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Acidic pH enables caeruloplasmin to catalyse the modification of low-density lipoprotein

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Abstract

LDL oxidation within the arterial wall may contribute to the disease of atherosclerosis. There is some evidence that elevated plasma levels of copper are associated with an increased risk of coronary artery disease. We have investigated the conditions under which caeruloplasmin (the plasma copper carrier protein) can catalyse the macrophage-mediated modification of LDL. Low concentrations of $CuSO_4$ (< 1 μM) could catalyse the macrophage-mediated modification of LDL. Native caeruloplasmin was unable to catalyse the modification of LDL at pH 7.4, but could do so after preincubation at acidic pH. After preincubation at acidic pH, concentrations of caeruloplasmin as low as 30 μg/ml (about one-tenth of the human plasma level) could catalyse significant LDL oxidation when added to macrophages. The activation of copper in caeruloplasmin in atherosclerotic lesions due to a localised acidic pH may help to explain why LDL oxidation occurs in these areas of the body.

Key words: Acidic pH; Atherosclerosis; Caeruloplasmin; Copper; Low-density lipoprotein; Macrophage; Oxidation

1. Introduction

The oxidation of low-density lipoprotein (LDL) in the arterial wall resulting in its rapid uptake by macrophages and their conversion into foam cells, the migration of monocytes into the arterial wall, cytotoxicity and many other effects may play an important role in the pathogenesis of atherosclerosis [1].

Caeruloplasmin has been recognised as the major copper-containing component of mammalian plasma since its isolation in 1947. Over 90% of the copper in plasma is generally believed to be present as an integral part of this protein. It is a glycoprotein of relative molecular mass 132,000 which contains 6 or 7 copper atoms per molecule [22]. Two of the copper sites are occupied by paramagnetic type-I copper atoms which endow the protein with its characteristic blue colour. The remaining coppers are paramagnetic type-II and diamagnetic type-III coppers [3]. These copper atoms are nondialysable [4] and nonexchangeable with copper atoms in serum [5]. The oxidation of lipoproteins in extracellular fluids during storage may possibly be due to caeruloplasmin promoting their oxidation [6].

A recent study in Indian men reported that those with coronary artery disease had 44% higher levels of caeruloplasmin than the controls, and that those with unstable angina possessed higher levels than those with stable angina [7]. Caeruloplasmin is an acute phase protein and the increased levels may possibly be due to the stress produced by angina or myocardial infarction [8]. In a recent prospective study in Finnish men, however, an elevated serum copper level was found to correlate to an increased progression of carotid atherosclerotic lesions [9]. In addition, an association between high serum copper levels and cardiovascular mortality was shown in a prospective case-control study in Dutch men [10]. Another recent study has shown that high serum caeruloplasmin levels correlate with an increased future risk of myocardial infarction [11]. Another study in Finnish men found an association between high copper content in drinking water and high coronary heart disease mortality [12].

We have investigated the ability of caeruloplasmin, of which plasma levels can apparently be a risk factor for coronary artery disease, to express catalytically active copper ions that can catalyse macrophage-mediated LDL modification.

2. Materials and methods

2.1. Isolation and radioiodination of LDL

LDL (1.019–1.063 g/ml) was isolated from normal pooled human blood by sequential density ultracentrifugation in KBr solutions at 4°C as described elsewhere [13] and radiolabelled with Na ^{125}I using iodine monochloride [14].

2.2. Caeruloplasmin preincubation

Caeruloplasmin was obtained from Calbiochem (Nottingham, UK). It had an A_{610}/A_{280} ratio of 0.048 (this is a spectrophotometric indication

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Abbreviations: DMEM, Dulbecco's modified Eagle's medium; LDL, low-density lipoprotein; PBS, phosphate-buffered saline.

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of the ratio of copper atoms to protein) which is consistent with an intact copper–protein complex [15,16] and ran largely as a single band on an SDS-polyacrylamide gel, with a molecular weight of approximately 132,000 kDa. Caeruloplasmin (3 mg/ml) was preincubated at 37°C in either a 50 mM sodium phosphate buffer (pH 5.0–7.4) or a 50 mM sodium acetate buffer (pH 4.5) for 24 h. It was then diluted 1 in 100 to give a final concentration of 30 μg caeruloplasmin/ml in Ham's F-10 medium specially formulated to be deficient in $FeSO_4$, $CuSO_4$ and $ZnSO_4$ (Irvine Scientific, Santa Anna, USA).

2.3. Modification of LDL by macrophages

Resident mouse peritoneal macrophages were isolated by peritoneal lavage of female Swiss T.O. mice as described elsewhere [14]. ^{125}I-labelled LDL (100 μg protein/ml) was incubated in triplicate with intact macrophages (1.25×10^6 peritoneal cells/ml) or cell-free wells in triplicate with either 30 μg native caeruloplasmin/ml, 30 μg preincubated caeruloplasmin/ml or $CuSO_4$ (up to 2 μM) for 18 h at 37°C in Ham's F-10 medium specially formulated to be deficient in $FeSO_4$, $CuSO_4$ and $ZnSO_4$.

2.4. Determination of oxidised LDL degradation by mouse peritoneal macrophages

Modified or control ^{125}I-labelled LDL was diluted to 10 μg protein/ml in DMEM containing 20% (v/v) foetal calf serum, 10 μg gentamicin/ml, 10 μg streptomicin/ml, 10 I.U. penicillin/ml and 5 μg amphotericin B/ml (Gibco, Middlesex, UK). It was then incubated for 20–22 h with a second set of macrophages (1×10^6 mouse peritoneal cells/well) or cell-free wells with 1 ml per well. The radioactive noniodide, trichloroacetic acid-soluble degradation products released into the medium were measured as described previously [13]. The degradation products in the cell-free wells were subtracted from those in the wells containing cells. The cells were washed in PBS containing Ca^{2+} and Mg^{2+}, lysed in 0.2 M NaOH as described elsewhere [14] and assayed for protein by a modified Lowry procedure [17].

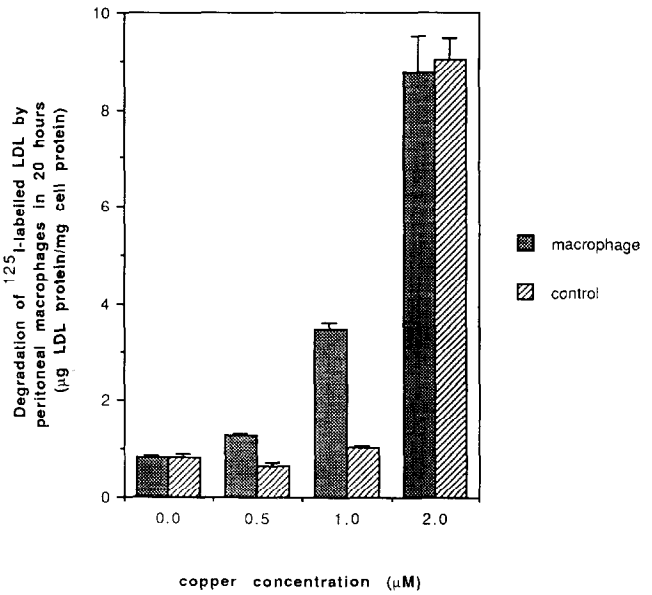

Fig. 1. The effect of increasing concentrations of $CuSO_4$ on macrophage-mediated LDL modification. ^{125}I-labelled LDL (100 μg protein/ml) was incubated for 18 h at 37°C with mouse peritoneal macrophages (1.25×10^6 peritoneal cells/ml) or in cell-free wells in Ham's F-10 medium specially formulated to be deficient in $FeSO_4$, $CuSO_4$ and $ZnSO_4$. Increasing concentrations of $CuSO_4$ were added from a stock 1 mM aqueous solution. The rate of degradation of the macrophage-modified LDL (solid histobars) and control LDL (hatched histobars) by a second set of mouse peritoneal macrophages was then determined. Each point is the mean ± S.E.M. of three wells.

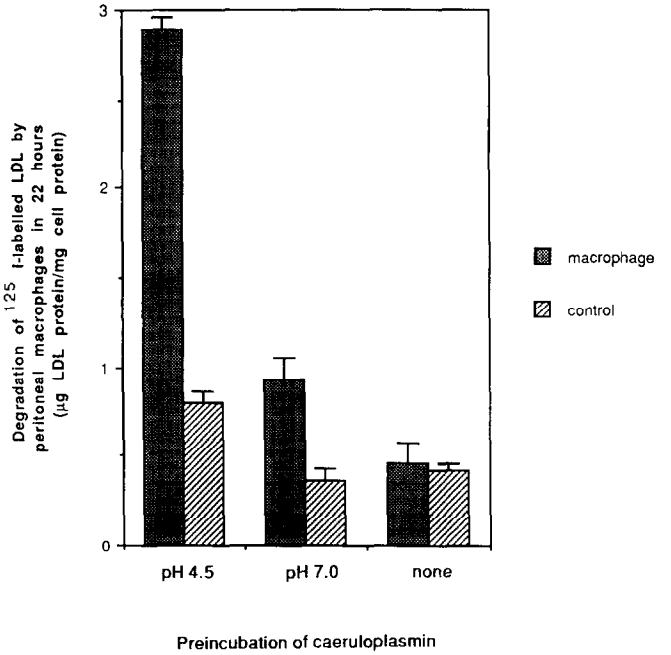

Fig. 2. The effect of preincubating caeruloplasmin at acidic pH in an acetate buffer on its ability to catalyse the modification of LDL by macrophages. Caeruloplasmin (3 mg/ml) was preincubated in a sodium acetate buffer, pH 4.5 or 7.0 for 24 h at 37°C. ^{125}I-labelled LDL (100 μg protein/ml) was incubated for 18 h at 37°C with mouse peritoneal macrophages (1.25×10^6 peritoneal cells/ml) or in cell-free wells in Ham's F-10 medium specially formulated to be deficient in $FeSO_4$, $CuSO_4$ and $ZnSO_4$. Preincubated or native caeruloplasmin was added to the macrophages at a final concentration of 30 μg/ml. The rate of degradation of the macrophage-modified LDL (solid histobars) and control LDL (hatched histobars) by a second set of macrophages was then determined. Each point is the mean ± S.E.M. of 3 wells.

3. Results

^{125}I-labelled LDL was incubated with mouse peritoneal macrophages in Ham's F-10 medium deficient in iron, copper and zinc ions in the presence of either $CuSO_4$, native caeruloplasmin or caeruloplasmin that had been preincubated at various pH values. The rate of degradation of these LDLs by a second set of mouse peritoneal macrophages was then determined.

The effect of increasing concentrations of $CuSO_4$ on LDL modification by macrophages is shown in Fig. 1. There was no apparent LDL modification in the absence of $CuSO_4$, but a progressive increase in LDL modification by the macrophages was observed as the concentration of $CuSO_4$ was increased. A concentration of 0.5 μM $CuSO_4$ resulted in the macrophages modifying the LDL so that it was degraded by a second set of macrophages around twice as fast as the control LDL. Increasing the $CuSO_4$ concentration to 1 μM resulted in the LDL being degraded by the macrophages around 3–4 times as fast as the control LDL. When 2 μM $CuSO_4$ was used, LDL incubated with macrophages and in cell-free wells was modified to a large and similar extent so that it was

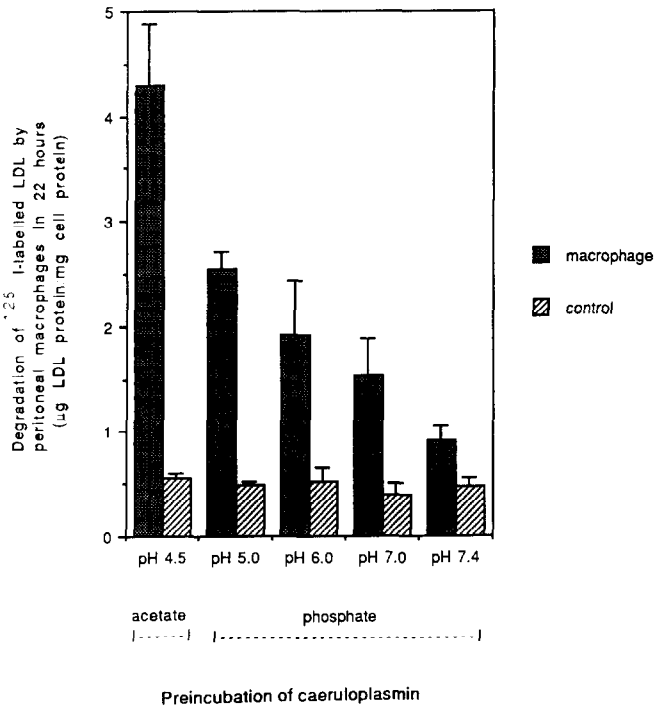

Fig. 3. The effect of preincubating caeruloplasmin at acidic pH in a phosphate buffer on its ability to catalyse the modification of LDL by macrophages. Caeruloplasmin (3 mg/ml) was preincubated in a sodium phosphate buffer, pHs 5.0, 6.0, 7.0 and 7.4, and in a sodium acetate buffer pH 4.5 for 24 h at 37°C. ^{125}I-labelled LDL (100 μg protein/ml) was incubated for 18 h at 37°C with mouse peritoneal macrophages (1.25×10^6 peritoneal cells/ml) or in cell-free wells in Ham's F-10 medium specially formulated to be deficient in $FeSO_4$, $CuSO_4$ and $ZnSO_4$. Preincubated caeruloplasmin was added to the macrophages at a final concentration of 30 μg/ml. The rate of degradation of the macrophage-modified LDL (solid histobars) and control LDL (hatched histobars) by a second set of mouse peritoneal macrophages was then determined. Each point is the mean ± S.E.M. of 3 wells.

degraded by a second set of macrophages around 9–10 times faster than unmodified LDL.

The macrophages were apparently unable to modify the LDL in the presence of native caeruloplasmin (30 μg/ml). Preincubation of caeruloplasmin in a sodium acetate buffer at pH 4.5, however, altered it in some way so that it became able to catalyse the macrophage-mediated modification of LDL, so that the LDL was degraded much faster by a second set of macrophages (Fig. 2). When the caeruloplasmin was preincubated in a sodium acetate buffer at pH 7.0, it became able to catalyse the oxidation of LDL by macrophages but to a much lesser extent than after preincubation at pH 4.5. Caeruloplasmin preincubated at pH 4.5, but not at pH 7.0, was able to modify LDL to a small extent in the absence of macrophages in this experiment, so that it was degraded faster than LDL incubated with native caeruloplasmin (Fig. 2), but this was not a consistent finding (Fig. 3). Caeruloplasmin was also preincubated in a sodium acetate buffer at pH 4.5 and in a sodium phosphate buffer at pHs 5.0, 6.0, 7.0 and 7.4 (Fig. 3). After preincubation at pH 4.5 in the acetate buffer, the caeruloplasmin was able to catalyse the macrophage-mediated modification of LDL so that it was degraded by a second set of macrophages 7–8 times faster than control LDL. A progressive decrease in macrophage-mediated LDL modification was observed as the pH of the caeruloplasmin preincubation step was increased, so that at pH 7.4 the macrophage-modified LDL was degraded only about twice as fast as the control LDL.

Caeruloplasmin was preincubated at pH 4.5 in a sodium acetate buffer and added in increasing concentrations to mouse peritoneal macrophages and LDL (Fig. 4). In the absence of caeruloplasmin, the macrophages were apparently unable to modify the LDL. A progressive increase in macrophage-mediated LDL modification was observed as the concentration of caeruloplasmin was increased. At a concentration of 30 μg/ml, the preincubated caeruloplasmin was able on this occasion to catalyse LDL modification by macrophages so that the LDL was degraded around 3 times faster than LDL incubated with macrophages in the absence of caeruloplasmin. As the concentration of preincubated caeruloplasmin was increased there was a progressive increase

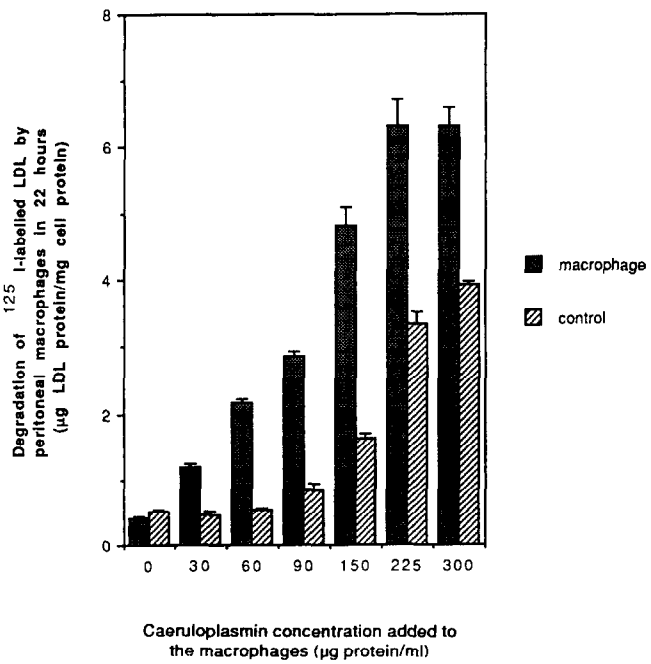

Fig. 4. The effect of increasing the concentration of preincubated caeruloplasmin on the macrophage-mediated modification of LDL. Caeruloplasmin (30 mg/ml) was preincubated in a sodium acetate buffer at pH 4.5 for 24 h at 37°C. ^{125}I-labelled LDL (100 μg protein/ml) was incubated for 18 h at 37°C with mouse peritoneal macrophages (1.25×10^6 peritoneal cells/ml) or in cell-free wells in Ham's F-10 medium specially formulated to be deficient in $FeSO_4$, $CuSO_4$ and $ZnSO_4$. Preincubated caeruloplasmin was added to the macrophages in increasing concentrations up to 300 μg/ml. The rate of degradation of the macrophage-modified LDL (solid histobars) and control LDL (hatched histobars) by a second set of mouse peritoneal macrophages was then determined. Each point is the mean ± S.E.M. of 3 wells.

in the modification of LDL by macrophages so that at 225 μg/ml and over the LDL was degraded around 16 times faster than LDL incubated with macrophages alone. The degradation of control LDL that had been incubated in cell-free wells was not increased until the caeruloplasmin concentration reached 90 μg/ml, when there was a small increase in the degradation of the control LDL. There was then a progressive increase in the degradation of control LDL as the concentration of caeruloplasmin was increased so that at a concentration of 300 μg/ml the control LDL was degraded 7–8 times faster than control LDL that had been incubated in the absence of caeruloplasmin. In all cases when preincubated caeruloplasmin was present, however, LDL was modified faster when macrophages were present than when they were absent.

4. Discussion

The mechanism by which LDL becomes oxidised to an atherogenic form in the arterial wall is as yet unknown, but seems likely to be dependent on the presence of catalytically active transition metal ions. There is some evidence that elevated plasma copper levels are associated with coronary artery disease [7,9–11]. In addition, canine aortas contain around 6 μmol copper atoms/kg wet issue [18] and human atherosclerotic plaques have been found to contain 0.11 g of caeruloplasmin/kg dry tissue (equivilant to 5.5 μmol copper atoms/kg dry tissue) [19]. Gruel taken from human advanced atherosclerotic lesions has been shown to contain up to 28 μM catalytically active copper ions [20].

We have shown here that low levels of $CuSO_4$ can catalyse the macrophage-mediated modification of LDL to increase its rate of degradation by a second set of macrophages. The macrophages were apparently unable, however, to modify the LDL in the presence of native caeruloplasmin. This suggests that the copper atoms are tightly bound in caeruloplasmin and are unable to redox cycle in a way that can catalyse LDL oxidation. When caeruloplasmin was preincubated at acidic pH prior to incubation with LDL and macrophages, however, it became able to catalyse the modification of LDL by macrophages. This effect was found to be pH dependent so that as the pH was lowered, the catalytic ability of the caeruloplasmin was increased progressively. This may possibly represent sequential release or activation of the different types of copper atoms in caeruloplasmin; type-I copper being more labile than the others [3] may be released or activated at mildly acidic pH whereas the more stable types of copper may be released or activated only at more acidic pH values. Preincubation of caeruloplasmin at pH 4.5 did not result in any apparent degradation of the protein as estimated by SDS-polyacrylamide gel electrophoresis (data not shown).

There appears to be a fairly large difference between preincubation at pH 4.5 and 5.0 on the catalytic abilities of caeruloplasmin (Fig. 3). This possibly reflects differences between phosphate and acetate buffers. It was not possible to find a buffer system that had a pH range of 4.5–7.4, was not toxic to macrophages, did not chelate copper ions and did not contain components possessing antioxidant properties, hence two systems were used to create the pH range desired. It is possible that in a phosphate buffer at acidic pH, some of the copper ions released into the medium were forming inactive complexes with phosphate thereby reducing the number of active copper ions able to catalyse the modification of LDL and hence limiting the overall degree of LDL modification. It was observed that adding saturated copper sulphate to these phosphate buffers resulted in visibly increasing precipitation as the pH of the buffer was lowered. This did not occur in the acetate buffer. This possibly explains the large difference between the extents of LDL oxidation at pH 4.5 (acetate) and pH 5.0 (phosphate) shown in Fig. 3.

The normal plasma concentration of caeruloplasmin is about 300 μg/ml [2]. The concentration of caeruloplasmin used throughout these experiments was 30 μg/ml, which is just 10% of the normal plasma level. This concentration of caeruloplasmin would contain 1.5 μM copper atoms (assuming an average of 6.5 copper atoms per molecule of caeruloplasmin based on its A_{610}/A_{280} ratio) which is more than sufficient in terms of 'free' copper ions to catalyse the modification of LDL in the presence of macrophages in this system (Fig. 1). Increasing the concentration of caeruloplasmin that had been preincubated at acidic pH resulted in a progressive increase of both macrophage-mediated and cell-free LDL modification, so that at a concentration of 300 μg caeruloplasmin/ml (the normal plasma concentration) both macrophage-incubated and cell-free LDL were extensively modified, probably to a maximal extent in the case of macrophage-incubated LDL since the degree of degradation of the macrophage-modified LDL appeared to have plateaued. Using the figure of 0.11 g of caeruloplasmin/kg dry tissue for human atherosclerotic plaques [19], it can be calculated that the extracellular fluid in these lesions should contain a sufficient concentration of caeruloplasmin to cause considerable LDL oxidation if the copper were to become catalytically active.

Caeruloplasmin at physiological concentrations or below can release or express sufficient catalytically active copper ions at moderately acidic pH to catalyse the modification of LDL to an atherogenic form in the presence of macrophages. But are these acidic conditions likely to occur within an atherosclerotic lesion? The media of large arteries is paradoxically one of the most poorly perfused tissues in the human body and hence experiences a low oxygen tension [21] and is therefore partially ischaemic which may result in a local lactic acidosis and

a depression of pH. In support of this, the extracellular pH at the centre of a necrosing tumour in a hyperglycaemic animal, also partially ischaemic, has been measured to be as low as 5.2 [22]. In addition, atherosclerosis is now often described as a chronic inflammatory disease, and by analogy to other inflammatory sites [23], the pH could be expected to fall below pH 7.4. Macrophages in atherosclerotic plaques have many of the characteristics of activated macrophages [24] and the pH measured near an activated macrophage can fall to as low as 3.6 [25]. Hence, it is possible for the pH of both early and advanced atherosclerotic plaques, particularly in the vicinity of macrophages, to fall below pH 7.4, although there is as yet no evidence that this occurs.

There is evidence that high serum copper or caeruloplasmin levels are associated with coronary artery disease. The local modification of LDL to an atherogenic form within the arterial wall by macrophages and other cells may possibly be mediated, at least in part, by copper ions released from caeruloplasmin at the low pH values that may be present within atherosclerotic lesions. As well as releasing copper from caeruloplasmin (and possibly iron from transferrin [26,27]), an extracellular acidic pH has also been shown to increase the rate of oxidation of LDL by macrophages [28].

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