Antioxidative Activity on Human Low Density Lipoprotein (LDL) Oxidation by Pentagalloic Acid

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Abstract The aim of this study was to investigate the efficiency of the pentagalloic acid compound in inhibiting the metal ions and cell lines that mediate in low density lipoprotein (LDL) oxidation. Pentagalloic acid prolonged the lag time preceeding the onset of conjugated diene formation. In chemically induced LDL oxidation by Cu^{2+} plus hydrogen peroxide or peroxyl radical generated by 2, 2'-azo-bis (2-amidino propane) hydrochloride (AAPH), pentagalloic acid inhibited LDL oxidation as monitored by measuring the thiobarbituric acid reactive substances (TBARS), malondialdehyde (MDA), and gel electrophoretic mobility. The physiological relevance of the antioxidative activity was validated at the cellular level where pentagalloic acid inhibited mouse macrophage J774 and endothelial cell-mediated LDL oxidation. When compared with several other antioxidants, pentagalloic acid showed a much higher ability than naturally occuring antioxidants, α-tocopherol and ascorbic acid, and the synthetic antioxidant, probucol.

Keywords: low density lipoprotein (LDL), antioxidant, pentagalloic acid

INTRODUCTION

The oxidative modification of LDL, that alters its physicochemical and biological properties is thought to play a central role in atherogenesis [1,2]. LDL is oxidized by free radicals generated from endothelial cells [3], monocyte-derived macrophages [4], and smooth muscle cells [5], resulting in several chemical and physical changes to LDL. Oxidized LDL is chemotactic for macrophages promoting their residence in the intima, cytotoxic to the endothelium, chemoattractant for monocytes, and rapidly accumulated by resident macrophages [6,7]. These changes depend on a common initiating step, the peroxidation of polyunsaturated fatty acid components, which leads to extensive fragmentation into degradative products, such as conjugated dienes, peroxy radicals, aldehydes, *etc* [8,9]. Oxidized LDL that has entered the artery wall and then accumulated in foam cells will then seemingly affect atherosclerotic progress. Therefore, it has been hypothesized that oxidized LDL initiates and promotes atherogenesis in several ways.

As a result, the intake of vegetable foods and beverages that contain phenolic antioxidant potentially protects against atherosclerosis. Phenolic compounds of vegetable origin commonly included in the diet, have antioxidant properties, and therefore, may be suitable for decreasing LDL susceptibility to oxidation and thereby preventing cardiovascular diseases [10]. In fact, this same phenomenon has been attributed to the regular consumption of red wine, and more specifically, to the high phenolic compound content of wine [11]. Furthermore, a high dietary intake of phenolic compounds has been associated with a decreased risk of developing cardiovascular diseases [12]. If oxidized LDL is crucial to atherogenesis, the potential role of antioxidants in the prevention of the oxidative modification of LDL assumes great importance. A class of compounds has recently been identified that appears to work by either mimicking or enhancing lipid oxidation action. Accordingly, there is a growing interest in understanding the role and mechanisms of phenolic compounds as inhibitors of deterious oxidative processes, particularly cancer and atherosclerosis [13]. Some have already been found *in vivo* and additional evidence suggests that they act as an antioxidants in the *in vivo* suppression of lipid peroxide levels and inhibition of LDL modification.

Recently, the development of natural antioxidants of phenolic compounds is expected to replace the synthetic antioxidants, which are widely used at the present time [13]. Antioxidants from natural substances such as edible plants, spices, and herbs are being extensively investigated, because these naturally occuring antioxidant compounds have been found to strengthen the resistence of LDL to oxidative modification *in vitro* and *in vivo*.

Pentagalloic acid was isolated from a higher phenol content of sumac has been reported as providing a strong resistance to oxidation [14].

Accordingly, the present study was designed to assess the effect of pentagalloic acid on the oxidation of human LDL by macrophages J774 and copper ions, as es-

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timated by measuring the formation of thiobarbituric acid reactive substances (TBARS) and conjugated diene.

MATERIALS AND METHODS

Materials

The pentagalloic acid was obtained from Dr. Greenspan, College of Pharmacy, University of Georgia. The 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH) was from the Wako Chemical Co. (Tokyo, Japan). The α-tocopherol and other chemicals were all purchased from the Sigma Co. (St. Louis, USA).

Isolation of Human Low Density Lipoprotein (LDL)

Human LDL was isolated from the blood of a healthy man by ultracentrifugation and dialyzed extensively against 0.9% (w/v) NaCl and 0.004% (w/v) at pH 7.4 [15]. Prior to oxidation, the LDL was dialyzed against phosphate-buffered saline, at pH 7.4, to remove the EDTA.

J774 Cultivation

The transformed mouse macrophage, J774, were maintained in Ham's F-10 supplemented with 10% (v/v) foetal calf serum, $NaHCO_3$ (2 g/L), and 4 mM Hepes, at pH 8.1. A series of antibiotics was included in the medium. The cells were routinely cultured in large dishes (90 mm diameter) in 10 mL of the medium and then plated out into smaller dishes (60 mm diameter) containing 2 mL of the medium. The cultures were maintained in a humidified incubator at 37°C with a fresh medium substituted every 48 h.

Detection of Conjugated Dienes

The formation of conjugated dienes associated with oxidized LDL was measured by monitoring the absorption at 234 nm using an UV-VIS spectrophotometer [16]. Briefly, 1 mL of an LDL solution (100 μg LDL, protein/mL) in phosphate-buffered saline, at pH 7.4, was incubated with 5 μM $CuSO_4$ at 37°C in the presence or absence of the tested compound, thereafter the absorbance at 234 nm was measured every 30 min. The formation of conjugated dienes in control solutions containing antioxidants in the absence of LDL and 5 μM $CuSO_4$ was also determined.

Oxidation of LDL

Oxidation was created by exposing LDL (10 μg/mL) to 10 μM Cu^{2+} in a 2 mM phosphate buffer at pH 7.5, containing 20 μM hydrogen peroxide or 2 mM AAPH, at 37°C. At certain time intervals, aliquots of the reaction mixture were taken to measure the extent of the lipid peroxidation through evaluating the TBARS and hydroperoxides. The entity of oxidation was expressed as malondialdehyde (MDA) equivalents using the MDA obtained by the acid hydrolysis of tetraethoxypropane as the standard [17]. The hydroperoxides (LOOH) were measured by the method of Cramer [18]. The extracts of 100 μL aliquots of the LDL solution diluted with 300 μL saline and acidified to pH 3.5 with citric acid; the triiodide ion was measured at 353 nm.

Assay of TBARS

The TBARS levels were determined spectrophotometrically 1 mL of 20% trichloroacetic acid and 1 mL of 1% thiobarbituric acid containing EDTA were added to 0.1 mL aliquot of the post incubation mixture and tetramethoxypropane standards. The tubes were then placed in a boiling water bath for 30 min. After cooling, the tubes were centrifuged at 1,500 *g* for 15 min. The absorbance of the supernatant was measured at 532 nm [19].

LDL Gel Electrophoresis

The electrophoresis of the oxidized and native LDL was carried out on an agarose gel in a barbital buffer at pH 8.6. The agarose plates were then stained with Nile red [20]. The results were expressed as the relative electrophoretic mobilities compared with the migration of the native LDL.

LDL Oxidation by Endothelial Cells

Primary cultures of human umbilical vein endothelial cells were obtained from a cord vein, after 15 min digestion by a 0.2% collagenase solution [21]. The cells were plated into tissue culture flask and allowed to grow to confluence in RPMI 1640 containing 20% fetal calf serum, 10 mM penicillin, 10 μg/mL streptomycin, and 2 mM L-glutamine at 37°C in a humidified atmosphere of 95% air and 5% CO_2. The resulting confluent human endothelial cell cultures in multiwell clusters(1.5×10^5 cells/cm^2) were then washed three times with a serum-free medium, supplemented with 5 μM $CuSO_4$, and incubated with LDL (200 μg/mL) in a serum-free medium containing 1% human serum albumin. Before being added to the endothelial cells, the LDL was loaded (30 min at 37°C) with vitamin E, pentagalloic acid or its vehicles (DMSO or ethanol respectively), and sterilzed by passage through 0.22 μm Millipore filters. After 18 h incubation at 37°C the medium was aspirated, centrifuged to remove any cell debris, and finally processed for the lipid peroxidation assay by the thiobarbituric acid reaction as described above.

Determination of Cellular Protein

The amount of cell protein was measured using bovine serum albumin as the standard [22].

Statistics

The data in the text and figures are mean±SD. The

statistical significance was examined rsing oneway analysis of variance. Significant differences were accepted at $P<0.05$.

RESULTS AND DISCUSSION

Effects of Pentagalloic Acid on Copper-mediated LDL Oxidation

Fig. 1 illustrates that the reaction kinetics of diene formation was composed of three phases. The first was the lag phase with a very low oxidation rate due to the counterbalance and consumption of endogenous antioxidants (Fig. 1(a)). The second phase corresponded to the maximal rate of oxidation and started after the antioxidants had been consumed (Fig. 1(b)). The third phase was the termination phase that was associated with a plateau in diene formation. The addition of antioxidants led to a prolongation of the lag phase, and this was also the case for pentagalloic acid (Fig. 1(c)). The inhibition period of diene formation in the presence of pentagalloyl glucose was concentration dependent, as shown by the plot of the pentagalloic acid concentration vs. the lag time (Fig. 1, inset); it is interesting to note that the regression line watched the zero inhibitor concentration value of the lag time. The current study is an extension of previously reported studies [14] that have have demonstrated that pentagalloic acid is a powerful inhibitor of LDL oxidation, induced chemically. To further examine this relationship, this study first investigated the affinity of pentagalloic acid for lipoprotein and the corresponding antioxidant effect. Pentagalloic acid was found to delay yet not stop the oxidation of lipoprotein, however, a further sstudy of LDL oxidation with different concentrations of pentagalloic acid is still needed. The inhibition of the lipid concentration would appear to be explained by a scavenging effect as the antioxidant for scavenge lipid peroxy radicals was formed in LDL during the lag phase. However, the LDL was depleted of its antioxidant compounds, the rate of lipid peroxidation rapidly increasesd. Therefore, the inhibition potency of LDL oxidation would seem to be related to the amount of antioxidant incorporated in LDL, which is likely related to the LDL drug affinity.

Fig. 2 shows the inhibitory potencies of pentagalloic acid and α-tocopherol in the AAPH-induced lipid peroxidation of LDL. LDL oxidation was induced chemically by copper ions in the absence or presence of hydrogen peroxide. The LDL was then incubated with 2 mM AAPH and various concentrations of α-tocopherol (5-50 μM) or pentagalloic acid (2-20 μM), and the TBARS levels measured after 3 h of incubation. Under these experimental conditions, pentagalloic acid and α-tocopherol both inhibited the AAPH induced LDL oxidation in a dose dependent manner. The 50% inhibitory concentrations of pentagalloic acid and α-tocopherol were 10 μM and 20 μM, respectively. A previous report proposed an antioxidative effect for pentagalloic acid,

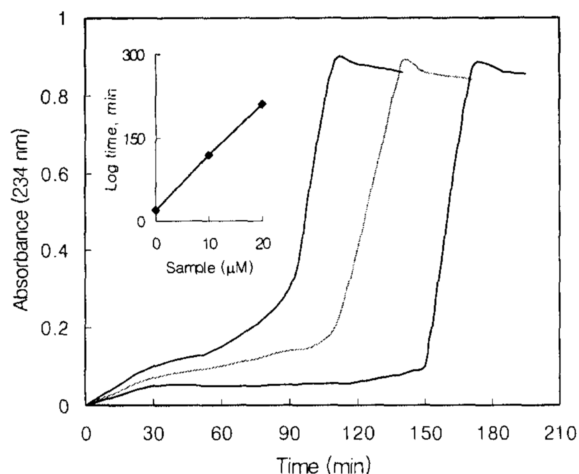

Fig. 1. Effect of pentagalloic acid on Cu^{2+} mediated LDL oxidation monitored by diene conjugation a, control; b, 10 μM pentagalloic acid, and c, 15 μM pentagalloic acid, Inset: concentration-dependence of inhibition period of diene formation. Each point represents the mean of three separate experiments with average variations.

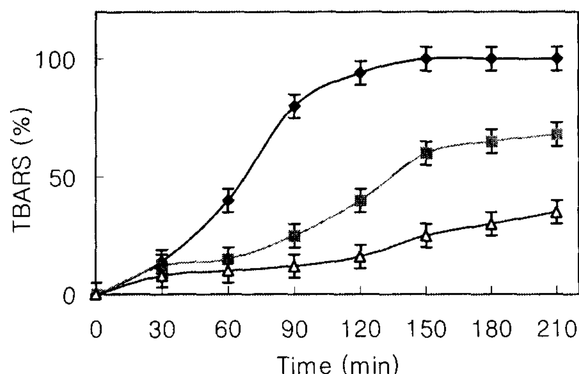

Fig. 2. Inhibition of LDL lipid peroxidation by pentagalloic acid and α-tocopherol. LDL (100 μg protein/mL) was subjected to oxidation with 2 mM AAPH in PBS in the absence (●) or presence (■) of pentagalloic acid and α-tocopherol (△). The TBARS contents are expressed as a percent of the maximum content observed after 3 h of incubation with 2 mM AAPH and the vehicle alone. The data are presented as the mean ±SD of 5 independent experiments.

which has a hindered phenol and is similar to flavonoids in structure [14]. This study demonstrated that pentagalloic acid exhibited an antioxidative effect on the oxidative modification of human plasma LDL, and that its antioxidative potency was significantly higher than that of α-tocopherol. The peroxy radicals derived from the AAPH abstract the hydrogen atoms from the polyunsaturated fatty acids in LDL, there by initiatino- and initiating the chain reaction [23,24].

The hindered phenol of α-tocopherol acts as a chain breaking antioxidant, whereas the major function of the phytyl side chain is to retain the molecule in LDL [23]. Although the mechanism of the enhanced antioxi-

Table 1. Effect of pentagalloic acid on LDL oxidation by macrophage, J774 as assessed by electrophoretic mobility

Incuation	Relative Electrophoreic Mobility	P
Native LDL	1.0	
LDL + Cell + Vehicle (control)	5.02 ± 0.04	
LDL + Cell + Pentagalloic Acid 5 μM	2.62 ± 0.03	
LDL + Cell + Pentagalloic Acid 10 μM	1.16 ± 0.04	< 0.05
LDL + Cell + Pentagalloic Acid 15 μM	1.12 ± 0.03	< 0.05
LDL + Cell + Pentagalloic Acid 20 μM	1.12 ± 0.01	< 0.01

[a] LDL (100 μg/mL) was incubated for 24 h in Ham's F-10 medium in 35-mm dishes containing macrophages in the presence or absence of pentagalloic acid. The electrophoretic mobility of LDL was determined in an agarose gel, as described in the text. The results are the means ± SD of three to five independent experiments.

dative activity of pentagalloic acid is still not clear, the phenol group of pentagalloic acid would seem to affect the stability of its molecule and its affinity for LDL.

In all cases, pentagalloic acid inhibited LDL oxidation with the characteristic induction of a concentration-dependent lag time, similar to classic antioxidants.

Effects of Pentagalloic Acid on LDL Oxidation and Assay Electrophoretic Mobility

Table 1 shows the effect of pentagalloic acid on the electrophoretic mobility of LDL submitted to oxidative modification by macrophages J774. An increase from 5.02±0.04 to 1.06±0.01 mm in the electrophoretic mobility of the LDL incubated with the macrophages for 24 h implies the lipid peroxidation of LDL and an increase in the negative charges in the LDL molecules. Pentagalloic acid reduced the relative electrophoretic mobility of LDL dose dependently. The LDL oxidized by $CuSO_4$ displayed a greater electrophoretic mobility in agarose gels compared to the native LDL. When LDL was incubated with 10 μM pentagalloic acid, the electrophoretic mobility of the oxidized LDL was only slightly greater than that of the native LDL. Pentagalloic acid inhibited the cell-induced oxidation of LDL as determined by the lipoperoxide content of the electrophoretic mobility of LDL in the agarose gels. Steinbrecher *et al.* [25] demonstrated that LDL could be modified by the addition of fatty acid peroxidation in the absence of cells. This modified LDL possesses an enhanced electrophoretic mobility without the oxidation of the lipid consitituents of LDL. It is also possible that the oxidation of LDL mediated by macrophages in Ham's F-10 culture medium may contribute to the modification of the LDL protein as determined by the enhanced electrophoretic mobility.

Effects of Pentagalloic Acid on Endothelial Cell Mediated LDL Oxidation

With the aim of substantiating whether pentagalloic acid can also act under more physiological conditions, experiments were at a performed a cellular level using human the endothelial cells to oxidize LDL. Pentagalloic acid successfully inhibited the endothelial cell-mediated LDL oxidation in a dose-dependent manner (Fig. 3), and a 50% inhibition was calculated to require about 15 μM. The antioxidant and vehicle concentrations applied did not induce any toxic effect in the endothelial cells, which kept the characteristic cobblestone morphology. Furthermore, no detachment was observed after the incubation period and after the cells were washed at the end of the incubation period they regained thein ability to oxidize LDL. Accordingly, it is evident that pentagalloic acid can inhibit LDL oxidation at the cellular level. Endothelial cells have been proposed as one of the sources responsible for LDL modification *in vivo* [3,5], by a free radical mediated mechanism. The inhibition of the oxidative modification of LDL is thus a crucial event in the suggested mechanism of atherosclerosis. One of the various biological characteristics of oxidized LDL is the uptake and degradation by a macrophage scavenger receptor, which causes the formation of foam cells, that are cytotoxic to most other cells [26].

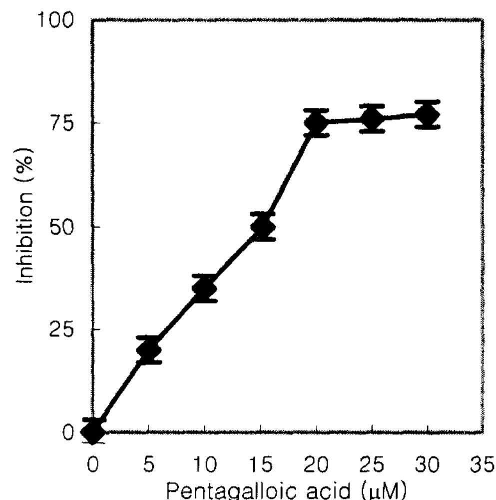

Fig. 3. Concentration-dependent inhibition of endothelial cell mediated oxidation by pentagalloic acid. Each data point represents the mean ± SD of three separate experiments.

Protective Activity of Pentagalloic Acid, Ascorbic Acid, Vitamin E, and Probucol

The protective activity towards LDL oxidation was compared with the activity exerted by the synthetic antioxidant, probucol, and the physiological antioxidants, ascorbic acid and vitamin E. The inhibitory activity was studied in LDL oxidation by copper, copper/hydrogen peroxide, and endothelial cells. The cumulative data, shown in Table 2, are based on an analysis of the MDA and hydroperoxide formation. The order of increasing activity was pentagalloic acid > probucol > ascorbic acid > vitamin E. The magnitude of the anti oxidant activity of pentagalloic acid was 3.2 times

Table 2. Relative antioxidative activity of pentagalloic acid, synthetic antioxidant probucol, and natural occuring antioxidants ascorbic acid and vitamin E

	Lipid Peroxidation (Cu^{2+}/H_2O_2)		Endothelial Cell Mediated Oxidation
	MDA	LOOH	
Pentagalloic Acid	11.5	12.2	12.8
Ascorbate	19.2	17.0	25.6
Probucol	20.4	15.0	9.8
Vitamin E	37.2	23.5	20.2

For each antioxidant the data was obtained from concentration-dependent inhibition experiments.

higher than that of vitamin E. In the endothelial cell-mediated LDL oxidation, the IC50 was 10 and 20 μM for pentagalloic acid and vitamin E, respectively. The inhibition of MDA and hydroperoxide formation required 1.9-3.0 times more vitamin E than pentagalloic acid. Compared to ascorbic acid, the activity of pentagalloic acid was 1.7 times higher, apart from the diene system where the difference was reduced to a factor of 1.4. Finally, pentagalloic acid was more potent than probucol by a factor ranging from 1.2 to 1.8 times, depending on the system.

To further support the physiological relevance of these results, activity of pentagalloic acid was compared to ascorbic acid and vitamin E, which are considered as reference antioxidants in biological systems, and probucol. Vitamin E, a normal constituent of LDL, is generally thought to function as the major lipid-soluble antioxidant, while ascorbic acid is considered to be the most important aqueous phase antioxidant in plasma [28]. Probucol was selected because most previous research on LDL oxidation has concentrated on the protection by this synthetic compound [29]. In all instances, pentagalloic acid produced the most potent inhibition of LDL oxidation. LDL oxidation is a complex mechanism involving initiator radicals generated by Fenton-like chemistry and the propagation of carbon-oxygen radicals, like peroxyl radicals. Thus, pentagalloic acid would appear to operate on two main levels, that is, the initiation and propagation of the lipid peroxidation. In addition, the superiority of pentagalloic acid can be explained in terms of partitioning the LDL lipids/aqueous phase and by better accessibility to sites of free radical attack. As demonstrated in this study, pentagalloic acid associates strongly LDL and is sufficiently water soluble to be able to intercept both the oxidation initiating radicals coming from the aqueous phase and the lipid radicals generated during the chain reaction.

These data show that pentagalloic acid is a powerful inhibitor of LDL oxidation *in vitro* and support the evaluation of this activity *in vitro*.

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