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The oxidation of low density lipoprotein by cells or iron is inhibited by zinc

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Abstract

We have examined the effect of zinc ions on low density lipoprotein (LDL) oxidation by macrophages, endothelial cells and iron ions in terms of the increased uptake of the LDL by macrophages. Zinc ions inhibited LDL modification by both cell types (which is dependent on the presence of iron ions in the culture medium) and by iron ions alone. As oxidised LDL is believed to be involved in atherogenesis, this raises the possibility that zinc may be an endogenous protective factor against atherosclerosis.

Key words: Atherosclerosis; Endothelial cell; Iron; Low density lipoprotein; Macrophage; Zinc

1. Introduction

The role of low density lipoprotein (LDL) oxidation in the pathogenesis of atherosclerosis is currently attracting a great deal of interest, with evidence for such a role accumulating from immunocytochemical and biochemical studies [1]. It has also been shown that antioxidants that prevent LDL oxidation in vitro also inhibit lesion development in hypercholesterolaemic animals [1], including primates [2]. All four cell types found in atherosclerotic lesions (endothelial cells, smooth muscle cells, macrophages and lymphocytes) are capable of oxidising LDL in vitro [3–6].

The trace element zinc has been shown to have antioxidant properties both in vitro and in vivo [7,8]. Kok et al. [9] have suggested that low serum levels of zinc may be associated with an increased risk of cardiovascular disease. It has been reported that zinc administration inhibits lesion development in cholesterol-fed rabbits, but the zinc decreased the serum cholesterol levels by 78% in this study and hence no conclusion can be drawn about any possible effects that zinc may have had at the level of the arterial wall [10]. Hooper et al. [11] have reported that pharmacological levels of zinc decrease the plasma high density lipoprotein–cholesterol levels in humans, whereas Shah et al. [12] have reported that zinc increases them. Klevay [13] has suggested that a high Zn/Cu ratio in the diet may be atherogenic, and the zinc level and zinc/copper ratio in hair have been shown to be higher in myocardial infarction patients than in control subjects [14]. There is therefore great uncertainty as to whether zinc is pro-atherogenic or anti-atherogenic.

In the present study, we have tested the effect of zinc ions on LDL oxidation by macrophages, endothelial cells or ferrous ions in the presence of a reduced thiol (cysteine). Under the conditions used, zinc strongly inhibited LDL oxidation by both cell types and by ferrous ions/cysteine.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM), foetal calf serum (FCS), gentamicin solution (10 mg/ml), penicillin/streptomycin solution (5,000 IU/5,000 μg per ml), amphotericin B solution (250 μg/ml) and glutamine solution (200 mM) were purchased from Gibco, Uxbridge, Middlesex, UK. Ham's F-10 medium and Nunc (25 cm^2) culture flasks were from ICN Flow, High Wycombe, Bucks., UK. Ferrous sulphate and Phenol red solution (0.5% (w/v)) were from Sigma Chemical Co., Poole, Dorset, UK, zinc sulphate (Analar) was from BDH Chemicals, Poole, Dorset, UK and Costar twelve-well cluster plates (22 mm diameter wells) were purchased from Northumbria Biologicals Ltd., Cramlington, Northumbria, UK.

2.2. Isolation of LDL and radioiodination

LDL (d = 1.019–1.063 g/ml) was isolated from the blood of healthy volunteers by sequential density ultracentrifugation, as previously described [15], in the presence of EDTA. LDL (2 mg protein) was then labelled with ^{125}I using an iodine monochloride method [16]. The radioiodinated LDL was mixed with non-iodinated LDL to obtain a preparation of specific activity 20–40 cpm/ng protein, and diluted to 2 mg protein/ml with buffer containing 100 μM EDTA. This concentration of ^{125}I-labelled LDL was standardised so as to give 5 μM EDTA during LDL modification at 100 μg protein/ml, as EDTA can either inhibit or promote LDL oxidation depending on its concentration [17]. LDL preparations were no longer used 1 month after isolation.

2.3. Isolation and culture of mouse peritoneal macrophages

Resident peritoneal macrophages were isolated from female Swiss T.O. mice (Tuck & Son, Battlesbridge, Essex, UK) by peritoneal lavage

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Abbreviations: DMEM, Dulbecco's modified Eagle's medium; EDTA, ethylenediamine tetraacetic acid; FCS, foetal calf serum; LDL, low density lipoprotein.

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[18]. The cells were plated at 1.5×10^6 peritoneal cells per well and incubated at 37°C under 5% CO_2 for 4 h to allow macrophage adherence, then washed with DMEM to remove lymphocytes before use in experiments.

2.4. Isolation and culture of endothelial cells

Bovine aortic endothelial cells were isolated and cultured as previously described [19] in 25 cm^2 culture flasks. The cells were subcultured into 22 mm wells and used to oxidise LDL.

2.5. Modification of LDL

^{125}I-Labelled LDL (100 μg protein/ml) was incubated for the specified time (16–22 h) at 37°C under 5% CO_2 with modifying macrophages (macrophage-modified LDL), or with confluent wells of bovine aortic endothelial cells (EC-modified LDL), or with 500 μM freshly dissolved cysteine or for the appropriate time in medium alone (control LDL). The incubation medium (0.75 ml/well) consisted of Ham's F-10 medium supplemented with additional 3 μM $FeSO_4$, Phenol red (12 μg/ml) and gentamicin (50 μg/ml). After incubation for the specified time, the medium was collected, centrifuged (13,000 rpm for 2.5 min in a MSE Microcentaur) to remove any detached cells, and then used for measurement of LDL uptake.

2.6. Measurement of modified LDL uptake

J774 mouse macrophage-like cells were plated at 1.5×10^5 cells/well and used the following day to measure LDL uptake. Modified or control LDL was diluted to 10 μg protein/ml in DMEM containing 20% (v/v) heat-inactivated FCS, penicillin/streptomycin (10 IU/10 μg per ml), amphotericin B (1 μg/ml) and glutamine (2 mM). The LDL was then incubated for 22–24 h with J774 cells or cell-free wells (1 ml/well) and its rate of degradation was determined by the measurement of radioactive non-iodide trichloroacetic acid-soluble degradation products [15]. The J774 cells were washed, lysed with 0.2 M NaOH and the cell protein and cell-associated radioactivity determined [16]. The low amounts of radioactivity remaining in the cell-free wells were subtracted from the cell-associated radioactivity. Values for degraded LDL and cell-associated LDL were combined to give total LDL uptake [20].

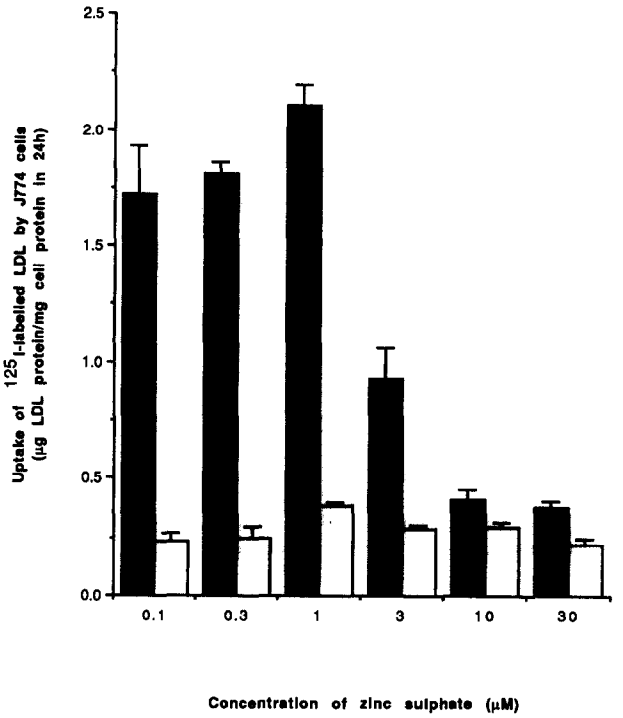

Fig. 1. The effect of zinc ions on macrophage-mediated LDL oxidation. ^{125}I-Labelled LDL (100 μg protein/ml) was incubated for 16 h with macrophages (the adherent cells from 1.5×10^6 peritoneal cells) (black bars) or cell-free wells (white bars) in Ham's F-10 medium with or without additional zinc ions (baseline = 0.1 μM). The LDL was then diluted to 10 μg protein/ml in serum-containing medium and its rate of uptake by J774 cells determined. Data are mean ± S.E.M. (n = 3 wells of 'modifying' macrophages), and are representative of 3 experiments.

3. Results

Ham's F-10 medium, as obtained from ICN Flow, contains zinc sulphate ($ZnSO_4 \cdot 7H_2O$) at 28.8 μg/l; hence all these studies have 0.1 μM as the base level of Zn^{2+}.

When the Zn^{2+} concentration was increased, a dose-dependent reduction in LDL modification by macrophages, as measured by LDL uptake by other macrophages, was observed, with a 50% reduction occurring at approximately 3 μM Zn^{2+} and a near total inhibition at 10 μM (Fig. 1). In other experiments, using a narrower range of zinc concentrations, the modification of LDL was reduced by 50% at 4–5 μM Zn^{2+}.

Similarly, when the Zn^{2+} concentration in incubations with bovine aortic endothelial cells was increased, a dose-dependent reduction in LDL modification was observed (Fig. 2). The concentration of Zn^{2+} required for 50% reduction of LDL modification was between 1 and 3 μM, and at 7.5 μM Zn^{2+} modification was totally inhibited. The modification of LDL by porcine aortic endothelial cells was similarly inhibited by zinc (data not shown).

The addition of a thiol (500 μM freshly dissolved cysteine) to Ham's F-10 medium, supplemented with $FeSO_4$ to raise the iron concentration to 6 μM, produced a substantial modification of LDL, as found previously by Parthasarathy [21]. When the concentration of Zn^{2+} was increased above the base level of 0.1 μM, a dose-dependent reduction of LDL was observed, with a 50% reduction between 3 and 10 μM (Fig. 3).

4. Discussion

Zinc is an essential trace element, required at about 15 mg/day [7], which has been proposed to have antioxidant properties both in vitro and in vivo [7]. It has been suggested that low serum zinc levels may result in a predisposition to cardiovascular disease [9], but this is highly controversial. We report here that, under the conditions used, zinc ions effectively inhibit the oxidation of LDL by macrophages, endothelial cells or iron/cysteine.

Iron ions have been shown to bind to LDL in 0.15 M NaCl in a form that is not removable by dialysis against 0.15 M NaCl [22]. Iron ions can also peroxidase lipid micelles derived from LDL as efficiently as intact LDL [23], suggesting that iron binding to the lipid region of the LDL particle (presumably the 'head groups' of the phospholipids) is important. The antioxidant activity of zinc ions, which are not redox active, in other oxidation

systems has been postulated to be due to displacement of copper or iron ions from their binding sites [7,8]. This would explain the inhibition we have observed with zinc or LDL oxidation by cells (which is dependent on iron in the medium [24]) and by Fe^{2+}/cysteine, since the iron may be displaced from its binding sites on LDL.

LDL oxidation by cells has an absolute requirement for cysteine [25]. Cysteine forms fairly strong complexes with iron, which may then react with molecular oxygen to form reactive oxygen species during the redox cycling of iron [26]. Zinc has been shown to inhibit the generation of active oxygen species in this system [26], probably by displacement of iron from cysteine, since cysteine has a higher affinity for zinc than for iron [27]. Hence, in our system Zn^{2+} may displace iron from LDL or cysteine, resulting in the inhibition of LDL oxidation.

Zinc is present in human atherosclerotic lesions [28] and is contained in plasma at a level of 15 μM, although almost all of it is bound to proteins (mainly α_2-macroglobulin and albumin), with the free plasma zinc totalling $\leq$ 15 nM [29]. LDL oxidation is thought to occur locally in the arterial wall, however, where iron and copper may be released from their normal sequestered states

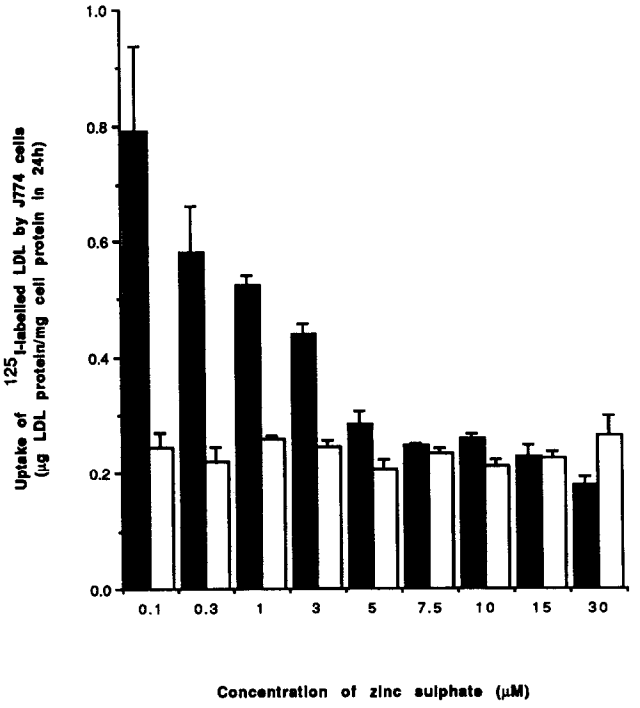

Fig. 2. The effect of zinc ions on LDL oxidation by bovine aortic endothelial cells. ^{125}I-Labelled LDL (100 μg protein/ml) was incubated for 22 h with confluent monolayers of cells (subculture number 10) (in 22 mm diameter wells) (black bars) or cell-free wells (white bars) in Ham's F-10 medium with or without additional zinc ions (baseline = 0.1 μM). The LDL was then diluted to 10 μg protein/ml in serum-containing medium and its rate of uptake by J774 cells determined. Data are mean ± S.E.M. (n = 3 wells of endothelial cells), and are representative of 7 experiments.

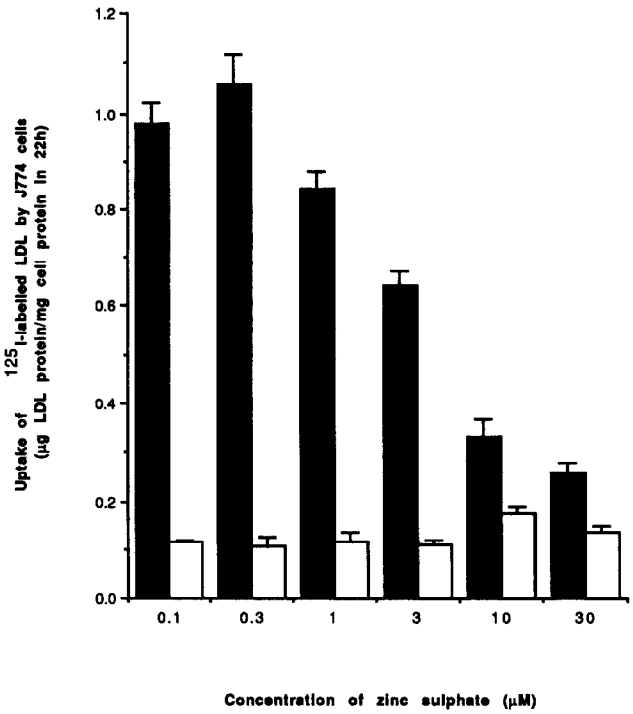

Fig. 3. The effect of zinc ions on iron/cysteine-mediated LDL oxidation. ^{125}I-Labelled LDL (100 μg protein/ml) was incubated for 18 h in Ham's F-10 medium supplemented with additional 3 μM $FeSO_4$ and 500 μM cysteine (black bars) or 3 μM $FeSO_4$ alone (white bars) with or without additional zinc ions (baseline = 0.1 μM). The LDL was then diluted to 10 μg protein/ml in serum-containing medium and its rate of uptake by J774 cells determined. Data are mean ± S.E.M. (n = 3 wells containing $FeSO_4$/cysteine). The results were confirmed in another experiment.

and generate an oxidative stress [1]. In such an oxidative microenvironment, 'free' zinc may possibly be released from plasma proteins or cellular proteins from damaged cells. Metallothionein, for instance, is known to release its bound metals under conditions of oxidative stress [30].

The inhibitory effect of zinc on LDL oxidation by iron-dependent processes at pH 7.4, both cell-free and cell-dependent, raises the possibility that zinc may act as an endogenous protective agent against atherogenesis. Much remains to be done, however, before we can 'decide' whether zinc is pro- or anti-atherogenic in humans, especially as we have very recently found that zinc can actually promote LDL oxidation by iron/cysteine at acidic pH (unpublished data).

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