

Non-oxidative modification of low density lipoprotein by ruptured myocytes

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Abstract In this study, the interaction of ruptured cardiac myocytes with low density lipoprotein (LDL) has been investigated and the consequent extent of uptake by macrophages. The results show that lysate released from ruptured myocytes is capable of inducing LDL oxidation and that the resulting modified form is recognised and degraded by macrophages. Peroxyl radical scavengers inhibit the LDL oxidation but not the macrophage uptake suggesting that LDL can be modified by mechanisms that are independent of oxidative processes by intracellular constituents of cardiac myocytes.

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1. Introduction

There is much evidence supporting the hypothesis that oxidative modification of low density lipoprotein (LDL) in the arterial wall may contribute towards the pathogenesis of atherosclerosis, although the mechanism by which this occurs in vivo is still far from clear. The presence of oxidatively modified LDL has been demonstrated in atherosclerotic lesions and LDL eluted therefrom, but not LDL from normal arteries, is rapidly taken up by the scavenger receptors of macrophages [1].

Studies in vitro have shown that LDL can be oxidatively modified by peroxynitrite [2], lipoxygenase [3], haem proteins [4,5] and all the major cell types found in the arterial wall: macrophages [6,7], monocytes [8], endothelial cells [9], smooth muscle cells [10] and lymphocytes [11], and recent work is demonstrating that the recognition properties of LDL can also be modified by non-oxidative mechanisms such that it is taken up by target macrophages [12–14].

Since release of myoglobin from ruptured cardiac myocytes is an early indicator of myocardial infarction, the interaction of ruptured cardiac myocytes with LDL has been investigated and the consequent altered recognition properties and uptake by macrophages. The results show that lysate released from ruptured myocytes is capable of inducing LDL oxidation and that the resulting modified form is recognised and degraded

by macrophages. Peroxyl radical scavengers inhibit the LDL oxidation but not the macrophage uptake suggesting that LDL can be modified by mechanisms that are independent of oxidative processes by intracellular constituents of cardiac myocytes.

2. Materials and methods

All chemicals used were of the analytical grade. Low density lipoproteins were isolated from human plasma using the modified method of Chung et al. [15] and dialysed in 10 mM phosphate buffered saline, pH 7.4 (PBS), containing EDTA (10 µM). The concentration of LDL protein was estimated according to Markwell et al. [16]. Low density lipoproteins were labelled with Na¹²⁵I using iodine monochloride as described by McFarlane [17] as modified by Bilheimer et al. [18]. The iodinated LDL was mixed immediately after labelling with the non-iodinated LDL to obtain a preparation of specific activity 30–60 cpm/ng protein. [¹²⁵I]LDL prepared by this method had the same electrophoretic mobility as the corresponding unlabelled native LDL from which it was derived.

Equine myoglobin was purified on a Sephadex G-25 column, after oxidation with excess of potassium ferricyanide, and the concentration of metmyoglobin determined spectrophotometrically [19]. Oxymyoglobin was purified on a Sephadex G-25 column, after reduction with excess sodium dithionite [20].

LDL oxidation was assessed by monitoring formation of lipid hydroperoxides and the altered surface charge in the apolipoprotein B-100. Relative electrophoretic mobility (REM) which is an index of the surface charge of apolipoprotein B-100 was measured using the Beckman Paragon LIPO electrophoresis kit and calculated as the ratio of the electrophoretic mobility of the treated samples to that of control incubated LDL.

Lipid peroxidation was measured by determining lipid hydroperoxides using the FOX assay [21]. Briefly, to 90 µl sample (125 µg LDL protein/ml) 10 µl of distilled water was added and the sample mixed. 900 µl of FOX reagent (250 µM ammonium ferrous sulphate, 100 µM xylenol orange, 25 mM H₂SO₄ and 4 mM butylated hydroxytoluene (BHT, in 90% (v/v) methanol) was added and the sample incubated at room temperature for 30 min. The sample was centrifuged and the absorbance read at 560 nm. Duplicate samples were treated with triphenylphosphine (a reductant of hydroperoxides to discriminate any constituents that give an absorbance at 560 nm but are not hydroperoxides), incubated for 30 min at room temperature and treated as described. The difference between the sample with and without triphenylphosphine gives the level of lipid hydroperoxide. The working reagent was routinely calibrated against solutions of H₂O₂ of known concentration.

Resident peritoneal macrophages were isolated from female Swiss T.O. mice (A. Tuck and Son, Battlesbridge, Essex, UK) by a modification of the method of Cohn and Benson [22]. The cells were plated in 22 mm diameter wells in 12 well cluster plates (Costar, from Northumbria Biologicals Ltd, Cramlington, Northumbria, UK) at 1 × 10⁶ peritoneal cells per well. The plated cells were incubated at 37°C under 5% CO₂ for 2–4 h to allow macrophage adherence, then washed in Dulbecco's modified Eagle's medium (DMEM; containing 10% fetal calf serum and 50 µg gentamicin/ml) to remove lymphocytes before use. J774 cells were obtained from Dr D.T. Hart (Life Sciences Division, King's College, London) and were grown in

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DMEM containing 20% (v/v) fetal calf serum, 10 µg streptomycin/ml, 10 IU penicillin/ml and 5 µg amphotericin/ml.

Cardiac myocytes were isolated from adult male Wistar rats by the method of Powell et al. [23]. The myocytes were prepared by the proteolytic disruption of Langendorff perfused hearts. The intact heart was perfused in the Langendorff mode with a low calcium buffer, in order to flush the coronary vasculature of blood. After 5 min, the heart was perfused with buffer containing a mixture of proteolytic enzymes [collagenase A (140 µg/ml; Sigma); protease XIV (30 µg/ml; Sigma)] in order to degrade the extracellular matrix and separate the intercellular junctions. The heart was finally perfused with buffer to remove any residual enzyme and the tissue disrupted mechanically. The myocytes in buffer were lysed by sonication (MSE Probe sonicator) for 2 min on ice. The membranes were removed from the myocyte lysate by centrifugation at 105 000×g for 1 h. The concentration of myoglobin was assessed by measurement of the absorbance of the supernatant at 414 nm after subtraction of the background absorbance at 700 nm [24].

Metmyoglobin and oxymyoglobin were prepared as described above. Following purification and quantification the myoglobin was sterilised by membrane filtration (0.22 µm pore size). Ferryl myoglobin was prepared by activation of metmyoglobin with hydrogen peroxide (molar ratio 1:1.25). PBS 10 mM used in the incubation was also sterilised by filtration.

¹²⁵I-Labelled LDL (100 µg protein/ml) was incubated with metmyoglobin 4 µM, oxymyoglobin 4 µM or ferryl myoglobin (formed from metmyoglobin 4 µM and hydrogen peroxide 5 µM) or myocyte lysate at a final concentration of myoglobin of 4 µM in PBS for 22 h at 37°C in a humidified 5% CO₂/95% air incubator. After 22 h the oxidised, control and native ¹²⁵I-labelled LDL were diluted in triplicate to 10 µg protein/ml in DMEM containing serum and gentamicin. The fetal calf serum in the medium prevents any further oxidation of the LDL at this stage [7]. The diluted ¹²⁵I-labelled LDL was added in triplicate to monolayers of macrophages or into cell-free 22 mm multiwell cluster plates and incubated for 22 h at 37°C in a humidified 5% CO₂ incubator. The rate of uptake followed by lysosomal degradation was estimated by the measurement of the release of radioactive non-iodide trichloroacetic acid-soluble degradation products into the medium [7].

The effects of antioxidants and of competition for the scavenger receptors on the uptake of ¹²⁵I-labelled LDL modified by a myocyte lysate by mouse peritoneal macrophages was assessed essentially as above. BHT (in ethanol) was added to inhibit oxidation at a final concentration of 15 µM. The effect of a protease inhibitor on the uptake by mouse peritoneal macrophages of ¹²⁵I-labelled LDL modified by a myocyte lysate was investigated by applying chymostatin to the lysates. Protease XIV is used in the isolation of the myocytes and it has been shown that proteolytic damage to LDL can result in uptake by macrophages [25]. Thus myocyte lysate was prepared by perfusion with collagenase alone, as well as with protease XIV plus collagenase.

3. Results

The oxidation of LDL (250 µg protein/ml) by lysate released from ruptured myocytes (10 µM myoglobin) was as-

Table 1
Effects of various myoglobin forms on the oxidation of LDL

	Lipid hydroperoxides (nmol/mg LDL protein)	REM
Control LDL	7 ± 0.005	1
Oxymyoglobin	418 ± 4	3.3
Metmyoglobin	342 ± 0.03	3.7
Ferryl myoglobin	319 ± 4	3.7
Myocyte lysate	18 ± 0.7	1.4

LDL (250 µg protein/ml) was incubated with 10 µM metmyoglobin, 10 µM oxymyoglobin, 10 µM metmyoglobin plus 12.5 µM hydrogen peroxide to generate ferryl myoglobin and myocyte lysate (final concentration of myoglobin 10 µM) at 37°C for 15 h for these comparison studies. The lipid hydroperoxides were then measured by the FOX assay. The results shown are the means of triplicate determinations ± S.D. but are representative of three independent experiments.

Table 2

Effects of myoglobins on the modification of LDL leading to increased macrophage uptake

	Degradation of ¹²⁵ I-labelled LDL (µg LDL protein/mg cell protein in 22 h)
Native LDL	0.341 ± 0.03
Control LDL	0.347 ± 0.017
Oxymyoglobin	0.556 ± 0.012
Metmyoglobin	0.693 ± 0.076
Ferryl myoglobin	1.018 ± 0.112
Myocyte lysate	1.50 ± 0.25

¹²⁵I-Labelled LDL (100 µg protein/ml) was incubated with myoglobin (Mb 4 µM) and myocyte lysate (Mb 4 µM) for 22 h at 37°C. Myoglobin-modified, control and native ¹²⁵I-labelled LDL were diluted to 10 µg protein/ml in serum-containing medium and its degradation by J774 cells determined. The means ± S.E.M. are shown but are representative of four independent experiments.

essed by production of hydroperoxides and the alteration in the surface charge on the apolipoprotein B-100 (Table 1) in comparison with the extent of oxidation promoted by ferryl, met- and oxymyoglobin with a similar myoglobin concentration. Under these conditions, myocyte myoglobin was less effective than free myoglobin preparations in catalysing oxidation of lipids. The apolipoprotein B-100 of LDL showed increased negative charge after oxy-, met- or ferryl myoglobin-promoted oxidation, but a considerably lower increase from myocyte lysate.

The recognition properties of the oxidised LDL by scavenger receptors on J774 macrophages was investigated. Uptake of ¹²⁵I-labelled LDL modified by myoglobin (4 µM) was measured by the release of radioactive non-iodide TCA-soluble degradation products into the medium (Table 2). LDL pre-oxidised by ferryl, met- and oxymyoglobin (4 µM) for 22 h was also taken up faster by macrophages, the hierarchy of degradation of the modified LDL paralleling the oxidation profile of ferryl, met and oxy. However, LDL modified by myocyte lysate was degraded more rapidly than the myoglobin-modified LDL and yet the extent of oxidation by myocyte lysate in terms of lipid hydroperoxides was much less than with myoglobin as shown in Table 1. Furthermore, the modification of the surface charge on the apolipoprotein B-100 was minimal with the same concentration of myocyte lysate as compared with met-, oxy- and ferryl myoglobin, thus the former might be expected to show limited uptake by macrophages.

In order to elucidate whether the greater extent of degradation of LDL modified by myocyte lysate was due to mechanisms that are independent of oxidation, the effect of BHT, a chain-breaking antioxidant, on the degradation by macrophages was investigated. At this stage the J774 cell line was replaced by mouse peritoneal macrophages in order that polyinosinic acid could be applied as a competitive inhibitor of the scavenger receptors. (The scavenger receptors on J774 cells may be different from those on mouse peritoneal macrophages, Stait and Leake, unpublished observations.) The results demonstrate that the addition of the antioxidant BHT to the myocyte lysate did not inhibit the modification of the LDL to a form that was subsequently degraded faster by macrophages (Table 3) and yet the increase in electrophoretic mobility was prevented. The increased uptake by macrophages therefore was apparently not as a result of lipid peroxidation but as a result of a non-oxidative process. The

Table 3

Effects of BHT and polyinosinic acid on the modification of LDL by a myocyte lysate and its degradation by macrophages

	Degradation of 125 I-labelled LDL (μ g LDL protein/mg cell protein in 22 h)	REM
Native LDL	0.6 ± 0.03	
Control LDL	0.7 ± 0.05	1
LDL+myocyte lysate	1.7 ± 0.08	1.6
LDL+myocyte lysate+BHT (15 μ M)	1.8 ± 0.09	1
LDL+myocyte lysate+Poly I (18 μ g/ml)	2.8 ± 0.02	

125 I-Labelled LDL (100 μ g protein/ml) was incubated with myocyte lysate (Mb 4 μ M) with or without BHT, for 22 h at 37°C. Myocyte lysate-modified, control and native 125 I-labelled LDL were diluted to 10 μ g protein/ml in serum-containing medium and incubated with mouse peritoneal macrophages (1×10^6 peritoneal cells/ml), with or without polyinosinic acid, or in cell-free wells for 22 h at 37°C. The means \pm S.E.M. are shown but are representative of three independent experiments.

addition of polyinosinic acid (18 μ g/ml) to the macrophages, to inhibit competitively the scavenger receptors of macrophages [7], did not reduce the degradation but increased it. It was deduced that the uptake of myocyte lysate-modified LDL was not via the type A scavenger receptor, cloned by Kodama et al. [26], but by another receptor.

The question might be posed as to whether a protease from within the myocytes or used in their preparation was damaging the LDL and increasing its uptake by macrophages. Previous studies have shown that proteolysis of LDL results in aggregation and enhanced uptake by macrophages [25,27] and others have shown that proteases especially cathepsin D in myocyte lysosomes may be released on sonication [28]. In addition, during the isolation of myocytes, protease (type XIV) is used during the digestion and it might be postulated that trace amounts may be present in the myocyte lysate, thus damaging the LDL and resulting in aggregation and its increased uptake by macrophages. Thus the effect of adding chymostatin, a protease inhibitor, to myocyte lysate was investigated on the subsequent degradation of the LDL by macrophages. The results show that there were no significant differences in the modification of 125 I-labelled LDL in the presence of chymostatin (Table 4). Once again LDL in the presence of myocyte lysate showed an increased rate of uptake, and the addition of BHT did not inhibit modification. In addition, myocyte lysate, prepared in the presence of collagenase only, was capable of modifying LDL to a form that had an increased rate of degradation by macrophages (results not shown).

To elucidate the possible mechanism of the non-oxidative

process involved, and whether protease or lipoprotein lipase activities were involved, the effect of pH on the modification of 125 I-labelled LDL by myocyte lysate, was investigated. The myocyte lysate was prepared without protease (type XIV) to eliminate any possible interference. 125 I-Labelled LDL (100 μ g protein/ml) was incubated with myocyte lysate at a final concentration of myocyte myoglobin of 4 μ M in PBS at pH 4, 5.5, 7.4, 8.5 and 10 for 22 h at 37°C. The results (Table 4) show that the modification of LDL by a myocyte lysate was pH dependent. The modification was favoured at acidic pH. At pH 7.4, the addition of 10 μ M EDTA and chymostatin (30 μ g/ml) had no effect on the rate of modification. Furthermore with 125 I-labelled LDL modified at pH 7.4, the addition of excess unlabelled LDL to the macrophages, to competitively inhibit the LDL receptor, had only a fairly small effect on the rate of degradation, thus the uptake was not via the LDL receptor.

4. Discussion

Previous studies have shown that LDL can be oxidatively modified by oxy-, met0 and ferryl myoglobin [4,29,30]. The results described here show further that such oxidatively modified LDL is recognised and taken up by macrophages. There was, as seen in previous studies, a hierarchy in the ferryl myoglobin-modified LDL was degraded more extensively than metmyoglobin-modified which was degraded more than oxy-myoglobin-modified LDL. It was reported [4] that the relative differences in the oxidation with met- and ferryl were indistinguishable after 14 h. However, these experiments clearly

Table 4

Effects of pH on the modification of LDL by a myocyte lysate

	Degradation of 125 I-labelled LDL by macrophages (μ g LDL protein/mg cell protein in 22 h)	
	without myocyte lysate	with myocyte lysate
pH 4	1.93 ± 0.17	8.45 ± 0.94
pH 5.5	2.06 ± 0.35	7.84 ± 0.06
pH 7.4	1.66 ± 0.19	4.97 ± 0.35
pH 8.5	1.49 ± 0.06	5.72 ± 0.19
pH 10	1.60 ± 0.02	5.16 ± 0.33
EDTA (10 μ M), pH 7.4		4.93 ± 0.38
Chymostatin (30 μ g/ml), pH 7.4		5.00 ± 0.05
Excess non-labelled LDL, pH 7.4		3.90 ± 0.49
Native (non-incubated) LDL	2.10 ± 0.19	

125 I-Labelled LDL (100 μ g protein/ml) was incubated with myocyte lysate (Mb 4 μ M) for 22 h at 37°C. Myocyte lysate-modified, control and native 125 I-labelled LDL were diluted to 10 μ g protein/ml in serum-containing medium and incubated with mouse peritoneal macrophages (1×10^6 peritoneal cells/ml) or in cell-free wells for 22 h at 37°C. Excess non-labelled LDL was added to some of the wells. The means \pm S.E.M. are shown but are representative of two independent experiments.

demonstrate that there are differences in the modification of the apolipoprotein B-100, which results in differential extents of degradation by macrophages.

Myocyte lysate modification of LDL results in only moderate modification of the surface charge on the apolipoprotein B-100. This can clearly be seen by agarose gel electrophoresis which shows only a small increase in electrophoretic mobility. Carpenter et al. [31] demonstrated that the electrophoretic mobility of oxidised LDL increases before the uptake by macrophages, being dependent on the modification of the apolipoprotein B-100, would be minimal. The oxidation and uptake by macrophages promoted by the various oxidation states of myoglobin was inhibited totally by the chain breaking antioxidant BHT whereas for the myocyte lysate-modified LDL, the increase in electrophoretic mobility was inhibited, but the increase in macrophage uptake was not.

Protease (type XIV) was used in the isolation procedure, a modification of that of Powell et al. [23]. It was a possibility that this protease was damaging the LDL and increasing the uptake by macrophages. However, myocyte lysate prepared without protease also resulted in increased LDL uptake by macrophages. Therefore protease activity from the isolation procedure was eliminated as a possibility. Leake et al. [25] reported that proteases present in sonicated macrophages partially degraded the apolipoprotein B-100 of LDL at acidic pH, and the resultant modified LDL was taken up much faster than control LDL, presumably because it was aggregated [27]. The use of protease inhibitors indicated that lysosomal cathepsin B and cathepsin D were involved. Similarly, in the myocyte lysate preparations lysosomal enzymes will be released on sonication and be present in the final myocyte lysate. Since lysosomes in myocytes contain cathepsin D [28], lysosomal proteolytic enzymes released by sonication, present in the myocyte lysate, may cause partial proteolysis of the apolipoprotein B-100 and result in aggregation of LDL particles and uptake by macrophages. Preliminary work involving Sepharose CL-4B columns indicated that the myocyte-modified LDL was not aggregated.

Further complications pertain since Bagby et al. [32] reported lipoprotein lipase activity in sonicated rat heart myocytes. The effects of enzymes which exist in the cells of the arterial wall have been studied by Aviram et al. [33]. Incubation of LDL and lipoprotein lipase led to modification of the lipoprotein core triglycerides. Lipoprotein lipase reduced the core triglyceride content without producing marked differences in the size, charge, or in the lipid peroxide content in comparison to native LDL. The triglyceride-depleted forms of LDL were degraded at approximately twice the rate of native LDL by macrophages [33]. Furthermore, phospholipase A₂ [34] and phospholipase D [35] can also modify LDL to increase its uptake by macrophages. The non-oxidative modification of LDL by myocyte lysate was pH-dependent, and this may once again indicate a role for acidic proteases and/or lipases. However, further extensive investigations would be required to elucidate the specific enzymes involved.

The addition of polyinosinic acid (a competing ligand for the type A scavenger receptor) did not result in a decrease in the degradation of myocyte-lysate modified LDL, therefore indicating the uptake was not via the scavenger receptor type A of mouse peritoneal macrophages that recognise ox-

idised LDL [7]. The addition of excess unlabelled LDL to competitively block the LDL receptor did not greatly reduce the degradation of ¹²⁵I-labelled myocyte lysate-modified LDL. It can therefore be concluded that uptake is via some other mechanism than the LDL receptor, unless myocyte lysate-modified LDL binds to the LDL receptor with much higher affinity than does LDL itself.

The mechanism of the uptake of myocyte lysate-modified LDL by macrophages is complex and it is not yet clear what mechanism of modification of apolipoprotein B-100 is involved, but what is clear is that it is not an oxidative process. Recent studies have been described in which non-oxidative modification of native LDL is induced by its oxidatively modified form [12] and the presence of these modified forms have been identified in the blood of patients with cardiovascular disease [13]. Other workers have shown the binding of angiotensin II to the surface of LDL and the uptake of angiotensin II-modified LDL by macrophages via the scavenger receptor [14].

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