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Gentisic acid, an aspirin metabolite, inhibits oxidation of low-density lipoprotein and the formation of cholesterol ester hydroperoxides in human plasma

Keiko Ashidate[a,b], Mitsunobu Kawamura[b,*], Daigo Mimura[c], Hisako Tohda[b], Shigeru Miyazaki[b], Tamio Teramoto[d], Yorihiro Yamamoto[e], Yukio Hirata[f]

[a]*Department of Internal Medicine, Kudanzaka Hospital, Tokyo, Japan*
[b]*Department of Internal Medicine, Tokyo Teishin Hospital, 2-14-23 Fujimi, Chiyoda-ku, Tokyo, 102-8798, Japan*
[c]*Department of Chemistry and Biotechnology Graduate School of Engineering, The University of Tokyo, Tokyo, Japan*
[d]*Department of Internal Medicine, Tokyo University School of Medicine, Tokyo, Japan*
[e]*School of Bionics, Tokyo University of Technology, Tokyo, Japan*
[f]*Department of Clinical and Molecular Endocrinology, Tokyo Medical and Dental University Graduate School, Tokyo, Japan*

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Abstract

Gentisic acid, an aspirin metabolite, has an antioxidant effect, although its detailed mechanism remains elusive. The present study was designed to determine whether it inhibits low-density lipoprotein (LDL) oxidation and the formation of lipid hydroperoxides in human plasma.

The susceptibility of LDL oxidative modification was investigated by a method using 2,2′-azobis or Cu^{2+}. To study the effect of gentisic acid on free radical-induced damage to plasma lipids, cholesterol ester hydroperoxides generated by incubating human fresh plasma with Cu^{2+} and gentisic acid was analyzed.

Gentisic acid inhibited LDL oxidation in a concentration-dependent manner. It significantly inhibited the formation of cholesterol ester hydroperoxides in plasma, and was consumed after the depletion of ascorbic acid and reduced form of coenzyme Q-10 ($CoQH_2$-10), whereas concentrations of other antioxidants remained unchanged. Gentisic acid had a potent free radical scavenging activity with a minimal chelating effect.

The potent antioxidant property of gentisic acid may partly account for the anti-atherogenic effects of aspirin.

Keywords: Gentisic acid; Aspirin; Antioxidant; LDL oxidation

1. Introduction

It is well known that oxidative modification of low-density lipoprotein (LDL) plays a pivotal role in the early stage of atherogenesis (Steinberg et al., 1989). Oxidized LDL has many potentially atherogenic effects, such as foam cell formation and cytotoxicity (Steinberg, 1997). Since the oxidation of LDL may facilitate the onset of atherosclerosis, the inhibition of this process with an antioxidant therapy is a promising strategy to prevent and/or retard the onset and progression of atherosclerosis (Heinecke, 1998).

Aspirin is widely used in the secondary prevention of thrombotic occlusive events, such as myocardial infarction and stroke (Awtry and Loscalzo, 2000). Aspirin acetylates cyclooxygenase in platelets, thereby irreversibly blocking the synthesis of thromboxane A_2 with platelet-aggregating and vasoconstricting properties. After oral administration, aspirin is rapidly converted to salicylic acid. Metabolism of salicylic acid occurs through glucuronide formation to produce salicyl acyl glucuronide and salicyl phenolic glucuronide, conjugation with glycine to produce salicyl

* Corresponding author. Tel.: +81 3 5214 7201; fax: +81 3 5214 7384.
E-mail address: mkawamura@tth-japanpost.jp (M. Kawamura).

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uric acid, and oxidation to gentisic acid (2,5-dihydroxybenzoic acid; Needs and Brooks, 1985).

Recently, gentisic acid is suggested to possess an antioxidant effect on LDL oxidation (Hermann et al., 1999; Exner et al., 2000) although its precise mechanism remains elusive. In human plasma, levels of 2 mM salicylic acid, which can be reached under aspirin therapy, lead to levels of about 20 μM gentisic acid (Forth et al., 1987). Therefore, the present study was designed to determine whether physiologically plausible concentration of gentisic acid, which can be reached under aspirin therapy, inhibits LDL oxidation in vitro and the formation of lipid hydroperoxides in human plasma.

2. Materials and methods

2.1. Materials

RPMI 1640 medium and 2,2′-azobis (4-methoxy-2,4-dimethylvaleronitrile [MeO-AMVN]) were obtained from GIBRO (Grand Island, NY) and Wako Pure Chemical Industries (Osaka, Japan), respectively. Gentisic acid (2,5-dihydroxybenzoic acid), α-tocopherol, isoluminol (6-amino-2,3-dihydro-1,4-phthalazinedione), and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were purchased from Sigma Chemical (St Louis, MO). Probucol was supplied by Daiichi Pharmaceutical (Tokyo, Japan).

2.2. Kinetics of azo compound (MeO-AMVN)- and Cu^{2+}-mediated LDL oxidation

LDL (d=1.019 to 1.063 g/ml) was separated from the pooled plasma drawn from normolipidemic, fasting subjects by ultracentrifugation using a vertical rotor (RP67-VF; Hitachi, Tokyo, Japan) according to the method of Chung et al. (1980). Centrifugation was performed at 65,000 × g for 90 min at 4 °C. The kinetics of MeO-AMVN- and Cu^{2+}-mediated LDL oxidation were measured by monitoring the changes of absorbance at 234 nm in a Shimazu UV-160A spectrophotometer (Kyoto, Japan) with 6 cuvette positions by modified method of Kondo et al. (1994) and Esterbauer

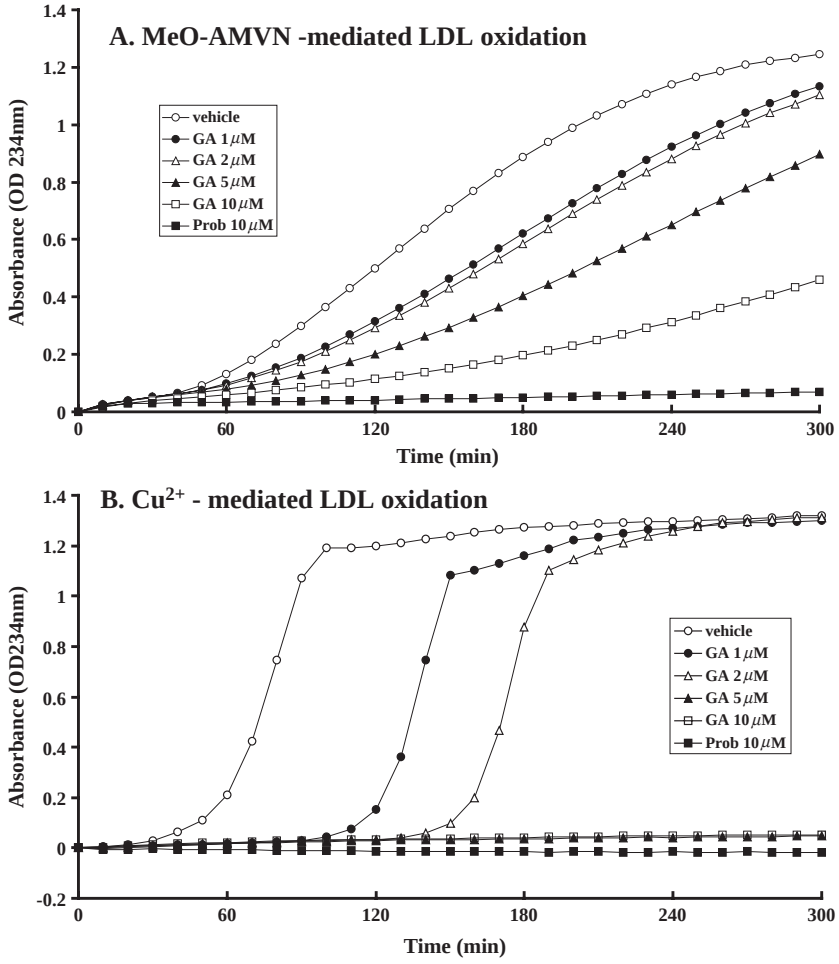

Fig. 1. Inhibition of azo compound- and Cu^{2+}-mediated LDL oxidation by gentisic acid. (A) LDL (70 μg protein/ml) and MeO-AMVN (400 μM), (B) LDL (100 μg protein/ml) and Cu^{2+} (5 μM) was incubated followed by the addition of gentisic acid (GA) in various concentrations (1, 2, 5, 10 μM) or probucol (Prob; 10 μM). Data are a representative of 3 experiments.

et al. (1989). After isolation, the oxidation of LDL (70 μg protein/ml) mediated by MeO-AMVN (final concentration, 400 μM) without dialysis against phosphate-buffered saline (PBS), pH 7.4, was immediately and continuously monitored at 37 °C for 5 h in the presence or absence of gentisic acid and probucol, because MeO-AMVN-mediated oxidation is not affected by EDTA (Kondo et al., 1994). Since MeO-AMVN, a lipid-soluble azo compound, is readily decomposed to produce peroxyl radicals at a constant rate within the lipid phase, it can induce the oxidation of LDL without interference with the absorbance for conjugated dienes at 234 nm (Kondo et al., 1994). In contrast, the LDL in Cu^{2+}-mediated oxidation was dialyzed for 20 h against PBS at 4 °C (Esterbauer et al., 1989). The oxidation of LDL (100 μg protein/ml) mediated by Cu^{2+} (final concentration, 5 μM) was monitored by the same method.

2.3. Cell culture

THP-1 cells were maintained essentially in RPMI-1640 containing HEPES (10 mM), supplemented with 10% FBS, L-glutamine (2 mM), penicillin (1×10^5 units/l), and streptomycin (100 mg/l) as previously described (Graham et al., 1994). Cells were seeded into 12-well plates(1×10^6 cells/ml) and transformed into macrophages by incubation with phorbol myristate acetate (PMA) for 24 h.

2.4. Cu^{2+}-mediated LDL oxidation in a cell-mediated system

LDL was preincubated for 30 min with various concentrations of gentisic acid at 37 °C, then aliquots of which (100 μg protein/ml) was incubated for another 24 h with the cells at 37 °C in the presence or absence of freshly prepared $CuSO_4$ (2.5 μM) in serum-free RPMI with different glucose concentrations (5, 15 mM). Probucol and α-tocopherol were used as positive controls. At the end of the incubation, Cu^{2+}-mediated oxidation was stopped by refrigeration (4 °C) and the addition of both diethylene triamine pentaacetic acid (final concentration, 50 μM) and butylated hydroxytoluene (final concentration, 25 μM; Kawamura et al., 1994; Gutteridge, 1994). The degree of LDL oxidation was measured by the thiobarbituric acid-reactive substances assay (Naito et al., 1993), and the results were expressed as malondialdehyde equivalents.

2.5. Analysis of lipid hydroperoxides and antioxidants

Pooled plasma prepared from fresh heparinized blood of healthy volunteers was incubated with gentisic acid (20 μM) and $CuSO_4$ (5 μM) at 37 °C in an incubator under air for 48 h; aliquots were extracted at 0, 1, 2, 5, 12, 24, 36, and 48 h. Plasma levels of ascorbic acid, uric acid, and unconjugated bilirubin were determined by high-pressure liquid chromatography (HPLC) equipped with an aminopropylsilyl column (Type Supelcosil LC-NH_2, 5 μm, 250 × 4.6 mm i.d., Supelco) and a UV detector (265 and 460 nm) as previously described (Yamamoto and Ames, 1987). The mobile phase consisted of methanol/40 mM sodium monobasic phosphate (= 9/1, v/v) delivered at a flow rate of 1.0 ml/min.

Plasma concentrations of reduced form of coenzyme Q-10 ($CoQH_2$-10), α-tocopherol, and total cholesterol were determined by a published procedure (Yamashita and Yamamoto, 1997) with modification. In brief, plasma treated with 2-propanol were analyzed with HPLC equipped with a guard column (Type Supelguard LC-ABZ, 5 μm, 33 × 4.6 mm i.d., Supelco), an analytical column (Type Supelcosil LC-8, 5 μm, 250 × 4.6 mm i.d., Supelco), a

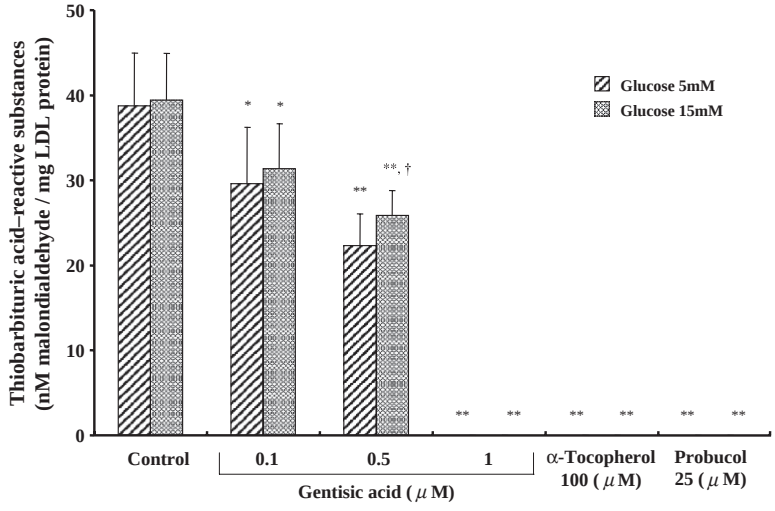

Fig. 2. Inhibition of Cu^{2+}-mediated LDL oxidation in a cell-mediated system by gentisic acid. LDL preincubated with various concentrations (0.1–1 μM) of gentisic acid, α-tocopherol (100 μM), or probucol (25 μM) were added to THP-1 cells and incubated for 24 h in media containing glucose concentrations of 5 mM (▨) and 15 mM (▩). After incubation, the medium was assayed for thiobarbituric acid-reactive substances. Each column expressed as nM malondialdehyde/mg LDL protein is the mean ± S.D. ($n = 6$) of three independent experiments. *$P < 0.01$, **$P < 0.001$ vs. control, $^{\dagger}P < 0.05$: glucose 5 mM vs. glucose 15 mM.

reduction column (Type RC-10-1, Irica, Kyoto), a UV detector (210 nm), and an amperometric electrochemical detector (ECD, Model Σ985, Irica). The ECD oxidation potential was +600 mV (vs. Ag/AgCl) on a glassy carbon electrode. The mobile phase consisted of 50 mM sodium perchlorate in methanol/*tert*-butyl alcohol (9/1, v/v) delivered at 0.8 ml/min.

Plasma was also extracted by shaking it vigorously with 4 volumes of methanol and 20 volumes of *n*-hexane, and the two phases were separated by centrifugation at 1500×*g* for 10 min. The hexane phase was evaporated under reduced pressure and the residue was redissolved in methanol/*tert*-butyl alcohol (1/1 by volume). A sample was injected into an octylsilyl column (5 μm, 250×4.6 mm, Supelco) to measure cholesterol ester hydroperoxides by chemiluminescence detection (Yamamoto and Niki, 1989). The eluent was methanol-*tert*-butyl alcohol (19/1 by volume). Methyl linoleate hydroperoxide was used as a standard for the measurement of lipid hydroperoxides.

2.6. Measurements of free radical scavenging and chelating activities

The free radical scavenging activity of gentisic acid was analyzed by 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay. Gentisic acid (20, 50 μM) was mixed with 1 ml of 0.1 mM DPPH (in ethanol). The time course of the change in the optimal density at 517 nm was then kinetically monitored (Blois, 1958). Alpha-tocopherol was used as a positive control.

The chelating copper ions activity of gentisic acid was measured by the changes in the ultraviolet absorption spectrum.

2.7. Statistical analysis

Results are expressed as the mean ± S.D. To compare thiobarbituric acid-reactive substances formation in the various groups in the cell-mediated system, Mann–Whitney's *U* test was performed. Comparisons of thiobarbituric acid-reactive substances between groups were made with analysis of variance (ANOVA) followed by Dunnett's post hoc test.

3. Results

3.1. Inhibition of azo compound- and Cu^{2+}-mediated LDL oxidation by gentisic acid

The effects of gentisic acid and probucol on the kinetic curves of conjugated dienes formation by the MeO-AMVN- and Cu^{2+}-mediated LDL oxidation are shown in Fig. 1. Gentisic acid inhibited the oxidation of LDL mediated by both the MeO-AMVN and Cu^{2+} in a concentration-dependent manner (1–10 μM), while probucol (10 μM) completely abolished the oxidative modification of LDL. Gentisic acid in the similar concentration almost completely inhibited Cu^{2+}-mediated LDL oxidation.

The experiments with aspirin and salicylic acid were performed under the same conditions. However, these two compounds did not have any antioxidant effects in the concentrations expected in vivo (data not shown).

3.2. Inhibition of Cu^{2+}-mediated LDL oxidation in a cell-mediated system by gentisic acid

Effect of gentisic acid on Cu^{2+}-mediated LDL oxidation incubated with THP-1 cells is shown in Fig. 2.

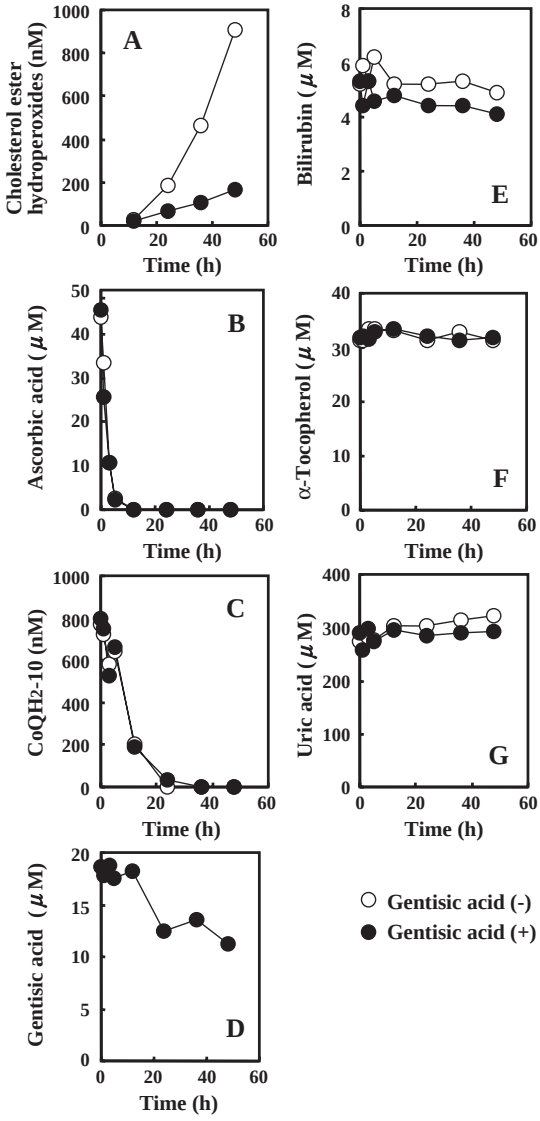

Fig. 3. Effects of gentisic acid on Cu^{2+}-mediated lipid peroxidation and various antioxidants in human plasma. Plasma was incubated with (●) or without (○) gentisic acid (20 μM) at 37 °C for 48 h in the presence of Cu^{2+} (5 μM). Changes in plasma concentrations of cholesterol ester hydroperoxides (A), ascorbic acid (B), $CoQH_2$-10 (C), gentisic acid (D), bilirubin (E), α-tocopherol (F), uric acid (G) are shown. Data are a representative of 5 experiments.

Gentisic acid dose-dependently (0.1–1 μM) and significantly inhibited LDL oxidation as measured by thiobarbituric acid-reactive substances. Thiobarbituric acid-reactive substances formation was completely inhibited by gentisic acid (1 μM) to the same extent by α-tocopherol (100 μM) and probucol (25 μM). The inhibitory effects by gentisic acid on thiobarbituric acid-reactive substances formation was observed in media containing high (15 mM) and normal (5 mM) glucose concentrations, although the inhibition by gentisic acid (0.5 μM) in high glucose was significantly less than that in normal glucose ($P<0.05$).

3.3. Inhibition of cholesterol ester hydroperoxides formation in human plasma by gentisic acid

To investigate the free radical-induced damage to plasma lipids and the kinetics of various plasma antioxidants, we measured cholesterol ester hydroperoxides and antioxidants with or without gentisic acid. As shown in Fig. 3A, gentisic acid (20 μM) significantly inhibited the formation of cholesterol ester hydroperoxides as a function of time. Plasma antioxidants were concomitantly decreased as a function of time in the order of ascorbic acid->$CoQH_2$-10>gentisic acid (Fig. 3B–D); gentisic acid decreased even after ascorbic acid and $CoQH_2$-10 had been depleted. In contrast, concentrations of bilirubin, α-tocopherol, and uric acid remained unchanged during 48 h of incubation (Fig. 3E-G).

3.4. Free radical scavenging and chelating activities by gentisic acid

To determine the underlying mechanism of antioxidant effect by gentisic acid, DPPH assay was performed. As shown in Fig. 4, gentisic acid showed radical scavenging activity in a dose-dependent manner (20–50 μM), although less potent than α-tocopherol.

To determine whether gentisic acid is capable of chelating copper ions, the changes of ultraviolet absorption spectrum of gentisic acid and copper ions were measured. Gentisic acid had only a minimal copper chelating activity (data not shown).

4. Discussion

The present study clearly demonstrates that physiologically plausible concentration of the aspirin metabolite, gentisic acid, effectively inhibits the oxidative modification of LDL and the formation of cholesterol ester hydroperoxides in human plasma. In this study, gentisic acid potently inhibits both the MeO-AMVN- and Cu^{2+}-mediated oxidation of LDL. Furthermore, the present study shows that gentisic acid has a potent radical scavenging activity with only a minimal copper chelating activity, suggesting that the antioxidant properties by gentisic acid are mainly due to its radical scavenging activity.

We have shown for the first time that gentisic acid inhibits the formation of cholesterol ester hydroperoxides, a primary product of lipid oxidation, in human plasma oxidized with Cu^{2+} ex vivo. Our results that cholesterol ester hydroperoxides appeared once ascorbic acid was completely consumed are consistent with those of Frei et al. (1988) which demonstrated that ascorbic acid is the first defense against lipid peroxidation in plasma. In our study, after the complete consumption of ascorbic acid, the formation of cholesterol ester hydroperoxides in the plasma was clearly decreased in the presence of gentisic acid. The consumption of gentisic acid appeared to start after the complete depletion of both ascorbic acid and $CoQH_2$-10, suggesting that these antioxidants initially prevented lipid

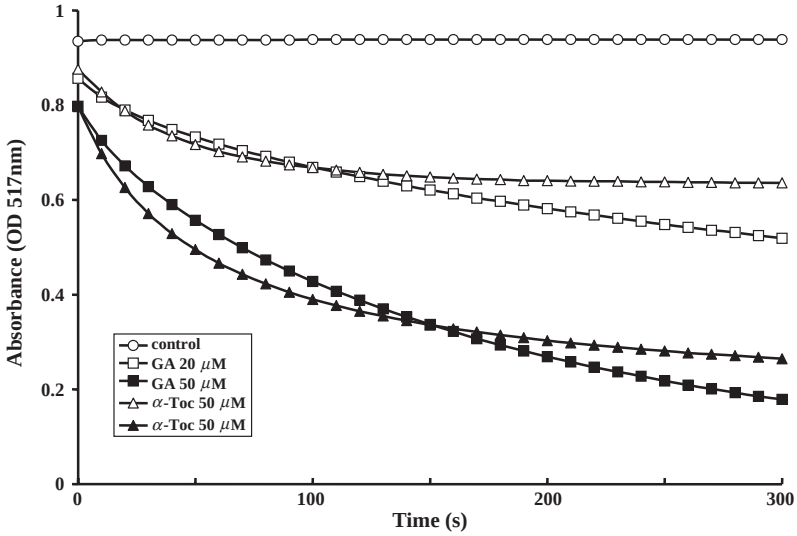

Fig. 4. Free radical scavenging activity of gentisic acid. Gentisic acid (GA) and α-tocopherol (α-Toc) at 20 and 50 μM was incubated with 0.1 mM DPPH and kinetic determination of the absorbance at 517 nm was performed. Data are a representative of 5 experiments.

oxidation. In contrast, other plasma antioxidants (bilirubin, α-tocopherol, uric acid) remained unchanged during 48 h of incubation. Thus, gentisic acid appears to be more effective than bilirubin, α-tocopherol, and uric acid in preventing lipid peroxidation in plasma.

It should be noted that the concentrations of gentisic acid used in our experiments were much lower than those used in previous studies (Hermann et al., 1999; Exner et al., 2000) showing its antioxidant effect on LDL oxidation. The present study showed that gentisic acid significantly inhibited thiobarbituric acid-reactive substances formation at concentration as low as 0.1 μM in the cell-mediated system. However, Hermann et al. (1999) have reported that gentisic acid (1 mM) inhibits LDL oxidation mediated by superoxide/nitric oxide radicals, and Exner et al. (2000) have reported that gentisic acid (20–50 μM) also inhibits glucose-autoxidation-mediated oxidation of LDL. The concentrations of gentisic acid used in the present in vitro study can be reached in vivo under aspirin therapy. In fact, it has been reported that concentrations of gentisic acid in human plasma was about 20 μM under aspirin therapy (Forth et al., 1987), and that plasma concentrations of gentisic acid reached to about 0.6 μM after oral administration of a single dose of 600 mg aspirin (Grootveld and Halliwell, 1986).

Gentisic acid produced in vivo by hydroxylation of salicylic acid accounts for less than 5% of the ingested salicylic acid (Shen et al., 1991). Despite the low production of gentisic acid in vivo, it has been suggested that gentisic acid is predominantly formed under inflammatory conditions by polymorphonuclear leukocytes producing reactive oxygen radicals (Hinz et al., 2000; Sagone and Husney, 1987; Davis et al., 1989). This raises the possibility that the local concentrations of gentisic acid at the site of inflammation could reach far higher than those in plasma.

The present in vitro study also showed that the antioxidant effect by gentisic acid in high glucose concentration was less than that in normal glucose concentration. This may be partly due to increased oxidative stress in high glucose media (Kawamura et al., 1994; Hunt et al., 1990).

In conclusion, the present study shows that gentisic acid, an aspirin metabolite, potently inhibits LDL oxidation in vitro, which may partly account for the anti-atherogenic effects by aspirin in vivo. In future studies, it will be important to determine whether gentisic acid also inhibits lipid peroxidation in vivo.

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