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Acidic pH increases the oxidation of LDL by macrophages

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We have investigated the effect of pH on LDL oxidation by macrophages (in the presence of iron ions), using a modification of Hanks' balanced salt solution. Increasing the acidity of the medium greatly increased the oxidation of the LDL by the macrophages as measured by thiobarbituric acid-reactive substances or increased uptake and degradation by a second set of macrophages. The rate of oxidation of LDL by iron ions alone, measured in terms of conjugated dienes, was also increased greatly even at mildly acidic pH. It is quite possible that atherosclerotic lesions have an acidic extracellular pH, particularly in the vicinity of macrophages, and the observation that LDL oxidation by macrophages is increased at acidic pH may therefore help to explain why atherosclerotic lesions are apparently one of the very few sites in the body where LDL oxidation occurs.

Atherosclerosis; Low density lipoprotein; Macrophage; Oxidation; Acidic pH

1. INTRODUCTION

Atherosclerotic lesions are often characterised by the presence of large number of foam cells filled with lipid droplets consisting largely of cholesteryl esters. Many of these foam cells are derived from macrophages [1–4]. Macrophages in culture take up low density lipoprotein (LDL) only slowly via their LDL receptors, which are present in relatively small numbers [5], but they take up oxidised LDL rapidly by means of their scavanger receptor(s) [6].

LDL can be oxidised in vitro under appropriate conditions by a number of cell types including endothelial cells [7], smooth muscle cells [8], macrophages [9,10] and lymphocytes [11] or by auto-oxidation catalysed by copper [12,13] or iron [10] ions in the absence of cells. Consequently, any relevant factor influencing this oxidative modification may play a potential role in the development of atherosclerosis. In this study, we have investigated the effect of pH on cell-mediated LDL oxidation or LDL oxidation mediated by iron ions.

2. MATERIALS AND METHODS

2.1. *Isolation and radioiodination of LDL*

LDL (1.019–1.063 g/ml) was isolated from normal human blood by sequential density ultracentrifugation in KBr solutions at 4°C as described elsewhere [14] and radiolabelled with $Na^{125}I$ using iodine monochloride [10].

2.2. *Modification of LDL by cells*

Resident macrophages were isolated by peritoneal lavage of female swiss T.O. mice (20–30 g) (A. Tuck and Son, Battlesbridge, Essex, UK) as described elsewhere [10]. ^{125}I-labelled LDL (100 μg protein/ml) was incubated with intact macrophages (1×10^6 peritoneal cells/well (22 mm multiwell dishes, Costar)) or cell-free wells for 18 h at 37°C in triplicate at the indicated pH value in modified Hanks' balanced salt solution (HBSS) (10 mM Na_2HPO_4 or 10 mM KH_2PO_4 was added to stock solutions of 1.26 mM $CaCl_2$, 0.81 mM $MgSO_4$, 5.36 mM KCl, 113 mM NaCl and 5.55 mM glucose, and then these two solutions mixed together to give the desired pH) supplemented with 6 μM $FeSO_4$ (1 ml per well). At the end of the incubations, viability of the cells was assessed by viewing under a phase-contrast microscope and by cellular protein estimations.

2.3. *Determination of oxidised LDL degradation by macrophages*

Modified or control ^{125}I-labelled LDL was diluted to 10 μg protein/ml in DMEM containing 10% (v/v) foetal calf serum and 50 μg gentamicin/ml. (The pH of the DMEM/serum medium under 5% CO_2 was not affected by the addition of the modified HBSS of various pHs, when added in a ratio of 1 vol. of HBSS to 9 vols. of DMEM/serum.) It was then incubated for a further 22 h with a second set of macrophages 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 [10]. Degradation products in the cell-free wells were subtracted from those in the wells containing macrophages. The cells were washed in PBS containing Ca^{2+} and Mg^{2+}, lysed in 0.2 M NaOH as described elsewhere [10] and assayed for protein by a modified Lowry procedure [15].

2.4. *Thiobarbituric acid-reactive substances assay [16]*

Samples of modified or control LDL (250 μl of 100 μg protein/ml) were taken and 3 ml of 0.335% (w/v) thiobarbituric acid in 10% (w/v) trichloroacetic acid was added to each sample and incubated at 95°C for 15 min. The absorbance was read at 535 nm. Standards of tetramethoxypropane (Sigma), up to 5 nmol per tube, were prepared in modified HBSS (pH 7.4).

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Abbreviations: DMEM, Dulbecco's Modified Eagle's Medium; LDL, low density lipoprotein; PBS, phosphate buffered saline; HBSS, Hanks' balanced salt solution; MDA, malondialdehyde; EDTA, ethylenediaminetetra-acetic acid; TBARS, thiobarbituric acid-reactive substances.

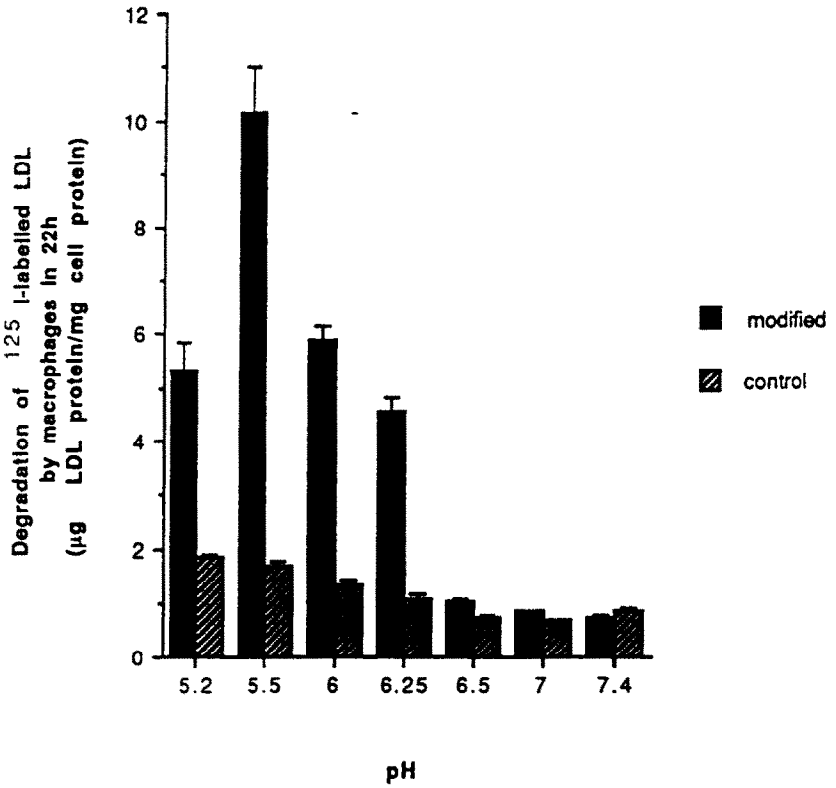

Fig. 1. ^{125}I-labelled LDL (100 μg protein/ml) was incubated for 18 h in triplicate at the indicated pH value with intact macrophages (macrophage-modified LDL) or in cell-free wells (control LDL) in modified Hanks' balanced salt solution supplemented with 6 μM $FeSO_4$. Modified or control LDL was diluted to 10 μg protein/ml in Dulbecco's modified Eagle's medium containing 10% (v/v) foetal calf serum and 50 μg gentamicin/ml and incubated with a second set of macrophages or cell-free wells for 22 h and its rate of degradation measured. The mean ± S.E.M. (n = 3) are shown. The results shown are representative of 3 independent experiments.

2.5. *Iron-mediated oxidation of LDL*

^{125}I-labelled LDL was dialysed against several changes of saline/phosphate buffer (pH 7.4) at 4°C to remove EDTA and its radioactivity was determined and its concentration adjusted to 50 μg protein/ml. It was then oxidised using 50 μM $FeSO_4$ at pH 7.4, 7.0, 6.5 or 5.5 in the modified HBSS at ambient temperature and monitored at 234 nm in a spectrophotometer [13].

3. RESULTS

By an adaptation of Hanks' balanced salt solution, we were able to maintain macrophages in a viable state over a wide pH range and study the effect of pH on cell-mediated oxidation of LDL, as measured by an increased rate of uptake and degradation by a second set of macrophages at pH 7.4. At all pH values, the macrophages had a normal appearance when viewed under the phase-contrast microscope and yielded similar cell protein contents when assayed at the end of the incubation (results not shown). Fig. 1 shows one experiment in which there was no LDL oxidation by macrophages at pH 7.4 but a progressive increase in oxidation as the pH was decreased with a pH optimum of 5.5. At this pH, there was a 6-fold increase in LDL modification relative to the control LDL (LDL incubated in cell-free dishes). The oxidation of the control LDL, probably due to the iron ions added to the modified HBSS, was also increased progressively at acidic pH, but to a more limited extent and with no evidence for an optimum pH over the pH range tested.

The modification of the LDL by macrophages at acidic pH was due to its oxidation, as shown by an increase in the levels in the medium of thiobarbituric acid-reactive substances (TBARS), products of lipid oxidation (Table I). Due to the necessity for transition metal ions to catalyse the oxidation of LDL, when $FeSO_4$ was excluded from the incubation medium little increase in TBARS or degradation by macrophages was observed in the macrophage-modified LDL compared to the control LDL. When 6 μM $FeSO_4$ was added, the TBARS and macrophage degradation increased for both the macrophage-modified LDL and control LDL, but the increases were greater for the macrophage-modified LDL. Further evidence that the modification process at acidic pH was an oxidative one was that the presence of the lipophilic antioxidant butylated hydroxytoluene (BHT) during exposure of the LDL to the modifying macrophages blocked the changes seen in the TBARS and in the degradation by a second set of macrophages (Table I). The addition of 20 μM polyinosinic acid (a competing ligand for the scavenger receptor)

Table I

	^{125}I-Labelled LDL degradation by macrophages (μg LDL protein/mg cell protein in 16 h)		TBARS (nmol MDA/mg LDL protein)	
	macrophage-modified LDL	control LDL	macrophage-modified LDL	control LDL
No addition	0.73 ± 0.04	0.67 ± 0.06	5.0 ± 1.0	2.0 ± 0.0
6 μM $FeSO_4$	2.39 ± 0.24	1.34 ± 0.08	29.0 ± 2.4	13.0 ± 2.7
6 μM $FeSO_4$ + 20 μM BHT	0.60 ± 0.04	0.66 ± 0.21	4.0 ± 0.0	4.0 ± 0.0

^{125}I-labelled LDL (100 μg protein/ml) was incubated for 24 h with macrophages (1×10^6 peritoneal cells/22 mm multiwell dish) or cell-free wells (control) in a modified Hanks' balanced salt solution at pH 5.5 with or without 6 μM $FeSO_4$ or 20 μM butylated hydroxytoluene (BHT). It was then assayed for MDA or MDA-like products by a TBARS assay or was diluted to 10 μg protein/ml in Dulbecco's modified Eagle's medium containing 10% (v/v) foetal calf serum and 50 μg gentamicin/ml and added to a second set of macrophages or cell-free wells and incubated for 16 h and its rate of degradation measured. The mean ± S.E.M. for 3 wells are shown. Another independent experiment gave similar results.

resulted in an 88% inhibition of the degradation by macrophages of LDL modified by macrophages at acidic pH (data not shown).

The formation of conjugated dienes was used as a measure of LDL oxidation by iron ions, in the absence of cells. LDL oxidation by iron ions alone in HBSS measured in this way was very slow at pH 7.4 (Fig. 2). The formation of conjugated dienes after the lag phase was much greater at pH 7.0 than at pH 7.4; at pH 6.5 it was greater still and with a shorter lag phase. It was much less, however, at pH 5.5 than at pH 6.5. (A similar result was found when an acetate buffer rather than a phosphate-based buffer was used.) The observed changes in absorbance were not an artefact due to an effect of pH on the molar absorption coefficient of conjugated dienes as this was unchanged over the pH range 7.4–5.5 (results not shown).

4. DISCUSSION

By an adaption of HBSS, we were able to study the effect of pH on both cell-mediated and metal ion-catalysed oxidation of LDL. Increasing the acidity of the medium greatly increased the oxidation of LDL by the

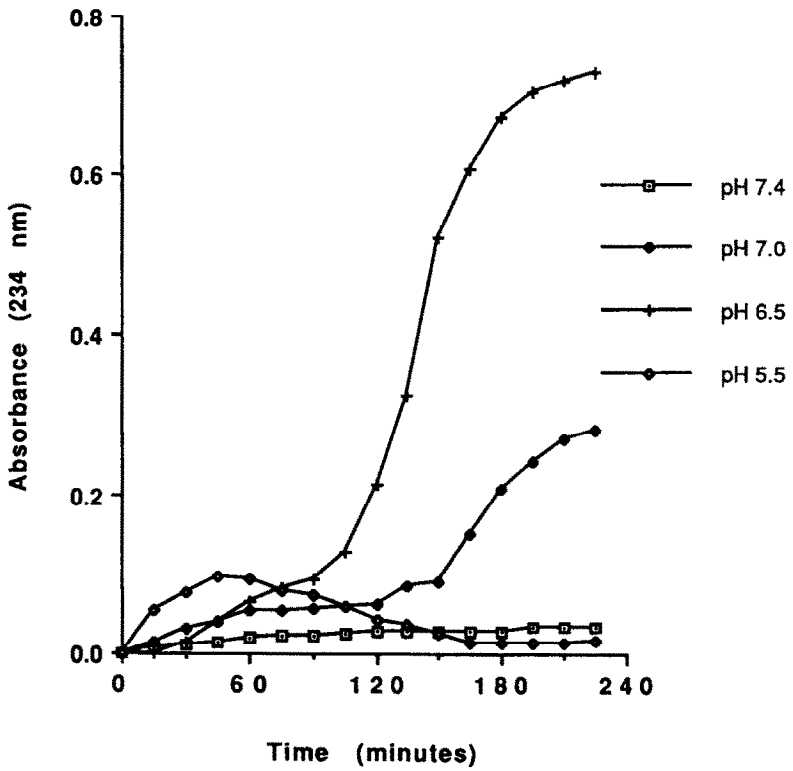

Fig. 2. Effect of pH on the oxidation of LDL by iron ions as measured by conjugated diene absorbance. Following dialysis to remove any EDTA, the LDL (50 μg protein/ml) was oxidised using 50 μM $FeSO_4$ at pH 7.4 (□), 7.0 (♦), 6.5 (+) or 5.5 (◇) in a modified Hanks' balanced salt solution at ambient temperature and monitored at 234 nm in a spectrophotometer. The results shown are representative of 5 independent experiments.

macrophages as measured by TBARS or macrophage degradation. A similar effect of pH was also seen when iron ions alone were used to catalyse the oxidation of LDL as measured by an increase in conjugated diene formation at pH 6.5 and 7.0 compared to 7.4. (When LDL was oxidised by copper ions alone, however, there was a large decrease in conjugated diene formation at acidic pH.)

There are many possibilities to explain why LDL oxidation by macrophages was increased by acidic pH. The macrophages may possibly produce more free radicals or oxidised lipids at acidic pH. Any superoxide anion produced by them would be converted more to hydroperoxyl radicals (which have a pKa of 4.8) and it is known that hydroperoxyl radicals generated by steady-state radiolysis are much more active in oxidising the lipids of LDL than superoxide anions are [17]. The dismutation of superoxide to H_2O_2 is more rapid at acidic pH [18] and if H_2O_2 and superoxide react together in a metal-catalysed Haber–Weiss reaction to form the highly reactive hydroxyl radical, this could conceivably help to explain why LDL oxidation by cells is faster at acidic pH. Another possibility is that the cellular release or extracellular oxidation of thiols, which may be involved in LDL oxidation [19], may be increased at acidic pH. Other possibilities to explain why macrophages or iron ions alone oxidise LDL faster at acidic pH are that the chain reaction of lipid peroxidation, the ability of iron ions to redox cycle, the solubility of ferric ions, the fragmentation of lipid hydroperoxides to produce aldehydes or the reaction of aldehydes with apolipoprotein B-100 may be increased at acidic pH.

It appears that the oxidation of LDL occurs in the arterial wall and not in the general circulation, as LDL oxidation is inhibited strongly by plasma [10]. Consequently, LDL oxidation may require the sequestered microenvironment present within atherosclerotic lesions, where the ratio of oxidative stress to antioxidants may be high and where the extracellular pH may be acidic. It is becoming increasingly clear that atherosclerotic lesions are analogous in many ways to chronic inflammatory sites. The extracellular pH has been known for a long time to be low in inflammatory sites [20]. In addition, atherosclerotic lesions are known to be ischaemic [21,22] and this may mean that cells in the lesions rely to a large extent on glycolysis for their energy production and thus produce a lot of lactic acid which may diffuse away only slowly due to the poor perfusion of atherosclerotic lesions and may thus acidify the extracellular space. The pH of the interstitial space of the ischaemic brain in hyperglycaemic rats has been shown to be 6.2 [23] and the extracellular pH of tumours (which are poorly perfused) in hyperglycaemic rats can be as low as 5.2 [24]. Macrophages extrude H^+ in part by a H^+-ATPase in the plasma membrane and in weakly buffered medium they can acidify their extracellular pH from 7.4–7.2 to 6.5 in only 25 min and it may well fall even more given longer [25]. Atherosclerotic lesions contain macrophages that have some of the characteristics of activated macrophages [26] and the pH near to the surface of activated macrophages is known to be as low as 3.6 [27]. Thus LDL oxidation may occur in microdomains of high oxidative stress near the surface of macrophages where the pH may be low.

The finding that LDL oxidation by macrophages takes place faster at acidic pH may help to explain why atherosclerotic lesions are apparently one of the very few sites in the body where LDL oxidation proceeds to a significant degree.

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