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Antioxidant α-Keto-carboxylate Pyruvate Protects Low-density Lipoprotein and Atherogenic Macrophages

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Oxidative modification of low-density lipoprotein (oxLDL) plays a pathogenic role in atherogenesis. Classical antioxidants such as L-ascorbic acid can inhibit formation of oxLDL. α-Keto-carboxylates such as pyruvate and congeners also display antioxidant properties in some cell-free and intact cell systems. We tested the hypothesis that pyruvate or α-keto-glutarate may function as antioxidants with respect to LDL incubated with 5 or 10 μM Cu^{2+} alone or in combination with THP-1-derived macrophages. α-Hydroxy-carboxylates (L-lactate), linear aliphatic mono-carboxylates (acetate/caprylate) and L-ascorbic acid served as controls. The oxLDL formation was ascertained by electrophoretic mobility and oxLDL cytotoxicity was judged by macrophage viability and thiobarbituric acid reactive substances (TBARS) formation. Cu^{2+} alone was not cytotoxic but increased electrophoretic mobility of cell-free LDL, stimulating TBARS. Millimolar pyruvate, α-keto-glutarate, or micromolar L-ascorbic acid partially inhibited oxLDL formation, while α-hydroxy-carboxylate or the aliphatic mono-carboxylates had no measurable antioxidant properties in cell-free LDL. Co-culture of LDL with macrophages and Cu^{2+} augmented TBARS release and resulted in 95% macrophage death. Pyruvate improved macrophage viability with 5 μM Cu^{2+} up to 60%. L-Ascorbic acid (≥100 μM) protected macrophages up to 80%. When ≥100 μM L-ascorbic acid was combined with pyruvate, oxLDL formation and macrophage death were fully prevented. Thus, α-keto-carboxylates, but not physiological α-hydroxy-carboxylates or aliphatic mono-carboxylates qualify as antioxidants in LDL systems. Since α-keto-carboxylates enhanced the antioxidant power of L-ascorbic acid, our findings may have implications for strategies attenuating atherosclerosis.

Keywords: Low-density lipoprotein oxidation; Macrophages; L-Ascorbic acid; Pyruvate; α-Keto-carboxylate; α-Hydroxy-carboxylate

INTRODUCTION

It has been established that oxidatively modified low-density lipoprotein (LDL) is causally involved in human atherosclerosis, even in the absence of hypercholesteremia.[1–3] LDL oxidation can occur within the vascular wall[2] where such changes may result in lipid accumulation, focal necrosis covered by smooth muscle cells and surrounded by macrophages, connective tissue proliferation due to chronic inflammation, and other subparenchymal events that sustain and promote the atherosclerotic process.[4,5]

The cellular mechanisms for the oxidized LDL (oxLDL) production comprise the lipoxygenase pathway[6] and the generation of superoxide radicals[7] by the mitochondrial electron transport chain most likely at the level of coenzyme Q[8] or via the NADH oxidase or xanthine oxidases.[9] LDL oxidation can also proceed via cell-dependent thiol output, possibly via the generation of thiyl radicals.[10] It has been recognized that thiol-dependent oxidative modification of LDL can be accomplished by superoxide-dependent and -independent mechanisms.[11] In cell-free and cell-containing systems, oxLDL particles can be generated from native LDL by trace amounts of transition metals such as copper (Cu^{2+}) and iron (Fe^{2+}), or by inorganic oxidants such as H_2O_2.[12] Based on the known mechanisms causing LDL to oxLDL transitions, it is likely that agents or antioxidants that inhibit production

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of or neutralize reactive oxygen species (hydroxyl radical, thiyl radicals, superoxide anion), may inhibit production of oxLDL *in vivo* and thereby decelerate atherosclerosis.

α-Keto-carboxylates such as pyruvate or α-keto-glutarate can directly neutralize H_2O_2 and other aqueous reactive oxygen species.[13–15] Pyruvate is the key substrate of mitochondrial pyruvate dehydrogenase and cytosolic lactate dehydrogenase. It is known to inhibit glycolysis and to metabolically stabilize cytosolic NAD^+ and GSH systems rendering pyruvate an antioxidant.[13,15–17] Due to intra-mitochondrial metabolism pyruvate also raises the cytosolic energy state and functions as anaplerotic precursor via the pyruvate carboxylase.[16,17] In the mitochondrial matrix, pyruvate raises NADH via the pyruvate-stimulated pyruvate dehydrogenase.[18]

In the present study, we tested the hypothesis that α-keto-carboxylates such as pyruvate or α-keto-glutarate exhibit antioxidant properties with respect to LDL metabolism. Accordingly, we determined whether Cu^{2+}-induced oxidative modification of LDL could be alleviated by pyruvate and/or α-keto-glutarate, compared to α-hydroxy- or linear aliphatic mono-carboxylates. LDL peroxidation status was judged by electrophoretic mobility and TBARS formation. In an oxLDL injury model of the atherosclerotic process, we examined the viability of human THP-1 monocytic leukemia cells derived macrophages exposed to LDL plus α-keto-carboxylates in the presence of added Cu^{2+} for 24 h. The results were compared with effects of L-ascorbic acid, the classic antioxidant. We observed that millimolar α-keto-carboxylates such as pyruvate could act as antioxidants with respect to LDL, both in the absence and presence of atherogenic macrophages. In addition, we observed that pyruvate boosted the antioxidant power of L-ascorbic acid in LDL systems co-cultured with human macrophages.

MATERIALS AND METHODS

Preparation of LDL

Human normolipidemic pooled plasma LDL ($d = 1.020–1.063$ g/ml) was prepared by a discontinuous density gradient ultracentrifugation as previously described elsewhere.[19,20] Isolated LDL was dialyzed at 4°C overnight against 2 l buffer (pH 7.4) of 0.154 M NaCl–0.01% EDTA. Any charge modification of the prepared LDL was detected by agarose gel electrophoresis,[21] and the presence of apo B100 in LDL was confirmed by 8–25% SDS gradient phastgel (Pharmacia Biotech. Inc., Piscataway, NJ). Protein contents in LDL were determined by the Lowry method,[22] and concentrations of triglycerides, total cholesterol, and phospholipids were measured by using diagnostic kits (Asan Pharmaceutical Co., Korea). Isolated LDL was used within 4 weeks after isolation procedure. An aliquot of EDTA-containing LDL (1 ml) was dialyzed overnight against 2 l EDTA-free buffer containing 0.154 M NaCl prior to experiments.

Electrophoretic Mobility Assay

To study the direct effects of pyruvate or α-keto-glutarate on Cu^{2+}-induced LDL oxidation in the cell-free system, the electrophoretic mobility pattern of LDL on agarose gel was examined using a previously published method with a minor modification.[21,23,24] Briefly, EDTA-free LDL (1 mg cholesterol/ml) was incubated in fresh F-10 medium (6.1 mM glucose, 0.01 μM $CuSO_4$, 3.38 μM $FeSO_4$) containing millimolar pyruvate or α-keto-glutarate for 24 h. The LDL mobility was also measured after incubation with millimolar L-lactate, acetate, caprylate (octanoate), and L-ascorbic acid. Aliquots of medium were run on a 0.8% agarose electrophoresis in pH 8.6-barbital buffer (Sigma Co., St. Louis, MO). The gel was immediately fixed in a 5% trichloroacetic acid solution and rinsed in 70% ethanol. Photographs of gel were obtained using Polaroid Type 667 positive/negative film.

Cell Culture

A human monocyte THP-1 cell line was obtained from the American Type Tissue Culture set (Rockville, MD) and grown in 25 mM HEPES-buffered RPMI-1640 (Sigma Co.) containing 10% fetal bovine serum (FBS), 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Cultures were maintained at 37°C in humidified atmosphere of 5% CO_2 in air, and cells were passaged weekly 1:4. To stimulate differentiation of THP-1 cells into macrophages, cells were plated at a density of 2×10^5 cells/ml and then incubated in RPMI-1640 containing 10% FBS for 24 h in the presence of 100 ng/ml phorbol 12-myristate-13-acetate (PMA) (Sigma Co.) dissolved in dimethylsulfoxide (final concentration 0.1%). After the 24 h incubation, cells were thoroughly rinsed with phosphate buffered saline (PBS) and continuously cultured in RPMI-1640 supplemented with 10% FBS in the absence of PMA for 7 days, as described elsewhere.[25] For the detection of macrophage differentiation, the non-specific esterase activity of THP-1 macrophages was measured cytochemically according to Suematsu *et al.*[25] 7 days after the PMA addition, and their capacity to take up DiI-labeled acetyl-LDL was examined. In all experiments, cells were pre-incubated for 24 h in fresh F-10 medium containing 5% FBS. THP-1-derived macrophages were then pre-treated with pyruvate (5–20 mM) and/or L-ascorbic

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acid (10–300 μM) in fresh F-10 medium plus 5% FBS for 4 h. Subsequently, EDTA-free LDL (1 mg cholesterol/ml) and 5 or 10 μM Cu^{2+} were added and incubation was continued for additional 24 h.

Cell Viability

The MTT [3-(4,5-dimetylthiazol-yl)-diphenyl tetrazolium bromide] assay was performed to estimate cellular viability.[26] After THP-1 macrophages were exposed to LDL and Cu^{2+} for 24 h, 100 μl of 40 mM EDTA was added for 3 min in order to prevent cell death due to further LDL oxidation. Cells were thoroughly rinsed with PBS and incubated in a fresh phenol red-free medium containing 1 mg/ml MTT for 3 h at 37°C. After removal of unconverted MTT, the purple formazan product was dissolved in 1 ml isopropanol with gentle shaking for 10 min. Absorbance of formazan dye was measured at $\lambda = 570$ nm with background subtraction using $\lambda = 690$ nm.

Thiobarbituric Acid Reactive Substances

Oxidative modification of LDL particles to oxLDL can be caused by transition metals such as Cu^{2+}.[12,27] To measure lipid peroxidation, thiobarbituric acid reactive substances (TBARS) were measured according to methods previously described.[28–30] After cells were incubated with LDL and Cu^{2+} for 24 h in the absence and presence of various concentrations of pyruvate and/or L-ascorbic acid, 100 μl of 40 mM EDTA was added to inhibit further lipid peroxidation, and the incubation medium was then collected. One-milliliter aliquots were centrifuged for 10 s to remove cell debris and then concentrated about five-fold in a Centricon tube with molecular cutoff at 10 kDa at 4°C. The filtrate was freeze-dried, and the resulting powder was dissolved in the concentrate obtained by previous filtration. Hundred microliter of the concentrate was used in the thiobarbituric acid assay, using the absorbance at $\lambda = 535$ nm.[28] The TBARS contents were expressed as μg malondialdehyde/ml, a product of lipid peroxidation. When recovery of MDA in samples (showing high TBARS values) concentrated from medium incubated with LDL in the presence of 10 μM Cu^{2+}, or medium incubated with cells in the presence of LDL and 10 μM Cu^{2+}, was examined, quantitatively similar TBARS values to those detected with unconcentrated medium were obtained.

Data Analysis

Data are presented as means ± SEM. Differences between groups were evaluated by two-way analysis of variance followed by the Tukey correction (SAS Institute Inc., Cary, NC). Statistical significance was set at $P < 0.05$.

RESULTS

Inhibition of Cu^{2+}-induced LDL Oxidation by α-Ketocarboxylates in Cell-free and Macrophage Containing Systems

Cell-free control cultures were performed using 1 mg/ml LDL in the presence of 5 or 10 μM Cu^{2+}. Data are compiled in Table I, rows 1–3. TBARS was formed from LDL particles in the absence of cells, and this oxidative modification of LDL was strongly stimulated by Cu^{2+} in the range between 0 and 10 μM. Table I, rows 4 and 5 show that LDL incubation with macrophages in the absence of Cu^{2+} did not appreciably increase the indices of LDL peroxidation; however, when Cu^{2+} was added in presence of the cells, TBARS formation was increased by 66 and 44% with 5 and 10 μM Cu^{2+}, respectively (rows 6 and 7 vs. 2 and 3, Table I). These observations demonstrated that Cu^{2+} readily induced LDL oxidation. Furthermore, macrophages substantially promoted LDL oxidation in the presence of Cu^{2+} (Fig. 1, panel A). Accordingly, close inspection revealed that the presence of macrophages enhanced lipid peroxidation of the combined system, possibly due to Cu^{2+}-induced peroxidation of membrane lipids of added cells and/or due to promotion of LDL oxidation triggered by the added cells.

The oxidative effect of Cu^{2+} on LDL particles was also evident from agarose electrophoretic mobility, a biochemical hallmark of LDL oxidation.[23,24] Table II summarizes the relative electrophoretic mobility

TABLE I Formation of medium thiobarbituric acid reactive substances (TBARS) from Cu^{2+}-stimulated oxidation of low-density lipoprotein (LDL) particles in cell-free and THP-1 macrophage containing systems

LDL culture protocols			TBARS
Cu^{2+} (μM)	Cells	LDL (1 mg/ml cholesterol)	
0	−	+	$0.42 \pm 0.12^{a,b}$
5	−	+	1.60 ± 0.51^{c}
10	−	+	2.44 ± 0.43^{d}
0	+	−	0.31 ± 0.04^{a}
0	+	+	0.54 ± 0.03^{b}
5	+	+	2.66 ± 0.29^{d}
10	+	+	3.58 ± 0.28^{e}

Values are given as means ± SEM, $n = 10$ separate experiments. THP-1 (2×10^5 cells/ml) derived macrophages were incubated in F-10 medium containing 5% FBS for 24 h, followed by another 24 h exposure to LDL in the absence and presence of either 5 or 10 μM Cu^{2+}. After 24 h formation of medium TBARS was measured (see "Materials and Methods" section). TBARS data are expressed as μg malondialdehyde/ml, a product of lipid peroxidation. The TBARS value of fresh unincubated native LDL was below detection value. Values with different superscripts are significantly different from each other at $P < 0.05$.

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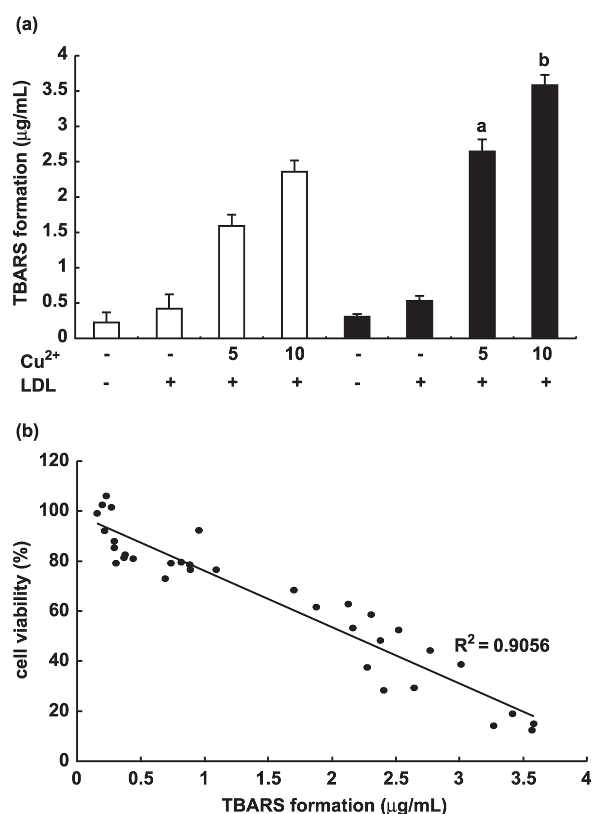

FIGURE 1 The rate of atherogenic low-density lipoprotein (LDL) oxidation in THP-1 (2×10^5 cells/ml) macrophage-free (open bars) and containing systems (closed bars) (A panel) and the relationship between macrophage viability and lipid peroxidation (measured as TBARS) induced by oxidative modification of LDL using 5 or 10 μM Cu^{2+} for 24 h. Panel A shows that the addition of macrophages accelerated the rate of LDL oxidation (means ± SEM of 10 different experiments). Panel B shows a linear inverse correlation between medium TBARS levels due to macrophages plus Cu^{2+}-oxLDL and macrophage cellular viability using all single data points. THP-1-derived macrophages were cultured in F-10 medium containing 5% FBS and exposed to LDL (1 mg cholesterol/ml) in the presence of either 5 or 10 μM Cu^{2+}. TBARS was measured to assess the degree of lipid peroxidation induced by Cu^{2+}-stimulated oxidative modification of LDL. TBARS data were expressed as μg malondialdehyde/ml, a product of lipid peroxidation. Viability data are expressed as percent of the viability of separate time controls without LDL and Cu^{2+} (viability = 100%). [a]$P < 0.05$, relative to cell-free controls at 5 μM Cu^{2+}. [b]$P < 0.05$, relative to cell-free controls at 10 μM Cu^{2+}.

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data of LDL particles incubated with Cu^{2+} in the absence and presence of the α-keto-carboxylate pyruvate, a known antioxidant in both cell-free and cellular systems.[13,15–17] When LDL was incubated in F-10 medium, LDL oxidation was minimal (1.19 ± 0.04 electrophoretic mobility relative to native LDL), demonstrating that spontaneous LDL oxidation occurred during incubation even without a Cu^{2+} addition. The addition of pyruvate did not influence this spontaneous LDL oxidation. As expected, there was a marked increase in the relative electrophoretic mobility when LDL was incubated for 24 h with 5 μM Cu^{2+}, compared to that observed in spontaneous oxLDL (rows 2 vs. 1, Table II). The electrophoretic mobility decreased dose-dependently when LDL was treated with ≥5 mM pyruvate plus 5 μM Cu^{2+}, relative to LDL exposed to Cu^{2+} alone (rows 3–5). The data show that millimolar concentrations of the α-keto-carboxylate pyruvate protect LDL against oxidation thus stabilizing LDL in its native state.

The effects of 10 mM α-keto-carboxylates (pyruvate, α-keto-glutarate) were compared with those of equimolar α-hydroxy-carboxylates (L-lactate) and linear aliphatic mono-carboxylates (acetate, caprylate). Only pyruvate and α-keto-glutarate decreased significantly the Cu^{2+}-stimulated electrophoretic mobility. In contrast, no such protection against LDL oxidation was observed with L-lactate or the aliphatic compounds; in fact, L-lactate and the fatty acids appeared to promote Cu^{2+}-induced oxLDL formation (Table II). In the experiments with LDL plus α-keto-glutarate the medium was slightly acidic (pH 5–6). When medium pH was adjusted to pH 7.4, α-keto-glutarate had a small but still significant effect on Cu^{2+}-stimulated LDL mobility, indicating that the direct LDL antioxidant activity of α-keto-glutarate was markedly pH-dependent (Table II). These findings are consistent with and predicted from acidic conditions affecting the rate of LDL oxidation.[31]

Inhibition of Cu^{2+}-induced LDL Cytotoxicity by Pyruvate

In the absence of LDL, Cu^{2+} alone even at a concentration of 10 μM did not induce THP-1 macrophage lipid peroxidation as evidenced by very low basal formation of TBARS (0.26–0.33 μg/ml) and conjugated dienes (4.1–5.3 μg/ml,[30]). In addition, Cu^{2+} did not decrease the macrophage viability under the same conditions (data not shown). Thus, Cu^{2+} *per se* was not cytotoxic in the absence of LDL. On the other hand, when macrophages were added to LDL-containing systems, the Cu^{2+}-induced formation of TBARS increased significantly (Table I).

In the absence of pyruvate, Cu^{2+}-induced oxLDL decreased the macrophage viability by 70% to near 90%; this massive cell death was associated with several-fold increase in TBARS formation, i.e. by 5–7 fold (Table I, rows 6 and 7). There was a highly significant inverse linear correlation between the degree of Cu^{2+}-stimulated LDL oxidation and macrophage viability (Fig. 1, panel B). Accordingly, it is obvious that macrophage viability was reflected by the rate of LDL oxidation measured as TBARS formation. Figure 2 depicts the dose-dependence of pyruvate protection against macrophage killing and lipid peroxidation in presence of LDL plus 5 or 10 μM Cu^{2+}. The cytotoxicity of oxLDL was substantially attenuated (60–80% viability)

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TABLE II Relative electrophoretic mobility of oxidatively modified low-density lipoprotein (LDL) particles

LDL culture protocols		Relative electrophoretic mobility (fold of mobility for native LDL)
Addition of Cu^{2+} (μM)	Pretreatment in presence of LDL	
Cu^{2+}-free	LDL alone	1.19 ± 0.04[a]
5	LDL alone	1.72 ± 0.04[b]
5	5 mM pyruvate	1.68 ± 0.06[b]
5	10 mM pyruvate	1.56 ± 0.05[c]
5	20 mM pyruvate	1.45 ± 0.05[d]
5	10 mM L-lactate	1.81 ± 0.09[b,e]
5	10 mM acetate	1.88 ± 0.08[e]
5	10 mM caprylate	1.87 ± 0.10[e]
5	10 mM α-keto-glutarate (pH 5–6)	1.26 ± 0.02[a]
5	10 mM α-keto-glutarate (pH 7.4)	1.58 ± 0.05[c]

Data are means ± SEM of the measurements of four independent LDL pools. LDL (1 mg cholesterol/ml) was incubated with 5 μM Cu^{2+} plus pyruvate, L-lactate, acetate, caprylate (octanoate) or α-keto-glutarate for 24 h in the cell-free system. Electrophoretic mobility was expressed relative to that of native LDL. Relative electrophoretic mobility of fully acetylated LDL was 1.95 ± 0.18. Values with different superscripts are significantly different from each other at $P < 0.05$.

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by millimolar pyruvate in a dose-dependent manner (Fig. 2, panel A); this pyruvate protection was associated with a dose-dependent reduction in lipid peroxidation (Fig. 2, panel B). However, even at 20 mM, pyruvate failed to fully protect the macrophages against oxLDL-induced death (Fig. 2, panel A). In addition, 20 mM pyruvate did not completely prevent Cu^{2+}-stimulated TBARS formation (Fig. 2, panel B).

We also noted that the spontaneous LDL oxidation was cytotoxic, as it lowered measurably THP-1 macrophage viability (Fig. 3, panel A) at measurably increased lipid peroxidation (Fig. 3, panel B). This underlying spontaneous peroxidation was not attenuated by α-keto-carboxylate treatment (Fig. 3), illustrating the limits of pyruvate protection against cytotoxic oxLDL.

Full Protection against Cytotoxic oxLDL Requires Pyruvate Plus L-Ascorbic Acid

At low micromolar concentrations, L-ascorbic acid is a natural and powerful antioxidant. It can directly scavenge aqueous reactive oxygen species and protect LDL particles against atherogenic modification by Cu^{2+}-dependent oxidation.[12,27] Table III shows the results of the electrophoretic mobility assay of Cu^{2+}-stimulated LDL pre-treated with 50 or 300 μM L-ascorbic acid and/or 5–20 mM pyruvate in the absence of macrophages. There was no noticeable effect on electrophoretic mobility when LDL was cultured with 5 mM pyruvate alone or 50 μM L-ascorbic acid alone (Table III). Only at the concentrations of ≥10 mM pyruvate alone or 300 μM L-ascorbic acid alone was there a measurable

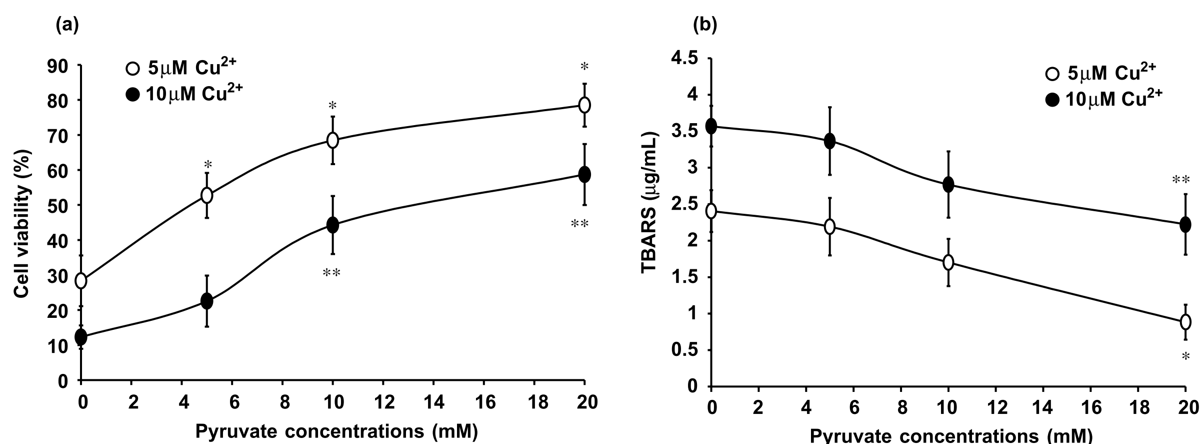

FIGURE 2 Dose-dependent pyruvate protection of THP-1 (2×10^5 cells/ml) macrophages (panel A) and lipid peroxidation (panel B) against atherogenic low-density lipoprotein (LDL) produced by oxidative modification with 5 or 10 μM Cu^{2+} for 24 h. Panel A shows the effects of Cu^{2+}-oxLDL on dose-response relationships between pyruvate and macrophage viability (means ± SEM of 10 different experiments). Panel B shows effect of pre-treatment with pyruvate on medium TBARS produced by macrophages plus Cu^{2+}-oxLDL over 24 h. THP-1-derived macrophages were cultured in F-10 medium containing 5% FBS with and without pyruvate for 4 h and then exposed to LDL (1 mg cholesterol/ml) in the presence of either 5 or 10 μM Cu^{2+}. Viability data are expressed as percent of the viability of separate time controls without LDL and Cu^{2+} (viability = 100%). TBARS was measured to assess the degree of lipid peroxidation induced by Cu^{2+}-stimulated oxidative modification of LDL. TBARS data were expressed as μg malondialdehyde/ml, a product of lipid peroxidation. *$P < 0.05$, relative to no pyruvate controls at 5 μM Cu^{2+}. **$P < 0.05$, relative to no pyruvate controls at 10 μM Cu^{2+}.

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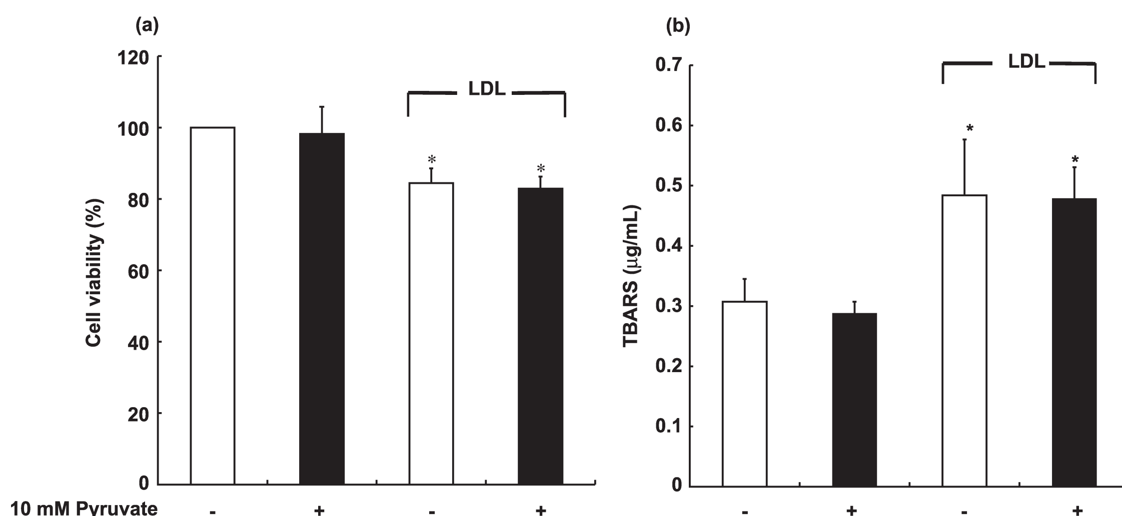

FIGURE 3 THP-1 macrophage viability (panel A) and TBARS formation (panel B) by low-density lipoprotein (LDL) in the absence and presence of 10 mM pyruvate. THP-1 (2×10^5 cells/ml)-derived macrophages were cultured in F-10 medium containing 5% FBS with LDL (1 mg cholesterol/ml). Cell viability was assessed by the MTT assay after 24 h. Viability data are expressed as percent of the viability of time controls without LDL and pyruvate. TBARS was measured as μg malondialdehyde/ml. The data were expressed as means ± SEM from four different experiments. $^*P < 0.05$, relative to no LDL controls.

improvement of Cu^{2+}-induced electrophoretic mobility (Tables II and III); nevertheless, either compound when applied alone failed to fully protect LDL against Cu^{2+}-induced oxidation. However, the electrophoretic mobility was completely restored when 20 mM pyruvate plus 300 μM L-ascorbic acid was tested. This could be concluded because the mobility of Cu^{2+}-free LDL (Table II, row 1) was not different from that observed in the combined treatment (20 mM pyruvate plus 300 μM L-ascorbic acid) group in the presence of Cu^{2+}-treated LDL (Table III, last row).

Figure 4 shows that in co-incubations of LDL with Cu^{2+} plus macrophages, a low physiological dose of ≈50 μM L-ascorbic acid was a powerful antioxidant, judged from the improvement of cell viability and marked inhibition of TBARS formation (Fig. 4, panels A and B). However, even at concentration of 300 μM, L-ascorbic acid did not completely prevent cell death and lipid peroxidation in presence of oxLDL, consistent with the electrophoretic mobility data (Table III).

To find out whether the effects of α-keto-carboxylate and L-ascorbic acid were additive with respect to protection against cytotoxicity of oxLDL, we incubated the macrophages with LDL, Cu^{2+} and 10 mM (Fig. 5, left panel) or 20 mM pyruvate (Fig. 5, right panel) and varied L-ascorbic acid from subphysiological to supraphysiological concentrations (10–100 μM, Fig. 5). In pyruvate-free control runs with L-ascorbic acid (Fig. 4), 10 μM L-ascorbic acid alone was not protective in presence of oxLDL. However, as demonstrated in Fig. 5, 10 μM L-ascorbic acid showed protective efficacy against oxLDL when applied in combination with ≥10 mM pyruvate. Similarly, 100 μM L-ascorbic acid alone (Fig. 4, panel A) inhibited oxLDL cytotoxicity by only about 80%, not 100%. However, combining 100 μM

TABLE III Effects of pyruvate or L-ascorbic acid, alone or in combination on relative electrophoretic mobility of oxidatively modified low-density lipoprotein (LDL) particles

Culture protocols with LDL in presence of 5 μM Cu^{2+}	Relative electrophoretic mobility (fold of mobility for native LDL)
LDL alone	1.72 ± 0.04^a
5 mM pyruvate	1.68 ± 0.06^a
20 mM pyruvate	1.45 ± 0.05^b
50 μM L-ascorbic acid	1.66 ± 0.11^a
300 μM L-ascorbic acid	1.49 ± 0.04^b
5 mM pyruvate + 50 μM L-ascorbic acid	1.62 ± 0.08^a
5 mM pyruvate + 300 μM L-ascorbic acid	1.27 ± 0.06^c
20 mM pyruvate + 50 μM L-ascorbic acid	1.41 ± 0.06^b
20mM pyruvate + 300 μM L-ascorbic acid	1.12 ± 0.06^d

Data (means ± SEM) from four different LDL pools. LDL (1 mg cholesterol/ml) was incubated with 5 μM Cu^{2+} plus pyruvate in combination with L-ascorbic acid as indicated (24 h, cell-free system). Electrophoretic mobility expressed relative to that of native LDL. Values with different superscripts are significantly different from each other at $P < 0.05$. There was no noticeable effect on electrophoretic mobility when LDL was cultured with 50 μM L-ascorbic acid alone compared to that observed with Cu^{2+}-alone-stimulated LDL. However, the electrophoretic mobility was restored to that of native LDL when co-incubated with 20 mM pyruvate plus 300 μM L-ascorbic acid.

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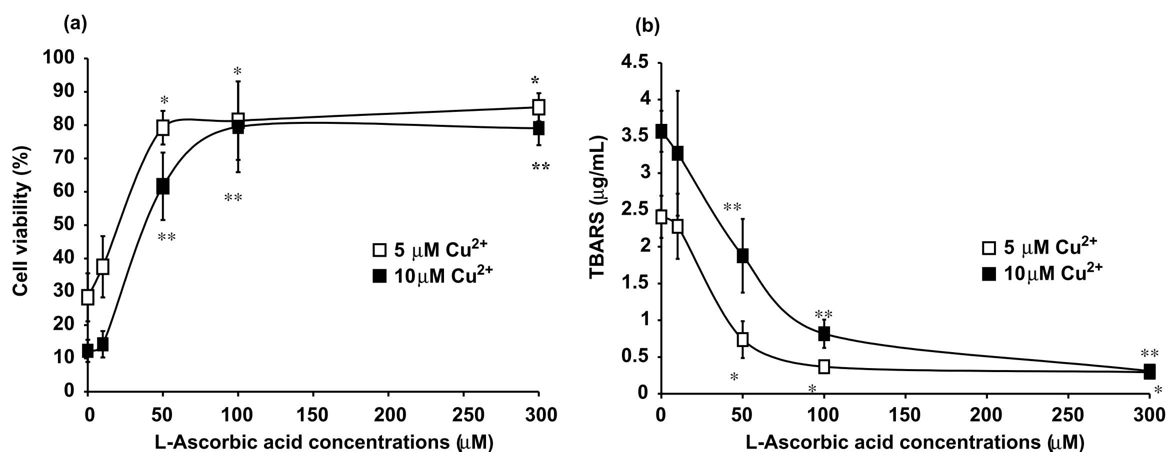

FIGURE 4 Effects of Cu^{2+}-induced oxidation of low-density lipoprotein (LDL) on dose-response relationships between L-ascorbic acid and THP-1 macrophage viability (panel A) or released TBARS (panel B) after 24 h. THP-1 (2×10^5 cells/ml) macrophages were pre-treated with or without various concentrations of L-ascorbic acid for 4 h and exposed to LDL (1 mg cholesterol/ml) in the presence of either 5 or 10 μM Cu^{2+}. The viability data are expressed as percent cell survival relative to the LDL- and Cu^{2+}-free time controls (means ± SEM of seven different experiments). TBARS was measured as malondialdehyde. Note that even at 300 μM L-ascorbic acid failed to fully protect macrophages from the toxicity of oxLDL, both in terms of macrophage viability and lipid peroxidation. *$P < 0.05$, relative to no L-ascorbic acid controls at 5 μM Cu^{2+}. **$P < 0.05$, relative to no L-ascorbic acid controls at 10 μM Cu^{2+}.

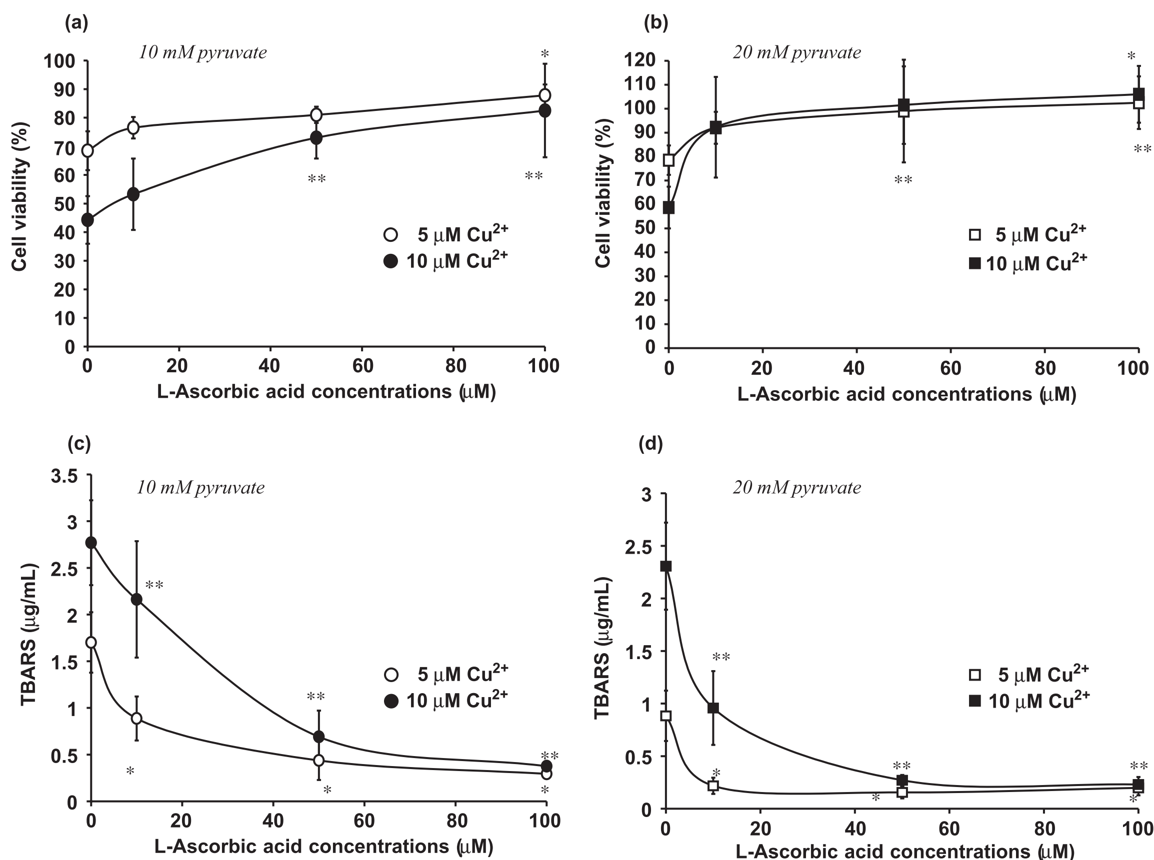

FIGURE 5 Synergistic protection by pyruvate and L-ascorbic acid against atherogenic Cu^{2+}-induced oxidized low-density lipoprotein (oxLDL), both in terms of cell viability (panel A, B) and formation of medium TBARS (panel C, D) from THP-1 (2×10^5 cells/ml) macrophages. Macrophages were cultured with either 10 or 20 mM pyruvate in F-10 medium plus 5% FBS containing L-ascorbic acid over the entire physiological range (10–100 μM) for 4 h, and then exposed to LDL (1 mg cholesterol/ml) in presence of either 5 or 10 μM Cu^{2+}. After 24 h macrophage viability and medium TBARS were determined. TBARS data (means ± SEM of four different experiments) expressed as μg malondialdehyde/ml, and viability expressed as percent of the time controls without LDL and Cu^{2+}. Neither millimolar pyruvate alone nor micromolar L-ascorbic acid alone did influence the cell viability. *$P < 0.05$, relative to no L-ascorbic acid controls at 5 μM Cu^{2+}. **$P < 0.05$, relative to no L-ascorbic acid controls at 10 μM Cu^{2+}.

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L-ascorbic acid with 20 mM pyruvate afforded full protection against cell death (Fig. 5, panels A and B) and, as expected, this protection was associated with extremely low levels of TBARS (Fig. 5, panels C and D).

DISCUSSION

The main findings of this study are that (1) micromolar amounts of Cu^{2+}, which are not cytotoxic *per se*, cause an oxidative modification of LDL particles that is highly cytotoxic to human THP-1 derived macrophages *in vitro*. (2) Millimolar pyruvate or α-keto-glutarate substantially attenuates but not fully protects native LDL against Cu^{2+}-induced oxidation, whereas physiological α-hydroxy-carboxylates (L-lactate) or linear aliphatic fatty acids (acetate, caprylate) are ineffective, if not even intensifiers of oxLDL formation. (3) Millimolar pyruvate also substantially improved macrophage viability in presence of oxLDL, but only ≥100 μM L-ascorbic acid protected macrophages by ≈80%, yet again not 100%. (4) The combined application of L-ascorbic acid plus pyruvate fully prevented Cu^{2+}-induced LDL oxidation and macrophage death. These findings highlight new antioxidant properties of pyruvate or related α-keto-carboxylates reported here for the first time. Although these data were derived from *in vitro* experiments, it is tempting to speculate that they might have metabolic significance for the *in vivo* situation and have pharmacological implications for strategies aimed at inhibiting atherosclerosis.

The antioxidant properties of α-keto-monocarboxylates and their anti-atherogenic potential was ascertained by measuring electrophoretic mobility of LDL and the production of membrane peroxidative products when added Cu^{2+} was used to oxidize LDL in a cell-free system. Conversely, in the cell-containing systems the THP-1 macrophages minimally oxidized native LDL in the absence of added Cu^{2+}. This residual LDL oxidation could be due to trace Cu^{2+} content of the F-10 incubation medium. However, only when the macrophages were co-incubated with added micromolar Cu^{2+} plus LDL, the lipid peroxidation was strongly stimulated. Our findings indicated that lipid peroxidation occurred both at the level of the LDL particles (see below) and that of the live macrophages. We observed that the atherogenic parameters were related to cellular viability clearly demonstrating direct relationships among oxLDL formation, decreased macrophage viability and increased lipid peroxidation (Fig. 1). This quantitative approach also produced new evidence that pyruvate and α-keto-glutarate appear to have the potential to reduce spontaneous LDL oxidation and in turn protect macrophages against peroxidative toxicity. Consequently, pyruvate and possibly other related α-keto-carboxylates appear to qualify as anti-atherogenic agents with respect to oxLDL formation and subsequent uptake by macrophages. However, we noted that even extremely high concentrations of pyruvate (20 mM) did not fully maintain the viability of the macrophages in presence of oxLDL (Fig. 3).

Pyruvate and especially α-keto-glutarate exhibited distinct antioxidant effects with respect to native LDL even in cell-free environments. It is not known whether this property to stabilize LDL in presence of Cu^{2+} *in vitro* translates into true anti-atherogenicity *in vivo*. On the other hand, the α-hydroxy-carboxylate L-lactate and the aliphatic short/medium chain fatty acids acetate or caprylate did not inhibit Cu^{2+}-induced LDL oxidation; in fact, based on the electrophoretic mobility data of Table II, L-lactate and fatty acids appeared to promote Cu^{2+}-induced oxLDL formation. Again, it is not clear from our current study whether this adverse property of L-lactate and fatty acids to destabilize native LDL *in vitro* translates into true atherogenicity *in vivo* by chronic lactemia and/or hyperlipidemia (i.e. high levels of serum FFA). Nevertheless, the obvious difference in native LDL protection between α-keto-carboxylates and α-hydroxy-carboxylates points to the α-keto configuration as a key intramolecular requirement for a direct non-enzymatic antioxidant efficacy of natural intermediates such as pyruvate, α-keto-glutarate and perhaps acetoacetate and congeners.[15]

Inhibition of Cu^{2+}-induced LDL oxidation by α-keto-carboxylates could be due to decreased Cu^{2+} binding to LDL, a mechanism that appears to be effective with L-ascorbic acid protection.[32] Pyruvate and related α-keto-carboxylates have been discussed to directly inhibit membranous enzyme systems such as NADH oxidase,[16] cyclooxygenase,[33] and cytochrome c oxidase,[34] i.e. systems that are involved in the initiation or propagation of peroxidative products/processes. In addition, since pyruvate markedly attenuated the cytotoxicity of oxLDL towards the macrophages and also enhanced the antioxidant effects of subphysiological doses of L-ascorbic acid, it appeared likely that the mechanism of pyruvate protection of macrophages plus LDL was at least in part related to cellular antioxidant metabolism. A combination of the following known effects of millimolar pyruvate may have boosted the macrophages antioxidant defenses: (a) increased GSH/GSSG ratio,[17] (b) reduced cytosolic $NADH/NAD^+$ ratio with inhibition of NADH oxidase,[16] (c) increased mitochondrial anaplerosis, (d) increased formation of mitochondrial and antiapoptotic *bcl-2* (unpublished observations).

L-Ascorbic acid is a powerful reductant of metal ions and one of the major natural antioxidants that

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directly scavenge or quench aqueous reactive oxygen species; it also protects LDL particles against atherogenic, i.e. oxidative modification by Cu^{2+}.[12,27,35] However, it has also been realized that L-ascorbic acid can be pro-oxidant by generating hydroxyl radicals in the presence of free metal ions *in vitro*.[36,37] Nevertheless, much like millimolar pyruvate, 100–300 μM L-ascorbic acid afforded strong yet incomplete protection of macrophages against oxLDL. Interestingly, the macrophage protection by L-ascorbic acid against oxLDL was enhanced by co-treatment with pyruvate, even when subphysiological doses of L-ascorbic acid were used (Fig. 5). The exact mechanisms of this synergism are presently unknown, but may involve direct intracellular and extracellular oxyradical scavenging combined with improved antioxidant defenses due to intensified pyruvate metabolism of the macrophages.

In summary, millimolar pyruvate as an antioxidant can directly inhibit Cu^{2+}-catalyzed LDL oxidation, a property related to the α-keto-carboxylate intramolecular configuration. In distinct contrast, the α-hydroxy-carboxylate intramolecular configuration (L-lactate) and linear aliphatic monocarboxylates appear ineffective. Neither pyruvate alone nor L-ascorbic acid alone provides full protection against the cytotoxicity of oxLDL in human macrophage systems. However, the combined use of L-ascorbic acid and pyruvate fully stabilized native LDL and prevented macrophage death with minimal signs of lipid peroxidation. Whether such or similar α-keto-carboxylate compositions may attenuate the oxLDL-triggered atherosclerotic process *in vivo* remains to be investigated. Furthermore, possible anti-atherogenic strategies of pyruvate and α-keto-carboxylate congeners remain to be delineated, based on inhibiting clinically and pharmacologically cholesterol metabolism.

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