed by polyacrylamide gel electrophoresis. The resolved proteins were electrophoretically transferred to nitrocellulose, which was stained with Fast Green to detect protein banding, and then blocked with 5% (w/v) nonfat dry milk in Tris-buffered saline (pH 7.4). Immunoblots were incubated with 1 μg/ml peroxidase-conjugated rabbit anti-goat serum (Sigma) and developed by the ECL method.

**Protein Kinase C and Protein Kinase A Activities**

The activities of protein kinase C (PKC) and protein kinase A were measured in the cytosolic or membrane fractions obtained from control and from glabridin-enriched macrophages using the MESACUP protein kinase assay kit, (PanVera Corp.).

**Mouse Studies**

Apolipoprotein E-deficient mice were generously provided to us by Dr. Jan Breslow (Rockefeller University, New York). Gene targeting in mouse embryonic stem cells was used to create these mice that lack apolipoprotein E (44). The mice (15 mice in each group) were fed with 9,4-dimethylglabridin in their drinking water (20 μg/g/day/mouse). At the end of glabridin feeding, MPM were harvested from the peritoneal fluid (after thioglycolate injection) for analyses of cellular glabridin content and MPM-mediated oxidation of LDL. Then the mice were sacrificed, and their hearts and entire aortas were dissected and fixed in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, with 0.01% calcium chloride. Following overnight fixation, the aortic arch was dissected free from the surrounding fatty tissue under a binocular stereomicroscope, and the first 4 mm of the ascending aorta (beginning with the aortic valves) was removed and cut transversely with razor blades into four blocks of approximately 1 mm each. The samples were then rinsed and stored in 0.1 M sodium cacodylate buffer containing 7.5% (w/v) sucrose prior to treatment with an unbuffered 1% aqueous solution of osmium tetroxide for 2 h. This was followed by a cacodylate rinse and dehydration in ascending ethanolos, prior to propylene oxide and embedding in epoxy resin (Eponate 12; Pelco International, Redding, CA). The blocks were orientated so that transverse sections of the aorta could be cut. After heat polymerization (18 h) at 60 °C, the blocks were trimmed, and 1-μm transverse sections cut with

**TABLE I**

The effect of macrophage enrichment with glabridin on cell-mediated oxidation of LDL: time study of LDL incubation with macrophages

| Cell-mediated oxidation of LDL | J-774A.1 | MPM |
|-------------------------------|----------|-----|
| 5 h                           | 20 h     | 20 h |
| Control cells                 | 6.0 ± 0.1| 24.5 ± 2.3| 5.80 ± 0.10| 28.5 ± 0.5 |
| With glabridin                | 1.1 ± 0.1| 12.8 ± 2.3| 0.58 ± 0.01| 9.8 ± 0.4 |

* p < 0.01 (versus control cells).

![Fig. 1](image-url) The effect of macrophage incubation with glabridin on its cellular accumulation and on cell-mediated oxidation of LDL: time and glabridin concentration studies. J-774 A.1 macrophages or MPMs were incubated with 20 μg of glabridin for 10, 30, or 120 min (A and B) or with increasing concentrations of glabridin (0–20 μM) for 2 h at 37 °C (C and D). A and C, at the end of the incubation period, the cells were washed, and cellular glabridin content was analyzed by HPLC, as described under “Experimental Procedures.” B and D, the cells were washed and further incubated with LDL (100 μg of protein/ml) in the presence of 2 μM CuSO4 for 5 h at 37 °C. The extent of LDL oxidation was measured in the incubation medium by the thiobarbituric acid-reactive substances assay. Results represent mean ± S.D. (n = 3).
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Glabridin inhibits macrophage-mediated oxidation of LDL by mouse peritoneal macrophages. MPMs were incubated with 0.2% ethanol (Control), with 20 μM of glabridin, or with 20 μM of 2',4'-O-dimethylglabridin for 2 h at 37 °C. A, molecular structures of glabridin and 2',4'-O-dimethylglabridin. B, at the end of the incubation period, the cells were washed, and cellular content of glabridin and of 2',4'-O-dimethylglabridin were determined by HPLC. C, the cells were washed and further incubated with LDL (100 μg of protein/ml) in the presence of 2 μM CuSO₄ for 5 h at 37 °C. Cell-mediated oxidation of LDL was determined by the thiobarbituric acid-reactive substances assay. Results represent mean ± S.D. (n = 5). * p < 0.01 (versus control).

RESULTS

We have recently shown that the licorice-derived isoflavon glabridin binds to LDL and substantially inhibits the oxidation of the lipoprotein, secondary to its ability to scavenge free radicals (12, 14–16). Consumption of flavonoids can affect LDL oxidation not only by a direct interaction of the flavonoids with the lipoprotein, but also secondary to their accumulation in cells such as arterial wall macrophages. In the present study, we analyzed the macrophage capability to take up glabridin and studied the mechanisms by which cellular accumulation of glabridin can affect macrophage-mediated oxidation of LDL.

Macrophage Enrichment with Glabridin and Cell-mediated Oxidation of LDL—Upon incubation of macrophages (J-774 A.1 cell line or MPMs) with glabridin (20 μM) for 20 h at 37 °C, cellular glabridin content increased from 0 to 1.8 ± 0.2 μg of glabridin/mg of cell protein (n = 3).

Incubation of MPMs or J-774 A.1 macrophages with LDL in the presence of 2 μM CuSO₄ for 5 h at 37 °C resulted in a 90% or 82% inhibition of LDL oxidation, respectively, by the glabridin-enriched macrophages in comparison with the control cells (Table I). Extension of the oxidation period up to 20 h resulted in only 65% and 48% inhibition of LDL oxidation by glabridin-enriched MPMs and J-774 A.1 cells, respectively, in comparison with the control cells (Table I).

In all of the following experiments, we have used 5 h for the studies of LDL oxidation by macrophages. To find out the time required for cell incubation with glabridin in order to reach a substantial cell enrichment with this isoflavon (which can result in a significant inhibition of macrophage-mediated oxidation of LDL), incubation time and glabridin concentration studies were carried out. Fig. 1 demonstrates a time-dependent increment in cellular accumulation of glabridin by both MPMs and J-774 A.1 macrophages with a major effect obtained already after 30 min of incubation (Fig. 1A). In parallel, cell-mediated oxidation of LDL was substantially inhibited by ~80% at this time period (Fig. 1B). After 2 h of incubation, there was about 90% inhibition in macrophage-mediated oxidation of LDL in both cell types (Fig. 1B). Glabridin was not cytotoxic to the cells, since no significant effect on cell count or on the release of lactic dehydrogenase could be shown (25 ± 1 unit/liter in control cells and 27 ± 2 units/liter in glabridin-treated cells). Cell fractionation revealed that about 60% of the glabridin was localized in the macrophage membrane, and the rest was in the cytosol (data not shown). Next, we analyzed the effect of glabridin concentrations (0–20 μM) on macrophage-mediated oxidation of LDL after 2 h of cell incubation with glabridin at 37 °C, followed by 5 h of cell incubation with LDL in the presence of copper ions. A glabridin dose-dependent stimulatory effect on cellular glabridin content was found, demonstrating a linearity between 5 and 20 μM of glabridin (Fig. 1C). In parallel, macrophage-mediated oxidation of LDL was reduced up to 90%, in comparison with control cells (Fig. 1D), and this effect was dependent on cellular glabridin content.

To find out the structural requirements for glabridin to act as a potent inhibitor of cell-mediated oxidation of LDL, we compared the effect of native glabridin to that of 2',4'-O-dimethylglabridin where the glabridin ring B hydroxyl residues were...
blocked by methyl groups (Fig. 2A). Recently, we have shown that unlike the potent inhibitory effect of glabridin on LDL oxidation, the above modified glabridin completely lost its antioxidant activity (16). Upon incubation of MPM with 20 μM of native glabridin or with 2′,4′-O-dimethylglabridin for 2 h at 37 °C, the macrophages accumulated similar amounts of these compounds (Fig. 2B). Analysis of macrophage-mediated oxidation of LDL, after 5 h of incubation, revealed that in comparison with a 90% inhibition of LDL oxidation by the glabridin-enriched cells, no inhibition of cell-mediated oxidation of LDL was obtained by the modified glabridin-enriched cells (Fig. 2C).

We have previously shown that glabridin binds to the LDL particle and, hence, inhibits its oxidation (12, 15). Thus, we next examined the possibility that glabridin can be released from the cells to the incubation medium and directly inhibit LDL oxidation by its binding to the LDL particle. For this purpose, MPMs were incubated with 20 μM of glabridin for 2 h at 37 °C. The cells were washed and further incubated in a fresh medium for 2 h at 37 °C. Medium samples were taken every 30 min for HPLC analysis of glabridin content. After 2 h of incubation, the amount of glabridin in the cells was 0.72 ± 0.10 μg/mg of cell protein, and the medium glabridin content (obtained from 1 mg of cell protein) was as high as 0.52 ± 0.05 μg (n = 3). When this medium was then incubated with LDL and copper ions in a cell-free system, only 10% inhibition in LDL oxidation was obtained (data not shown). Thus, the substantial (~90%) inhibition of LDL oxidation by glabridin-enriched cells, in comparison with control cells, is mainly due to the effect of glabridin on the cellular oxidative state rather than a direct effect of glabridin on the lipoprotein.

Mechanisms for the Inhibition of LDL Oxidation by Glabridin-enriched Macrophages—NADPH oxidase, which is found in arterial wall cells (including macrophages), can convert native LDL into oxidized LDL (19, 22). We have previously demonstrated that under oxidative stress, induced by PMA or by the addition to the cells of LDL in the presence of copper ions, the macrophage NADPH oxidase was activated (19). The activated NADPH oxidase complex in the plasma membrane is responsible for the production and release of superoxide anions, which can then lead to cell-mediated oxidation of LDL. Since some flavonoids were found to inhibit NADPH oxidase-induced superoxide release (33), we next analyzed the effect of cellular accumulation of glabridin on superoxide production and release. Upon incubation of MPM with increasing glabridin concentrations (0–20 μM) for 2 h at 37 °C, the release of superoxide...
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Fig. 5. The effect of macrophage enrichment with glabridin or with 2',4'-O-dimethylglabridin on the translocation of the NADPH oxidase cytosolic component P-47 to the plasma membrane. Results represent SDS-polyacrylamide gel electrophoresis immunoblot analysis of P-47 in MPM cytosol and membrane fractions. Results are shown for resting cells, control cells, glabridin (20 μM)-treated cells, and 2',4'-O-dimethylglabridin (20 μM)-treated cells, following cell stimulation with 50 ng/ml PMA or with LDL (100 μg of protein/ml) in the presence of 2 μM CuSO4. A, resting cells; B, control cells plus LDL plus Cu2+; C, control cells plus PMA; D, 2',4'-O-dimethylglabridin-treated cells plus LDL plus Cu2+; E, 2',4'-O-dimethylglabridin-treated cells plus PMA; F, glabridin-treated cells plus LDL plus copper; G, glabridin-treated cells plus PMA.

Table II
The effect of macrophage enrichment with glabridin or with 2',4'-O-dimethylglabridin on the translocation of the NADPH oxidase cytosolic component P-47 to the plasma membrane: densitometric analysis of membrane immunoblots

The experiment is described in the legend to Fig. 5. The determination by densitometry of the relative amounts of P-47 in the macrophage membrane is given as mean ± S.D. from three different experiments. Arbitrary units of density are presented, with the higher numbers representing darker bands on the immunoblot.

| Cells                        | P-47     |
|------------------------------|----------|
| Untreated cells              | 5 ± 1    |
| Control cells                |          |
| With LDL + Cu2+              | 57 ± 5   |
| With PMA                     | 64 ± 6   |
| 2',4'-O-Dimethylglabridin-treated cells |           |
| With LDL + Cu2+              | 56 ± 5   |
| With PMA                     | 59 ± 6   |
| Glabridin-treated cells      |          |
| With LDL + Cu2+              | 9 ± 1    |
| With PMA                     | 25 ± 3   |

* p < 0.01, glabridin-treated cells stimulated with LDL plus copper ions versus control cells stimulated with LDL plus copper ions.

** p < 0.01, glabridin-treated cells stimulated with PMA versus control cells stimulated with PMA.

Anions from the cells to the medium was determined following 30 min of cell stimulation with either LDL (100 μg of protein/ml) and 2 μM CuSO4 or with the NADPH oxidase activator PMA (50 ng/ml). A glabridin dose-dependent inhibitory effect on the release of superoxides was found, with up to 61% or 56% inhibition in response to PMA or to LDL plus CuSO4, respectively (Fig. 3, A and B). The modified form of glabridin (2',4',D-dimethylglabridin) had no significant effect on macrophage release of superoxides (Fig. 3, A and B). Kinetic analysis also demonstrated that glabridin accumulation in macrophages reduced superoxides release (Fig. 3, C and D). In both systems (PMA and LDL plus CuSO4), glabridin-enriched macrophages showed a time-dependent reduction in the release of superoxides in comparison with control cells, with up to 75% and 80% inhibition observed in the PMA and in the LDL plus CuSO4-treated cells, respectively (Fig. 3, C and D).

Superoxide production by cellular NADPH oxidase involves translocation of the enzyme cytosolic components (P-47, P-67, and Ras-related GTP-binding protein) to the plasma membrane, where they interact with cytochrome b559 to form an active complex that can convert oxygen into superoxides (46, 47). To find out whether glabridin directly inhibits the active NADPH oxidase complex or the translocation of the cytosolic components to the plasma membrane, we assayed the production of superoxide in a cell-free system by reconstitution of macrophage membrane and cytosol fractions. MPMs (105 cells) were incubated with 20 μg glabridin for 2 h at 37 °C. Control cells were incubated with 0.2% ethanol. PKC and protein kinase A (PKA) activities were then measured in the cytosol. Results represent mean ± S.D. (n = 3).

Fig. 6. Protein kinase C and A activities in control MPMs, glabridin-enriched MPMs, and 2',4'-O-dimethylglabridin-enriched MPMs. MPMs (2 × 105 cells) were treated with 0.2% ethanol (Control), with 20 μg glabridin, or with 20 μg 2',4',D-dimethylglabridin for 2 h at 37 °C. The cells were then washed and stimulated with 50 ng/ml PMA or with LDL (100 μg of protein/ml) in the presence of 2 μg CuSO4 for 2 min at 37 °C. The cytosolic fractions were obtained as described under "Experimental Procedures." PKC and protein kinase A (PKA) activities were then measured in the cytosol. Results represent mean ± S.D. (n = 3).

These results suggest that glabridin has no direct inhibitory effect on the active NADPH oxidase complex, but rather an inhibitory effect on cellular processes that lead to NADPH oxidase activation. Thus, we next examined the effect of cellular glabridin on the translocation of P-47 from the cytosol to the plasma membrane, by Western blot analysis, using a specific antibody against P-47. Translocation of P-47 from the cytosol to the plasma membrane occurs upon cell incubation with 50 ng/ml of PMA or with LDL (100 μg of protein/ml) plus 2 μg CuSO4 (Fig. 5). P-47 translocation in response to LDL plus...
**Table III**

PKC activity in cytosolic and membrane fractions obtained from control MPMs or from glabridin-treated MPMs

| PKC activity | Cytosol | Membrane | Cytosol | Membrane |
|--------------|---------|----------|---------|----------|
| Glabridin-treated cells | 0.87 ± 0.07<sup>a</sup> | 0.05 ± 0.01<sup>a</sup> | 0.33 ± 0.06<sup>a</sup> | 0.18 ± 0.02<sup>a</sup> |
| Control cells | 1.80 ± 0.08 | 0.08 ± 0.01 | 1.10 ± 0.05 | 0.90 ± 0.03 |

<sup>a</sup> p < 0.01 versus control cells.

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PKC activity was measured in unstimulated cells and in cells stimulated with 50 ng/ml PMA. PKC activity was measured in the cytosolic and membrane fractions that were obtained from 2 × 10³ MPMs following their incubation with 0.2% ethanol (control cells), or with 20 µM glabridin for 2 h at 37°C. Results are given as the mean ± S.D. of three different experiments.

The results so far suggest that macrophage accumulation of glabridin caused inhibition of macrophage-mediated oxidation of LDL via an inhibitory effect of glabridin on the translocation of the NADPH oxidase P-47 cytosolic protein to the plasma membrane and, hence, on macrophage superoxide production.

Since PKC was shown to be required for LDL oxidation by activated human monocytes (48) and since phosphorylation of the NADPH oxidase cytosolic component is involved in the enzyme activation (47, 49), we next questioned whether cellular accumulated glabridin could affect PKC activity. For this purpose, we used the PKC inhibitors calphostin C, staurosporine, and H-7. Calphostin C has been reported to compete with phorbol ester for binding to the regulatory domain of PKC, and it has been shown to be more potent in inhibiting PKC than any other protein kinase, by at least 3 orders of magnitude (50). It has been shown to be more potent in inhibiting PKC than any other protein kinase, by at least 3 orders of magnitude (50).

H-7 is thought to act competitively at the ATP-binding site (51), whereas staurosporine has been reported to have both competitive and noncompetitive effects with respect to ATP (52). We have previously demonstrated that PKC inhibitors inhibited PMA-stimulated NADPH oxidase activity, as well as phosphorylation and translocation of P-47, in a dose-dependent manner (49), and these results are in accordance with other studies (53, 54).

The addition of J-774 A.1 macrophages of PKC inhibitors (such as 1 µM calphostin C, 40 ng/ml staurosporine, or 100 µM H-7) inhibited cell-mediated oxidation of LDL in comparison with its oxidation by macrophages in the absence of the inhibitors by 65%, 90%, and 60% (from 5.8 ± 0.2 to 2.0 ± 0.1, 0.6 ± 0.1, and 1.7 ± 0.2 mmol of malondialdehyde/mg of LDL protein, respectively). The addition of 50–100 µM HA1004 (protein kinase A inhibitor) to J-774 A.1 macrophages in the presence of LDL plus copper ions had no effect on cell-mediated oxidation of LDL (data not shown), suggesting the involvement of PKC, but not of protein kinase A, in LDL oxidation by macrophages. The inhibitors used had no effect on cell viability as analyzed by trypan blue assay (data not shown). The activities of protein kinases were next directly measured in the cytosolic fractions obtained from glabridin-enriched MPMs or from 2',4'-O-dimethylglabridin-enriched MPMs in comparison with control cells, following their activation with either PMA (50 ng/ml) or LDL (100 µg of protein/ml) in the presence of copper ions. Macrophase PKC activity was reduced by ~70% (Fig. 6A), whereas protein kinase A activity was not significantly affected (Fig. 6B) in the glabridin-enriched MPMs, in comparison with control cells (Fig. 6). In 2',4'-O-dimethylglabridin-enriched MPMs, however, both PKC and protein kinase A activities were not affected (Fig. 6). These results further indicate the importance of the glabridin hydroxyl groups on its flavonoid B ring for the inhibition of PKC and NADPH oxidase activation and, hence, for the attenuation of macrophage-mediated oxidation of LDL.

In order to examine the possibility that the reduced PKC activity in the cytosol obtained from glabridin-enriched MPMs is associated with its increased translocation to the plasma membrane, we measured PKC activity in membrane fractions isolated from control cells or from glabridin-treated cells, before or after their stimulation with 50 ng/ml PMA (Table III). Total PKC activity (cytosol plus membrane) was reduced by 50% in glabridin-treated unstimulated cells and by 70% in glabridin-treated cells that were prestimulated with 50 ng/ml PMA (Table III). Finally, we questioned whether glabridin has a direct inhibitory effect on macrophage PKC activity. For this purpose, we measured PKC activity in unstimulated MPMs in the absence or presence of increasing concentrations of glabridin (0–20 µM) during an in vitro PKC assay (Table IV). Under these conditions, glabridin directly inhibited PKC activity in a dose-dependent fashion by up to 80% at a glabridin concentration of 20 µM (Table IV).

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**Table IV**

The effect of glabridin on PKC activity in unstimulated MPMs: glabridin concentration study

| Glabridin concentration (µM) | PKC activity (A<sub>492</sub>)<sup>a</sup> |
|----------------------------|----------------------------------------|
| 0                          | 1.75 ± 0.05                            |
| 2.5                        | 1.23 ± 0.04*                           |
| 5                          | 0.82 ± 0.03*                           |
| 10                         | 0.61 ± 0.02*                           |
| 20                         | 0.36 ± 0.02*                           |

<sup>a</sup> p < 0.01 (versus 0 concentration).
Glabridin had little effect on lesion content in lesions from the control mice (Fig. 7). However, 56%, respectively, was noted in comparison with the oxysterol oxycholesterol, and 7-ketocholesterol by 54%, 58%, 51%, and (1011)

In lesions from the glabridin-treated mice, reduced content of 7a-hydroxycholesterol, 7b-hydroxycholesterol, a-epoxycholesterol, and 7-ketocholesterol at 54%, 58%, 51%, and 56%, respectively, was noted in comparison with the oxysterol content and for lesion size analysis.

Fig. 7 demonstrates detailed analysis of the lesion oxysterol derivatives. In lesions from the glabridin-treated mice, reduced content of 7a-hydroxycholesterol, 7b-hydroxycholesterol, a-epoxycholesterol, and 7-ketocholesterol by 54%, 58%, 51%, and 56%, respectively, was noted in comparison with the oxysterol content in lesions from the control mice (Fig. 7). However, glabridin had little effect on lesion b-epoxycholesterol content (Fig. 7).

Glabridin supplementation to the atherosclerotic mice resulted in about 50% reduction in the lesion area compared with the lesion areas in aortas from placebo-treated mice (52 ± 8 versus 106 ± 11 μm² × 10⁴, respectively; *, p < 0.01, n = 14). The photomicrographs of the lesions (Fig. 8) demonstrate only few foam cells in the glabridin-treated mice compared with the placebo-treated mice (Fig. 8).

These results demonstrated that in vivo, glabridin accumulates in the mice peritoneal macrophages, and these glabridin-enriched macrophages possess reduced capability to oxidize LDL. The above characteristics were associated with a remarkable attenuation in macrophage foam cells and in the development of the atherosclerotic lesions in the apolipoprotein E-deficient mice.

**DISCUSSION**

In the present study, we have demonstrated for the first time that glabridin, a lipophilic isoflavon isolated from licorice root, can be taken up by and accumulated in macrophages. Glabridin cellular accumulation substantially inhibited macrophage-mediated oxidation of LDL, secondary to the inhibition of NADPH oxidase.

We have previously demonstrated the potent inhibitory effect of glabridin on LDL oxidation (12, 14–16). When consumed, glabridin is absorbed and binds to plasma lipoproteins (12, 15). Absorbed glabridin, however, can also be taken up by cells, including arterial wall macrophages and, hence, can affect the cellular oxidative state. Indeed, the present study demonstrated that glabridin was taken up by macrophages, accumulated in the cells’ plasma membrane, and exerted a potent inhibitory effect on macrophage-mediated oxidation of LDL. This effect was time- and dose-dependent and required similar structural elements (the hydroxyl groups on the isoflavon B ring), as previously shown for the antioxidative effect of glabridin against LDL oxidation (16). This last similar requirement suggests that the inhibitory effect of glabridin on the macrophage machinery, which is required for LDL oxidation, may be related to the antioxidative properties of this isoflavon. We have demonstrated, however, that the inhibitory effect of glabridin, which accumulated in the cells, on LDL oxidation was mainly related to its effects on the cells rather than a phenomenon that could be related to the release of some glabridin from the cells, followed by its interaction with the LDL.
particle and scavenging of reactive oxygen species. In addition, the inhibitory effect of glabridin on cell-mediated oxidation of LDL was still a substantial one, even after 20 h of LDL oxidation, suggesting that the glabridin in the cells was stable and preserved its activity along this relatively long time of incubation.

Cellular release of superoxide anions by arterial wall cells was suggested as a possible mechanism for LDL oxidation (19, 20, 55–58). Several flavonoids were shown to inhibit the release of reactive oxygen species by stimulated human leucocytes or neutrophils (33, 59, 60). This inhibitory effect was attributed to the structure of the flavonoid, to its hydrophobicity, and to the number and/or position of the hydroxyl groups on the flavonoid B ring (59). The flavonoid’s free radical scavenging capability (61, 62) may have a role in the prevention of cellular production of free radicals and, thus, in the inhibition of the formation of oxidized LDL, but glabridin enrichment of macrophages reduced superoxide release from these cells and inhibited macrophage-mediated oxidation of LDL, also via a direct effect on NADPH oxidase activation. We have previously shown that activation of the macrophage NADPH oxidase can lead to a substantial LDL oxidation (19). The inhibitory effect of glabridin on superoxide release was detected only when it was added to intact cells and not when present in a cell-free system, indicating that this agent does not directly affect the NADPH oxidase complex, but rather it affects the signaling that leads to NADPH oxidase activation. An important role for PKC was shown in the phosphorylation and translocation of P-47 from the cytosol to the plasma membrane to form an active NADPH oxidase complex (49, 63–66). Continuous phosphorylation by PKC and translocation of P-47 is necessary in order to maintain NADPH oxidase in an activated state (53, 66). The results of the present study demonstrated that PKC activity induced by either PMA or by LDL plus copper ions was inhibited by cellular glabridin. Thus, inhibition of the macrophage PKC activity inhibited the translocation of cytosolic P-47 to the plasma membrane to form the assembled active NADPH oxidase.

These effects of glabridin were found to be specific to the glabridin hydroxyl groups on the isoflavan B ring, since 2’,4’-O-dimethylglabridin was not active anymore.

It was previously shown that methylation of the hydroxyl group at the 4’-position of the phenyl ring of hesperetin also contributes to the loss of its PKC-inhibitory activity (34). We have demonstrated direct inhibitory effect of glabridin on PKC activity in unstimulated cells. This inhibitory effect of glabridin occurred only when glabridin was added prior to the addition of ATP, suggesting that glabridin is a competitive inhibitor with respect to ATP binding. Flavonoids that inhibited PKC activity were indeed found to be competitive inhibitors with respect to ATP binding (34).

Dimethylglabridin, in which the hydroxyl groups on the isoflavan B ring were protected, had no inhibitory effect on PKC activity, on P-47 translocation, on cellular superoxide release, and on macrophage-mediated oxidation of LDL. These results point to the importance of the glabridin unique structure in the inhibition of the sequence of events that leads to cell-mediated oxidation of LDL. In order to assess the physiological significance of our in vitro results, we questioned the relationship between macrophage glabridin content, cell-mediated oxidation of LDL, and the extent of atherosclerosis by using the atherosclerotic apolipoprotein E-deficient mouse model. These mice demonstrate increased LDL oxidation and develop extensive atherosclerosis within a few months of age. The present study clearly demonstrated a significant reduction in MPM-mediated oxidation of LDL, in lesion oxysterol content, and in the atherosclerotic lesion area in the glabridin-treated mice, in comparison with placebo-treated mice. These results indicate that inhibition of atherosclerosis in these mice by glabridin consumption is related to reduced macrophage-mediated oxidation of LDL. Glabridin may possess this property not only because of its binding to LDL (12) but also by its accumulation in cells such as macrophages, where it reduces cellular oxidative stress by inhibiting protein kinase C and, hence, reducing NADPH oxidase activation, an important inducer of macrophage-mediated oxidation of LDL (19).

Flavonoids, as potent antioxidants against LDL oxidation, as well as inhibitors of macrophage oxygenases, may be used as important intervention means to inhibit cell-mediated oxidation and to attenuate atherosclerosis (67–69).

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