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Dipicolinic acid prevents the copper-dependent oxidation of low density lipoprotein

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Abstract

Effect of dipicolinic acid (pyridine 2,6-dicarboxylic acid) and pyridine compounds on the copper-dependent oxidation of human low density lipoprotein was analyzed in relation to the inhibition of copper reduction. Dipicolinic acid inhibited copper-dependent LDL oxidation completely, but the LDL oxidation was slightly inhibited by pyridine compounds with one carboxyl group at 2 or 6-position. Reduction of copper by LDL itself and ascorbate was inhibited completely by dipicolinic acid, but only partially by picolinic acid, quinolinic acid and isocinchomeronic acid with 2- or 6-carboxylic group. Pyridine compounds without 2- or 6-carboxyl group did not show any inhibitory effect on the LDL oxidation and the copper reduction. Protective effect of dipicolinic acid on the LDL oxidation was closely correlated with the copper-reducing activity. Dipicolinic acid shows an antioxidant action by the formation of a chelation complex with copper. This may have implications in understanding mechanisms of preventing LDL oxidation during the early phase of atherosclerosis.

Keywords: Dipicolinic acid; Pyridine compounds; Low density lipoprotein; Antioxidant

1. Introduction

Oxidation of LDL may be involved in the pathogenesis of atherosclerosis. Oxidative modification of LDL is likely a prerequisite for macrophage uptake and cellular accumulation of cholesterol, causing the formation of early atherosclerotic lesions [1]. Therefore, studies directed at examining preventive effects on LDL oxidation are important in strategies of atherosclerosis prevention. Ingestion of various dietary factors such as carotenoids and tea polyphenols, which act as an antioxidant, can prevent the oxidation of LDL *ex vivo* [2,3], leading to reduction in the risk of cardiovascular diseases. Considerable attention has been focused on the role of transition metals such as copper and iron, which can induce the oxidation of LDL [4]. Recently, we showed that dipicolinic acid (pyridine 2,6-dicarboxylic acid), a potent metal-chelator, which is synthesized in large amount in the spore of genus *Bacillus* [5], acted as an antioxidant: dipicolinic acid inhibits lipid peroxidation [6] and protects glutathione reductase from the copper-dependent inactivation [7]. A habit in Japan of eating Natto, a traditional fermented food made from soybeans with *Bacillus* may cause intake of a large amount of dipicolinic acid [8]. In this communication, we examined the protective effect of dipicolinic acid on LDL oxidation in relation to the copper reduction. Antioxdant effect of dipicolinic acid can be explained by the formation of the inactive chelation complex with copper.

2. Materials and methods

2.1. Materials

Following pyridine compounds were obtained from Tokyo Fine Chemical Co. (Tokyo, Japan): dipicolinic acid (pyridine 2,6-dicarboxylic acid), quinolinic acid (pyridine 2,3-dicarboxylic acid), lutidinic acid (pyridine 2,4-dicarboxylic acid), cinchomeronic acid (pyridine 3,4-dicarboxylic acid), isocinchomeronic acid (pyridine 2,5-dicarboxylic acid), and picolinic acid. Human low densiy lipoprotein, bathocuproine disulfonate and neocuproine were products

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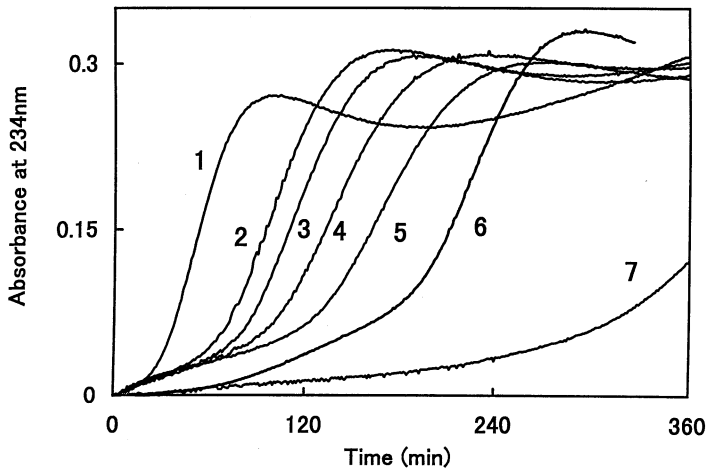

Fig. 1. Effect of pyridine compounds on the copper-dependent oxidation of low density lipoprotein. LDL was diluted to the concentration of 60 μg/ml with 10 mM potassium phosphate buffer (pH 7.4) containing 1.5 μM EDTA and 0.15 M NaCl, and LDL oxidation was started at 37°C by adding 2 μM $CuSO_4$ in the absence and presence of 5 μM pyridine compounds or trolox. The progress of oxidation was monitored spectrophotometrically equipped with a thermostatic control (37°C) and an automatically exchangeable six-positions cuvette holder, operating at 234 nm [6]. Curve 1, no addition; Curve 2, isocinchomeronic acid; Curve 3, quinolinic acid; Curve 4, lutidinic acid; Curve 5, picolinic acid; Curve 6, trolox; Curve 7, dipicolinic acid.

of Sigma-Aldrich (Tokyo, Japan). Other chemicals were obtained from commercial sources.

2.2. Oxidation of low density lipoprotein

Oxidation of LDL was determined as described previously [9]. LDL was diluted to the concentration of 60 μg/ml with 10 mM potassium phosphate buffer (pH 7.4) containing 1.5 μM EDTA and 0.15 M NaCl. LDL oxidation was performed at 37°C in 1 ml of the phosphate buffer (pH 7.4) containing 2 μM $CuSO_4$, 5 μM pyridine compounds or trolox and 0.15 M NaCl. The progress of oxidation was monitored spectrophotometrically (Shimadzu 1600, equipped with Peltier-thermostatted six cell holder) by the formation of conjugated dienes at 234 nm [9].

2.3. Effect of pyridine compounds on the reduction of Cu^{2+} ion by LDL and ascorbic acid

Copper reduction was followed by determining the cuprous ion concentration with bathocuproine disulfonate. For the analysis of the reduction of copper by LDL, the mixture of 0.3 ml containing 10 mM potassium phosphate buffer (pH 7.4), 0.15 M NaCl, 25 μM $CuSO_4$, 6 μM EDTA, 0.5 mM bathocuproine disulfonate and 0.1 mg/ml LDL in the presence of 0.1 mM pyridine compounds was incubated at room temperature, and the increase in the absorbance at 492 nm was recorded. For the analysis of the action of pyridine compounds on the copper reduction by ascorbic acid, the samples of 0.3 ml containing 10 mM Tris-HCl (pH 7.1), 0.1 mM $CuSO_4$, various concentrations of ascorbic acid and 0.5 mM bathocuproine disulfonate in the presence of pyridine compounds were incubated at room temperature, and the absorbance at 492 nm was recorded. Effect of various concentrations of pyridine compounds on the copper reduction by ascorbic acid was further analyzed. The mixture of 0.32 ml contained 20 mM Tris-HCl buffer (pH 7.1), 0.1 mM $CuSO_4$, 0.25 mM ascorbic acid and 0.5 mM neocuproine in the presence of various concentrations of dipicolinic acid, quinolinic acid, lutidinic acid or picolinic acid. The mixture was incubated for 1 hr at room temperature, and the absorbance at 450nm was recorded.

3. Results

3.1. Effect of pyridine compounds on the LDL oxidation

Effect of pyridine compounds on the oxidation of low density lipoprotein was examined. Addition of Cu^{2+} ion caused the oxidation of LDL with the concomitant formation of conjugated dienes (Figure 1). The time course of a slow oxidation (the lag period) followed by a rapid rise in the rate of formation of conjugated dienes. As shown in Figure 1, dipicolinic acid markedly increased the length of the lag-period of LDL oxidation. Other pyridine compounds with 2- or 6-carboxylic group also showed a weak inhibitory effect on the LDL oxidation. The order of effectiveness of pyridine compounds as inhibitors on LDL oxidation was dipicolinic acid $>>$ picolinic acid $>$ lutidinic acid $>$ quinolinic acid $>$ isocinchomeronic acid. Other pyridine compounds including nicotinic acid and cinchomeronic acid without carboxylate at 2- or 6-position did not show any inhibitory effect on LDL oxidation (data not shown). Trolox effectively increased the length of the lag-phase, but showed only a little effect on the propagation rate of LDL oxidation (Figure 1). Effect of pyridine compounds on the propagation rate of LDL oxidation was summarized in Table.

Table 1
Effect of pyridine compounds and trolox on the propagation rate of LDL oxidation

	ΔAbsorbance/min	% of the control	
None	0.0055 ± 0.00033		
Dipicolinic acid	0.0008 ± 0.00042	14.0 ± 7.6	$p < 0.001$
Picolinic acid	0.0021 ± 0.00067	37.5 ± 11.6	$p < 0.01$
Quinolinic acid	0.0028 ± 0.00088	51.4 ± 14.4	N.S.
Lutidinic acid	0.0032	57.6	
Isocinchomeronic acid	0.0041	72.5	
Trolox	0.0050	92.7	

Experimental conditions were similar to those of the experiments in Fig. 1. Data were expressed as mean ± SD with four independent experiments.

3.2. Reduction of copper by LDL and ascorbic acid

Oxidative modification of LDL requires redox active metal ions, and reduced copper ion is an important mediator of LDL oxidation [10]. Here we examined the effect of pyridine compounds on the reduction of copper by LDL itself. Dipicolinic acid completely inhibited the reduction of copper by LDL, and quinolinic acid and picolinic acid showed only a little inhibition of copper reduction (Figure 2). We further examined the inhibitory effect of dipicolinic acid and pyridine compounds on the ascorbate-mediated reduction of copper. Ascorbate-dependent copper reduction was inhibited by dipicolinic acid completely, and by quinolinc acid and lutidinic acid to a lesser extent (Figure 3). Picolinic acid and isocinchomeronic acid did not show any inhibitory effect. These results suggest that dipicolinic acid inhibits LDL oxidation by chelating copper and by the formation of the complex that is inert to oxygen molecule. The concentration necessary for 50% inhibition, $I_{0.5}$ value of dipicolinic acid for ascorbate-dependent copper reduction was 0.5 mM (Figure 4). The $I_{0.5}$ values of quinolinic acid and picolinic acid were also determined to be 1.25 and 5 mM, respectively (Figure 4).

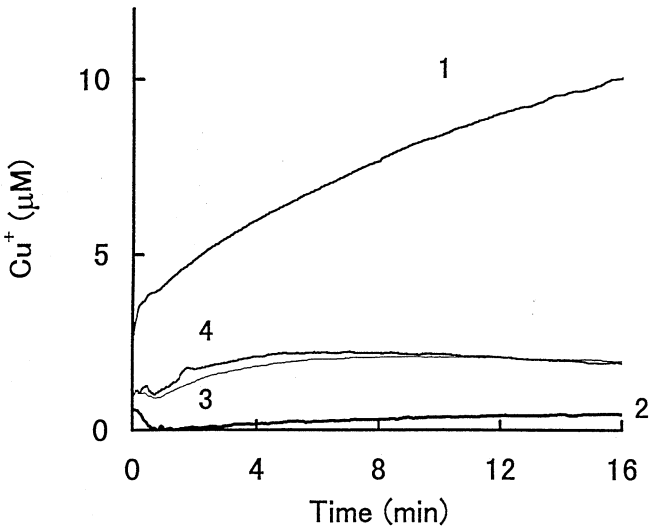

Fig. 2. Effect of pyridine compounds on the reduction of copper by LDL. Copper reduction was followed by determining the cuprous ion concentration with bathocuproine sulfonate. The samples of 0.3 ml contained 10 mM potassium phosphate buffer (pH 7.4), 0.15 M NaCl, 25 μM $CuSO_4$, 6 μM EDTA, 0.5 mM bathocuproine disulfonate and 0.1 mg/ml LDL in the presence of 0.1 mM pyridine compounds. The mixture was incubated at room temperature, and the increase in the absorbance at 492 nm was recorded. Curve 1, No addition; Curve 2, 0.1 mM dipicolinic acid; Curve 3, 0.1 mM picolinic acid; Curve 4, 0.1 mM quinolinic acid.

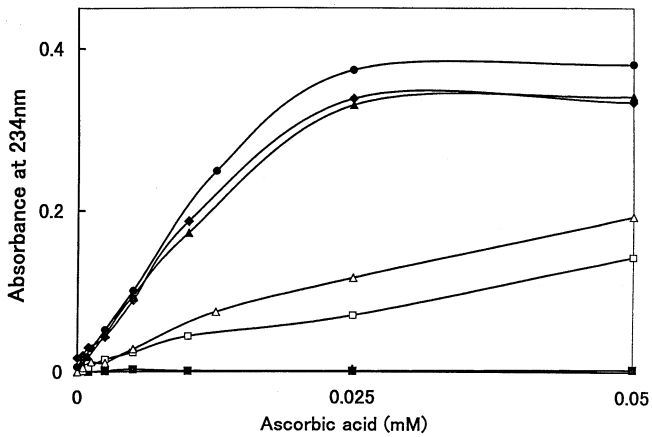

Fig. 3. Effect of pyridine compounds on the reduction of copper by ascorbic acid. The samples of 0.3 ml contained 10 mM Tris-HCl (pH 7.1), 0.1 mM $CuSO_4$, various concentrations of ascorbic acid and 0.5 mM bathocuproine disulfonate in the presence of pyridine compounds. The mixture was incubated at room temperature, and the absorbance at 492 nm was recorded. ◆, No addition; ■, Dipicolinic acid; ▲, Picolinic acid; △, Lutidinic acid; ●, Isocinchomeronic acid; □, Quinolinic acid.

4. Discussion

Oxidative modification of LDL takes place *in vivo*, and a number of experimental and clinical evidences have been

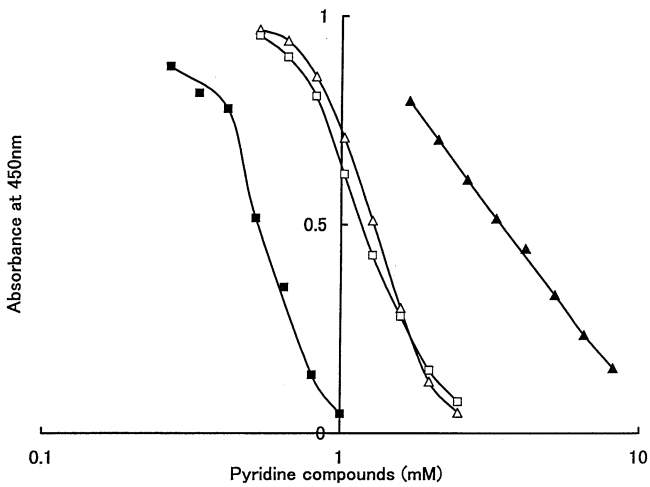

Fig. 4. Inhibitory effects of pyridine compounds on the copper reduction by ascorbic acid. The mixture of 0.32 ml contained 20 mM Tris-HCl buffer (pH 7.1), 0.1 mM $CuSO_4$, 0.25 mM ascorbic acid and 0.5 mM neocuproine in the presence of various concentrations of pyridine compounds. The mixture was incubated for 1 hr at room temperature, and the absorbance at 450nm was recorded. ■, Dipicolinic acid; □, Quinolinic acid; ▲, Picolinic acid; △, Lutidinic acid.

obtained indicating a close association between LDL oxidation and the onset and progression of atherosclerosis [1]. Copper-promoted LDL oxidation is assumed to be initiated by the reduction of Cu^{2+} to Cu^{+} [1, 10, 11]. Our recent finding on the inhibition of LDL oxidation by addition of superoxide dismutase implies that superoxide radical plays an essential role in the copper-mediated LDL oxidation [9]. Reduced copper ion may react with molecular oxygen to form superoxide radical as an initiator of LDL oxidation. However, identification of the sources of reducing equivalents is still an open question. Different mechanisms of copper reduction for the conversion of Cu^{2+} to Cu^{+} are progressively recruited during copper-induced LDL oxidation: α-tocopherol-dependent and lipid peroxide-dependent copper-reduction mechanisms are involved in the LDL oxidation [11]. Products of LDL oxidation have been shown to mimic several pathological changes in the vascular wall that are typical of the atherosclerotic process [1]. In recent years considerable attention has been devoted to some antioxidants that potentially prevent the development of diseases for which lipid oxidation has been demonstrated as a pathogenetic factor. Dietary antioxidants including carotenoids, flavonoids and other polyphenolics play important roles in preventing atherosclerosis: tea polyphenols inhibit the oxidative modification of LDL under the *in vivo* conditions [2,3,12].

Our previous study presented some evidences that dipicolinic acid can act as an antioxidant, which inhibits lipid peroxidation [6] and protects glutathione reductase from the copper-dependent inactivation [7]. Inhibitory effects of dipicolinic acid on the iron-dependent lipid peroxidation and the copper-mediated inactivation of glutathione reductase are related to the electron deficient nature of pyridine ring. Pyridine ring has the unshared pair of electrons on the nitrogen atom, and introduction of electron-attracting carboxylic groups to 2- and 6-positions further causes more higher electron deficiency. Copper binds to dipicolinic acid, which can attract electron from Cu^{+} as the prooxidant to form Cu^{2+}, resulting in the inhibition of formation of reactive oxygen species. As shown in this paper, dipicolinic acid potently inhibited the copper-mediated LDL oxidation. Pyridine compounds with 2-carboxyl group also inhibited the oxidation to a lesser extent, and thus, 2-carboxyl group is essential for the inhibition of LDL oxidation. Dipicolinic acid and pyridine carboxylates show higher chelating activiy toward most metals such as copper and iron [13,14]. Cupric ion bound to dipicolinic acid was not at all reduced by LDL and ascorbic acid (see, Figures 2 and 3). Inhibitory effect of dipicolinic acid on the copper-mediated LDL oxidation is essentially related to the inhibition of copper reduction by this compound, and thus, can be explained by the copper binding, and by the inhibition of its reduction. Some discrepancy of the potency order of pyridine compounds was shown in the relationship between the inhibitory activity against LDL oxidation and copper-reducing ability (see, Figures 1 to 4). This may be related to the difference in binding affinity of the pyridine/copper complex to LDL or ascorbic acid. Oxidation of LDL can be induced by copper and iron, and in particular, iron widely distributed in large amount may participate in the substantial role in the initiation of LDL oxidation *in vivo.* Inhibitory effect of dipicolinic acid is equally effective for copper- and iron-mediated LDL oxidation. Thus, dipicolinic acid effect may be physiologically relevant.

Dipicolinic acid is synthesized in the spore of *Bacillus*, and the traditional fermented food made from soybeans with *Bacillus,* that is Natto in Japan, contain a large amount of dipicolinic acid [8]. Dipicolinic acid shows various biological functions including activation/inactivation of some metalloenzymes [13,14] and inhibition of electron transport system [15], and acts as a potent inhibitor of LDL oxidation. Absorption of dipicolinic acid in human and animals has not been examined. However, picolinic acid-metal complex was demonstrated to be taken up by intestine [16], and furthermore, dipicolinic acid was found to be incorporated to malaria-infected human red blood cells [17]. Chelating actions of dipicolinic acid and the subsequent prevention of LDL oxidation may have therapeutic implications in the treatment of atherosclerosis.

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