

A moderate reduction in extracellular pH protects macrophages against apoptosis induced by oxidized low density lipoprotein

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Abstract We investigated the effect of pH on macrophage apoptosis induced by oxidized low density lipoprotein (OxLDL), as human atherosclerotic lesions have regions of low pH. Hydroperoxide-rich and oxysterol-rich LDL caused 38% and 74% apoptosis of J774 macrophages, respectively, at 24 h, as measured by the externalization of phosphatidylserine. Native LDL, however, did not cause apoptosis. Reducing the pH of the culture medium from 7.4 to 7.0 inhibited apoptosis induced by hydroperoxide-rich or oxysterol-rich OxLDL by 61% and 46%, respectively ($P < 0.001$). These data were confirmed by semiquantitative analysis of cytochrome *c* release from mitochondria. Decreasing the extracellular pH to 7.0 reduced the uptake of hydroperoxide-rich and oxysterol-rich ¹²⁵I-labeled LDL by 82% and 42%, respectively, and reduced cell surface binding of oxysterol-rich LDL by 31%. This may explain the reduced apoptosis. Additionally, low pH did not affect OxLDL-induced apoptosis of human monocytes, which do not possess scavenger receptors for OxLDL, but reduced apoptosis of human monocyte-derived macrophages, which do possess them. Our investigations suggest that the presence of areas of low pH within atherosclerotic lesions may reduce the uptake of OxLDL and reduce macrophage apoptosis, thus affecting lesion progression.—Gerry, A. B., and D. S. Leake. A moderate reduction in extracellular pH protects macrophages against apoptosis induced by oxidized low density lipoprotein. *J. Lipid Res.* 2008. 49: 782–789.

Supplementary key words atherosclerosis • inflammation • monocyte • necrosis • scavenger receptor

We proposed in 1990 that a low extracellular pH may play a role in the pathogenesis of atherosclerosis (1–5). Atherosclerosis is a chronic inflammatory disease (6), and inflammatory sites are known to have a low extracellular pH (7). Naghavi et al. (8) showed in 2002 that the pH of lipid-rich areas of atherosclerotic lesions in human carotid endarterectomy specimens was 7.15 ± 0.01 , significantly less than in the calcified areas or in normal human

umbilical arteries (7.73 ± 0.01 and 7.24 ± 0.01 , respectively). Some lipid-rich areas had a pH of 7.0 or less.

The oxidation of LDL is widely believed to be an important event in the pathogenesis of atherosclerosis (9). We have shown that a low extracellular pH can increase the oxidation of LDL by cells (2, 3). Oxidized low density lipoprotein (OxLDL) may induce the apoptosis of macrophages and other cells in atherosclerotic lesions (10). In general, plaques that rupture demonstrate apoptosis of both smooth muscle cells and macrophages (11). Apoptosis is a prominent feature of atherosclerotic lesions, occurs in all lesion types, and may be associated with the instability of plaques (12–16). Apoptosis of macrophages may have both beneficial and harmful implications in lesions. Apoptosis with efficient removal of apoptotic bodies of lipid-laden foam cells may decrease the lipid content and inflammatory cell content of the arterial wall, but impaired removal of these apoptotic bodies in advanced lesions may lead to necrosis and deposition of lipid (17).

Reducing the apoptotic activity of macrophages in mice with a Bcl2-associated X protein (Bax) deficiency promoted the development of atherosclerosis, indicating that macrophage apoptosis can sometimes suppress atherosclerosis (18). It has also been shown that induction of smooth muscle cell apoptosis by a selective diphtheria toxin-sensitive mechanism in apolipoprotein E-deficient mice induced the features of plaque instability (16). p53-deficient mice had reduced apoptosis overall and increased atherosclerosis, although the situation was complex because p53 appeared to promote apoptosis in macrophages but to protect smooth muscle cells from apoptosis (19).

The true role of apoptosis in the atherogenic process is still not fully understood. Apoptosis is supposed to be a noninflammatory death process; however, as discussed above, efficient apoptosis may be hindered in complex inflammatory situations, such as in atherosclerotic lesions. An understanding of how apoptosis is affected by low pH

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within lipid-rich areas of lesions might help us understand how to stabilize lesions.

Here, we demonstrate that a small reduction in extracellular pH inhibits OxLDL-induced apoptosis of macrophages. This inhibition of apoptosis is attributable at least in part to a decrease in the binding of OxLDL to cell surface receptors at low pH, thus reducing endocytosis of the OxLDL.

MATERIALS AND METHODS

Materials

Unless stated otherwise, all chemicals were obtained from Sigma-Aldrich or Invitrogen in their highest purity.

Isolation of LDL

LDL ($d = 1.019\text{--}1.063$ g/ml) was isolated from the blood of healthy human volunteers as described previously (20). Protein content was determined by a modified Lowry assay (21).

Oxidation of LDL to defined extents

Native LDL was dialyzed against MOPS buffer containing $10\ \mu\text{M}$ CuSO_4 for 24 h at either 4°C (termed hydroperoxide-rich LDL) or 37°C (termed oxysterol-rich LDL) (22). Hydroperoxide-rich LDL was defined as containing maximum hydroperoxides, and oxysterol-rich LDL was defined as having almost complete hydroperoxide decomposition and high levels of oxysterols, such as 7-ketocholesterol. OxLDL species were filter-sterilized, assayed for protein, and stored at 4°C under argon. Concentrations of cholesterol, cholesteryl esters, and oxidation products were determined by reverse-phase HPLC, as described previously (22).

Electrophoresis of LDL

Native LDL or OxLDL was subjected to electrophoresis on preformed 0.5% agarose gels (Lipogels; Beckman Coulter) according to the manufacturer's instructions. The relative electrophoretic mobility (REM) was calculated as the distance the OxLDL migrated from the origin compared with that for native LDL.

Iodometric hydroperoxide assay

Total hydroperoxides were quantified using a method based on that described by El-Saadani et al. (23). The hydroperoxide content of LDL was determined by comparison with a H_2O_2 standard plot and was expressed as nanomoles of H_2O_2 equivalents per milligram of LDL protein.

Cell culture

J774 monocyte-macrophages were maintained in DMEM [containing glucose (1 g/l), sodium pyruvate, and pyroxidine, supplemented with 20% FBS (v/v), Glutamax (2 mM), penicillin (20 IU/ml), streptomycin (20 $\mu\text{g}/\text{ml}$), and amphotericin B (0.95 $\mu\text{g}/\text{ml}$)] at 37°C and 5% CO_2 . For growth curves, both adherent and suspension cells were collected and counted using a Beckman ZM series Coulter counter. Human blood-derived monocytes were isolated using Nycoprep 1.068 solution (Robbins Scientific, Solihull, UK) as described previously (24). The monocyte-rich layer was plated at 1×10^6 cells/ml and cultured at 37°C and 5% CO_2 in serum-free DMEM overnight to adhere to the culture plates, then washed gently three times with serum-free DMEM to remove any contaminating lymphocytes. The cells

were confirmed to be monocytes by microscopic examination of their gross morphology. Monocytes were cultured in serum-free medium during treatment to prevent differentiation or incubated for 7 days in medium containing FBS (20%, v/v) to allow differentiation into macrophages before treatment. The pH of the culture medium was altered using 5 M HCl and equilibrated at 37°C and 5% CO_2 for at least 5 days. The pH of the culture medium was measured before and after incubation with the cells, and little or no change was seen.

Radiolabeling of LDL and uptake by macrophages

J774 cells were plated overnight at 1.5×10^5 cells/well on 12-well culture plates, followed by 18 h of incubation with 10 $\mu\text{g}/\text{ml}$ of native or oxidized ^{125}I -labeled LDL (labeled as described previously) (20). Further oxidation of the LDL was prevented by the presence of serum in the culture medium (25). After incubation, the noniodide trichloroacetic acid-soluble degradation products in the medium and cell-associated radioactivity were determined, as described previously (20). Total uptake of ^{125}I -labeled LDL was taken to be equal to the sum of cell-associated LDL and degraded LDL, expressed as micrograms of LDL protein per milligram of cell protein.

Binding of LDL to cell surface receptors was assayed by measuring cell-associated ^{125}I -labeled LDL (50 $\mu\text{g}/\text{ml}$) after 2 h on ice (in a 5% CO_2 incubator with culture medium pH corrected as necessary to account for the temperature change) (26).

Measurement of apoptosis by flow cytometry

J774 cells were incubated for 24 h in culture medium of the required pH values with or without native LDL or OxLDL. The externalization of phosphatidylserine to the cell surface, which is an early marker of apoptotic cell death (27), was detected by flow cytometry using ApoAlert Annexin V-FITC apoptosis kits (Clontech) as described in the manufacturer's instructions. Data acquisition and analysis were performed with a Becton Dickinson FACScan flow cytometer, using CellQuest software. Apoptosis was measured in both adherent cells and cells in suspension, and no differences were seen between these populations. The data presented are for pooled adherent and suspended cells.

Visualization of cytochrome *c* release by confocal microscopy

J774 cells were incubated on glass coverslips for 48 h in culture medium of the required pH values with or without native LDL or OxLDL. Cells were fixed with 4% (w/v) paraformaldehyde and permeabilized with methanol at -20°C . Cells were incubated with rabbit anti-mouse cytochrome *c* polyclonal antibody (5 $\mu\text{g}/\text{ml}$; Santa Cruz) and a FITC-conjugated goat anti-rabbit IgG secondary antibody (5 $\mu\text{g}/\text{ml}$; Immunologicals Direct). Microscope slides were analyzed using a Leica TCSNT confocal microscope (Leica Microsystems) with a $63\times$ immersion objective lens. Semiquantitative analysis was performed on 16 cells each from three independent experiments chosen as the nearest cell to the center of each square of a 4×4 square grid. Cytoplasmic fluorescence intensity was measured with Quantity One software.

Statistical analysis

Graphs represent means \pm SEM of the indicated number of independent experiments. Statistical analysis was performed where necessary using Student's *t*-test to determine differences between two observations, or using two-way ANOVA to test effects of multiple parameters in combination, using the SPSS sta-

tistics package. Differences between observations were deemed significant at $P < 0.05$.

RESULTS

The effects of OxLDL upon cells have been studied extensively, but the interpretation of these results has sometimes been difficult because of the diverse nature of the OxLDL species produced by various laboratories. Therefore, we devised a method to generate OxLDL rich in hydroperoxides and low in oxysterols (produced by dialysis against copper at 4°C) or conversely rich in oxysterols and low in hydroperoxides (produced by dialysis against copper at 37°C). In summary, native LDL con-

tained 39 ± 13 nmol total hydroperoxide/mg LDL protein ($n = 4$ independent experiments) and 0.2 ± 0.1 nmol 7-ketocholesterol/mg LDL protein. Hydroperoxide-rich LDL contained 626 ± 98 nmol hydroperoxide/mg, 3 ± 1 nmol 7-ketocholesterol/mg, and had a REM of 2.60 ± 0.55 . Oxysterol-rich LDL contained 50 ± 14 nmol hydroperoxide/mg, 143 ± 20 nmol 7-ketocholesterol/mg, and had a REM of 4.20 ± 0.42 (22).

Effects of pH and OxLDL on macrophage proliferation

J774 cells had a cell doubling time of 24–36 h (Fig. 1). Treatment with native LDL had no significant effect upon the rate of proliferation (Fig. 1A). Both hydroperoxide-rich LDL (Fig. 1B) and oxysterol-rich LDL (Fig. 1C), however, inhibited proliferation ($P < 0.001$). The increase in cell number over 72 h was reduced by 37% when the pH was decreased from 7.4 to 7.0 in the absence of LDL and by 32% in the presence of native LDL. At pH 7.4, hydroperoxide-rich LDL inhibited proliferation by 76% and oxysterol-rich LDL inhibited proliferation by 78%. At

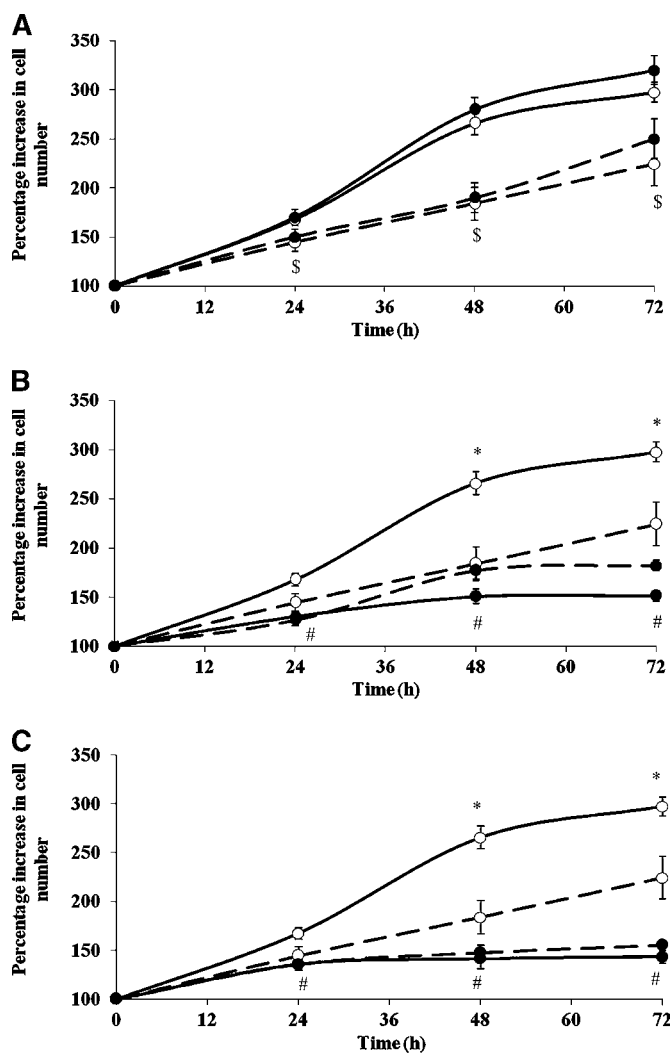


Fig. 1. Low pH reduced oxidized LDL (OxLDL)-induced inhibition of proliferation. J774 cells were incubated at pH 7.4 (continuous lines) or pH 7.0 (broken lines) either with no LDL (open circles) or with native LDL (A), hydroperoxide-rich LDL (B), or oxysterol-rich LDL (C) (each 200 μ g protein/ml; closed circles). The data for the incubations without LDL are repeated in each panel for clarity. $^{\$}$ $P < 0.001$, low pH inhibits proliferation without LDL; * $P < 0.05$, OxLDL reduces proliferation less at low pH; $^{\#}$ $P < 0.001$, OxLDL reduces proliferation at both pH values. Graphs represent means \pm SEM of three independent experiments.

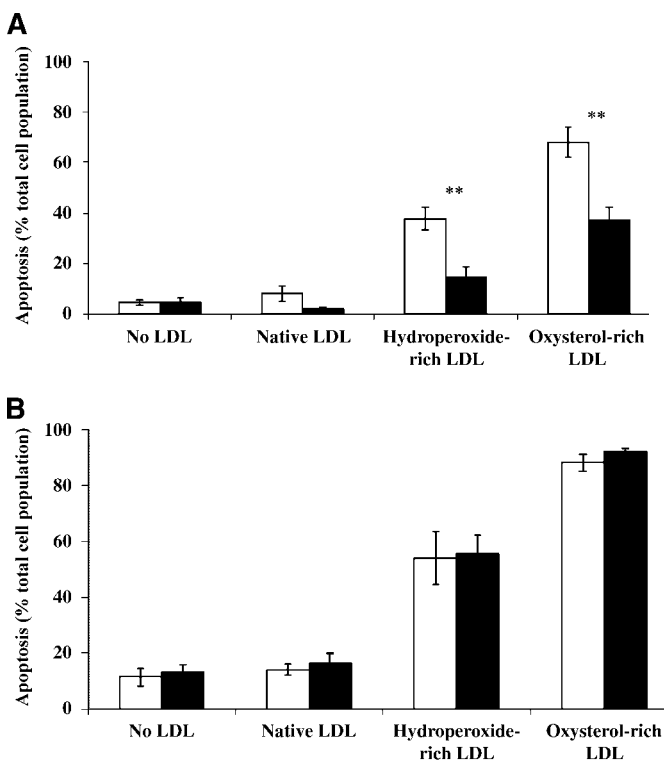


Fig. 2. Low pH reduced OxLDL-induced apoptosis: phosphatidylserine externalization. A: J774 cells were treated for 24h with or without native LDL, hydroperoxide-rich LDL, or oxysterol-rich LDL (200 μ g LDL protein/ml) at either pH 7.4 (white bars) or pH 7.0 (black bars). Cells were assayed for phosphatidylserine externalization by flow cytometry. ** $P < 0.001$, percentage apoptosis significantly different at pH 7.0 compared with pH 7.4. The graph represents means \pm SEM of at least three independent experiments. B: Low pH pretreatment alone did not protect from subsequent OxLDL-induced apoptosis. J774 cells were incubated at either pH 7.4 (white bars) or pH 7.0 (black bars) for 24 h before treatment with OxLDL (200 μ g protein/ml) for 24 h at pH 7.4. Phosphatidylserine externalization was determined by flow cytometry. The graph represents means \pm SEM of three experiments.

pH 7.0, OxLDL also inhibited J774 proliferation, but the effects were not as pronounced as at pH 7.4. Indeed, cell number after 72 h of treatment with OxLDL tended to be higher at pH 7.0 than at pH 7.4, although the effects were only statistically significant for hydroperoxide-rich LDL after 48 h. Hydroperoxide-rich LDL and oxysterol-rich LDL significantly reduced the cell number compared with that without LDL within 24 h at pH 7.4 ($P < 0.001$); however, cell number was not reduced significantly compared with the cell number without LDL until 72 h of OxLDL treatment at low pH. Additionally, cell number (when expressed as a percentage of that without LDL) was significantly higher at pH 7.0 than at pH 7.4 from 48 h onward (data not shown).

Effects of pH and OxLDL on apoptosis

Our experiments demonstrated that the reduced rate of increase of macrophage numbers by OxLDL was attributable, in least in part, to an increase in cell death by

apoptosis. Externalization of phosphatidylserine to the cell surface is an early marker of apoptotic cell death. This change can be detected using FITC-conjugated Annexin V, which binds to phosphatidylserine residues. Propidium iodide, a marker of membrane permeability, can be used to distinguish early apoptosis from necrosis. Plasma membrane integrity is lost during both primary necrosis and secondary necrosis (necrosis occurs after apoptosis when apoptotic cells are not removed by phagocytosis), allowing propidium iodide to enter the cell and stain the nuclear material. Incubation of J774 cells at pH 7.4 with no LDL caused no visible change in morphology (as determined by phase-contrast microscopy) and no increase in apoptosis or necrosis for up to 72 h (data not shown). Treatment of cells for 24 h with both OxLDL species caused a significant increase in apoptosis compared with control cultures ($P < 0.001$) (Fig. 2A), whereas native LDL had no significant effect. Hydroperoxide-rich LDL and oxysterol-rich LDL caused $37.7 \pm 4.4\%$ and

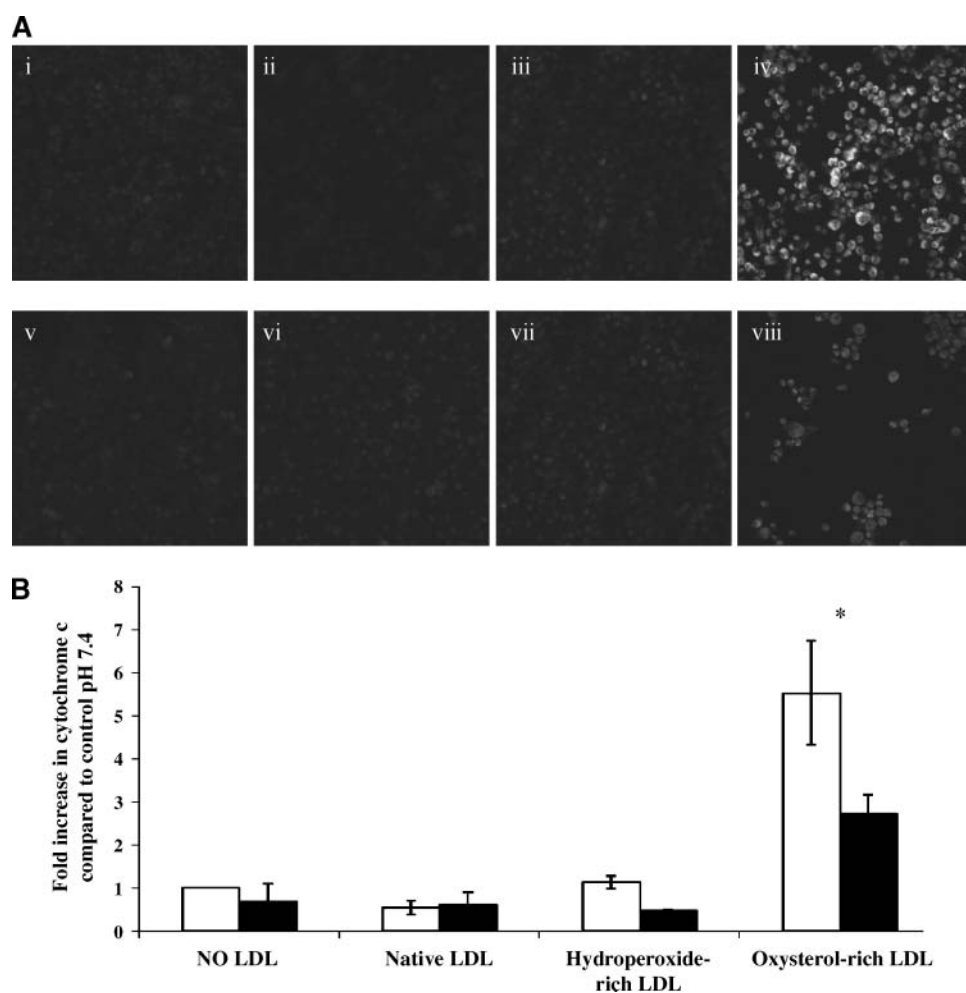


Fig. 3. Low pH reduced OxLDL-induced apoptosis: cytochrome *c* release. **A:** J774 cells were incubated without (panels i, v) or with native LDL (panels ii, vi), hydroperoxide-rich LDL (panels iii, vii), or oxysterol-rich LDL (panels iv, viii) (200 μg protein/ml) at either pH 7.4 (panels i–iv) or pH 7.0 (panels v–viii). Cells were assayed for cytochrome *c* release by confocal microscopy. **B:** The data are shown graphically. White bars, pH 7.4; black bars, pH 7.0. Error bars represent means \pm SEM of three independent experiments. * $P < 0.05$, difference in effect between pH values.

68.0 ± 6.0% apoptosis, respectively, at 24 h. The increased toxicity of oxysterol-rich LDL over hydroperoxide-rich LDL ($P < 0.001$) may be attributable to either oxysterols (or other oxidized components in oxysterol-rich LDL) being more toxic to these cells than are hydroperoxides or as a result of increased endocytosis of oxysterol-rich LDL over hydroperoxide-rich LDL. OxLDL species caused no significant changes in the percentage of cells undergoing primary necrosis with up to 48 h of treatment, but an increase in secondary necrosis was detected with oxysterol-rich LDL treatment at 48 h (11.2 ± 1.2% secondary necrosis at 48 h vs. 4.9 ± 1.2% at 24 h; $P < 0.005$) (data not shown).

As well as low pH reducing the antiproliferative effects of OxLDL, low pH reduced OxLDL-induced apoptosis. Figure 2A demonstrates that reducing the pH of the culture medium from 7.4 to 7.0 reduced hydroperoxide-rich LDL-induced apoptosis from 37.7 ± 4.4% to 14.7 ± 3.7% ($P < 0.001$) and reduced oxysterol-rich LDL-induced apoptosis from 68.0 ± 6.0% to 36.8 ± 5.3% in 24 h ($P < 0.001$). Additionally, low pH pretreatment followed by treatment with OxLDL at pH 7.4 was not protective; thus, the protection is dependent upon the presence of low pH during OxLDL treatment (Fig. 2B). A low pH also reduced the release of cytochrome *c* from mitochondria induced by OxLDL, as shown by semi-quantitative confocal microscopy (Fig. 3). OxLDL also caused apoptosis of human monocytes (Fig. 4A) and human monocyte-derived macrophages (Fig. 4B). We incubated the OxLDLs with human monocytes for 24 h and with human monocyte-derived macrophages for 48 h, because this gave a comparable degree of apoptosis at pH 7.4 for both types of cells. No serum was added to the culture medium of the monocytes, as we did not want the monocytes to differentiate into macrophages. Interestingly, low extracellular pH inhibited OxLDL-induced apoptosis of the monocyte-derived macrophages but not of the monocytes, suggesting that low pH may reduce scavenger receptor-mediated endocytosis, as human monocytes express low levels of scavenger receptors, which increase during differentiation to macrophages (28, 29). It may also be of interest that low pH alone caused a moderate increase in apoptosis of human monocyte-derived macrophages in the absence of OxLDL (Fig. 4B), for some unknown reason.

Effects of pH on the endocytosis of OxLDL

The reduction in OxLDL-induced apoptosis by low pH could have been attributable to a reduction in the uptake of OxLDL. J774 macrophages endocytosed 0.5 ± 0.1 µg native ¹²⁵I-labeled LDL protein/mg cell protein at pH 7.4 in 18 h (Fig. 5A). Vastly more hydroperoxide-rich and oxysterol-rich ¹²⁵I-labeled LDL was endocytosed by these cells (5.5 ± 0.8 and 14.1 ± 1.2 µg LDL protein/mg cell protein, respectively; $P < 0.001$), which correlated with the level of modification of the apolipoprotein B-100 moiety of LDL, as measured by REM. Reducing the extracellular pH from 7.4 to 7.2 and 7.0 significantly reduced the uptake of all LDL species ($P < 0.001$). A decline

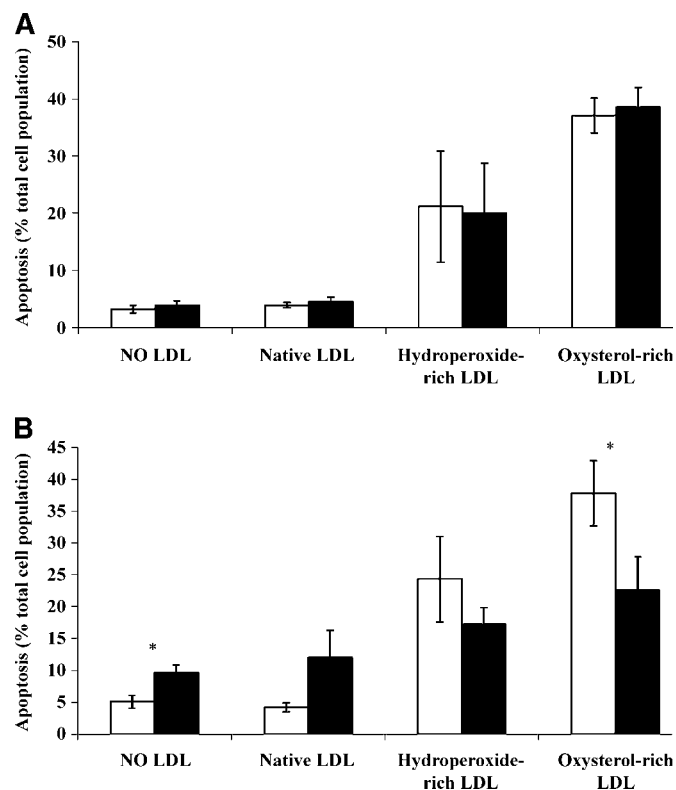


Fig. 4. Low pH reduced OxLDL-induced apoptosis in human monocyte-derived macrophages but not in human monocytes. Analysis of phosphatidylserine externalization by flow cytometry. **A:** Human monocytes were incubated in serum-free DMEM without or with native, hydroperoxide-rich, or oxysterol-rich LDL (200 µg protein/ml) for 24 h at pH 7.4 (white bars) or pH 7.0 (black bars). **B:** Human monocytes were allowed to differentiate into monocyte-derived macrophages by incubation for at least 7 days with DMEM containing 20% (v/v) serum, followed by native, hydroperoxide-rich, or oxysterol-rich LDL (200 µg protein/ml) for 48 h (with serum present) at pH 7.4 (white bars) or pH 7.0 (black bars). The graphs represent means ± SEM of at least three independent experiments. * $P < 0.05$, difference in effect between pH 7.4 and pH 7.0.

in pH from 7.4 to 7.0 reduced native LDL uptake by 59%, hydroperoxide-rich LDL by 82%, and oxysterol-rich LDL by 42%. These effects of low pH may result from decreases in scavenger receptor expression, conformation, or charge (hence, altering their functionality), which may help explain why low pH reduces the adherence of J774 macrophages to culture plates (A. B. Gerry, unpublished data).

It was observed that the degree of oxidation of LDL affected its intracellular degradation as well as its rate of uptake ($P < 0.002$) (Fig. 5B). A greater fraction of cell-associated oxysterol-rich and hydroperoxide-rich LDL than native LDL remained cell-associated after 18 h (57, 47, and 18%, respectively). Similar observations have been made previously (30, 31). Low pH reduced the ability of macrophages to degrade native LDL but not OxLDL ($P < 0.001$). At pH 7.4, 82 ± 5% of the native LDL endocytosed was degraded, but at pH 7.0, only 50 ± 7% was degraded (Fig. 5B). Additionally, the reduction in uptake

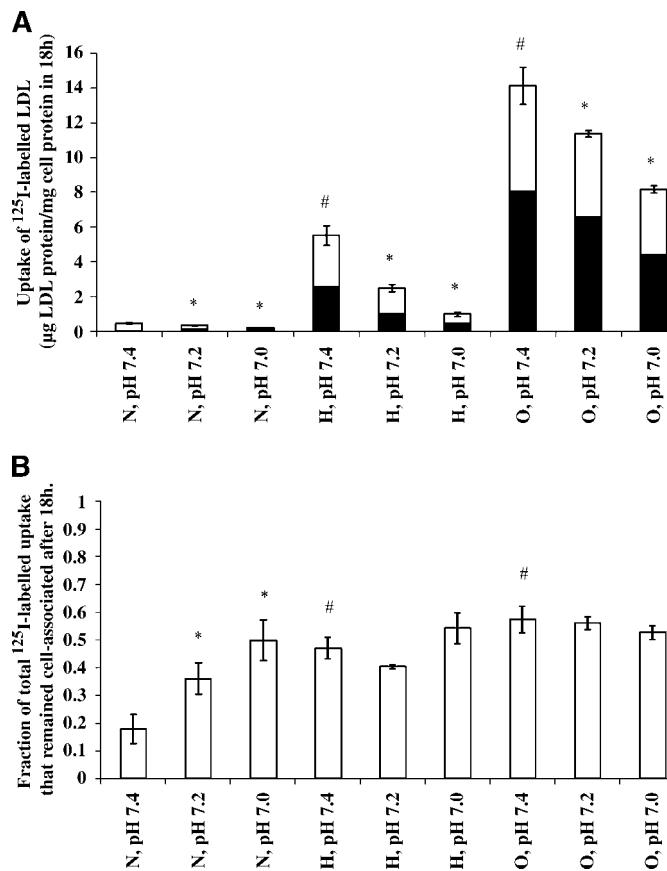


Fig. 5. Low pH reduced oxidized ¹²⁵I-labeled LDL uptake by J774 cells. **A:** Total uptake of ¹²⁵I-labeled LDL was determined as described in Materials and Methods. An increase in the degree of oxidation of ¹²⁵I-labeled LDL increased its uptake by J774 macrophages ([#] $P < 0.001$ by two-way ANOVA). Reduction in extracellular pH reduced the uptake of native (N), hydroperoxide-rich (H), and oxysterol-rich (O) LDL ($*P < 0.001$). The graph represents means \pm SEM of four independent experiments. The lower bar (black) represents cell-associated radioactivity, and the upper bar (white) represents noniodide trichloroacetic acid-soluble radioactivity in the medium. The total height of the bar represents the total uptake of ¹²⁵I-labeled LDL. **B:** J774 macrophages could not degrade OxLDL as efficiently as native LDL ([#] $P < 0.002$). J774 macrophages were less able to degrade native LDL at pH 7.0 or 7.2 than at pH 7.4 ($*P < 0.001$). The graph represents means \pm SEM of four independent experiments and shows the fraction of total ¹²⁵I-labeled LDL uptake that remained cell-associated after 18 h.

could be attributable to a reduction in the binding of OxLDL to scavenger receptors. We measured the binding of ¹²⁵I-labeled OxLDLs to macrophages at 0°C using a concentration of 50 µg protein/ml, so as to measure the nearly maximal binding capacity of the cells for the OxLDLs. **Figure 6** demonstrates that low pH cotreatment reduced the cell surface binding of oxysterol-rich LDL by 31% (Fig. 6A), but pretreatment at low pH had no effect (Fig. 6B). It is also of note that little hydroperoxide-rich LDL bound to the cell surface compared with oxysterol-rich LDL and that a reduction in pH had no significant effect upon the binding of hydroperoxide-rich LDL (Fig. 6A).

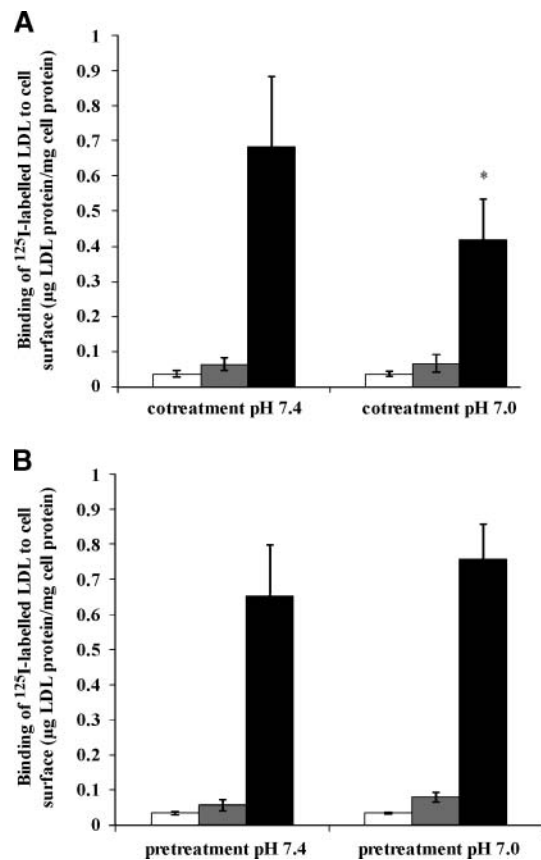


Fig. 6. Low pH inhibited cell surface binding of oxidized ¹²⁵I-labeled LDL. **A:** Low extracellular pH reduced the binding of oxysterol-rich ¹²⁵I-labeled LDL to the cell surface. Cells were incubated with native (white bars), hydroperoxide-rich (gray bars), or oxysterol-rich (black bars) ¹²⁵I-labeled LDL at either pH 7.4 or 7.0 on ice for 2 h before determination of cell-associated radioactivity. **B:** Low extracellular pH pretreatment of cells for 24 h before incubation with ¹²⁵I-labeled native LDL or OxLDL at pH 7.4 for 2 h did not reduce cell-associated radioactivity. The graphs represent means \pm SEM of three independent experiments ($*P < 0.01$).

DISCUSSION

Atherosclerosis is both a lipid disorder, with a plethora of roles described for OxLDL, and a chronic inflammatory disease, with central roles for infiltrating macrophages. The responses of macrophages to defined OxLDL species at varying extracellular pH values in terms of proliferation, death, and endocytosis of LDL were investigated in an attempt to elucidate how macrophages may respond to oxidative stress in vivo in atherosclerotic lesions, where there is much heterogeneity of extracellular pH (8).

We have demonstrated that reducing the extracellular pH by 0.4 reduced the proliferation of J774 cells. This inhibition of proliferation by low pH could be the result of an increase in cell death or a decrease in cell division. Regardless of the mechanism for this effect, the implications of a decrease in cell number for atherosclerotic lesion pathogenesis are multiple. There is evidence that macrophages proliferate in atherosclerotic lesions (19, 32). It is known that mammalian cell proliferation is

decreased by a modest decline in extracellular pH (33). The inhibition of macrophage proliferation by low pH may possibly have a beneficial effect on certain aspects of atherosclerosis if it decreases the burden of inflammatory cells in the lesions. Low pH, however, reduced the inhibitory effects of the OxLDL species upon cell proliferation (Fig. 1), perhaps as a result of reduced OxLDL-induced apoptosis.

Both hydroperoxide-rich and oxysterol-rich LDL-induced apoptosis was reduced by incubation of cells at pH 7.0 rather than at pH 7.4, as demonstrated by both phosphatidylserine externalization and cytochrome *c* release from mitochondria. OxLDL has been shown previously to release cytochrome *c* from mitochondria in macrophages (34). Interestingly, oxysterol-rich LDL caused much more cytochrome *c* release from mitochondria than did hydroperoxide-rich LDL. This observation suggests that oxysterol-rich LDL caused apoptosis by a mitochondrial pathway, whereas hydroperoxide-rich LDL-induced apoptosis may have involved other pathways.

We found previously that hydroperoxide-rich LDL induced more apoptosis than did more highly OxLDL in human arterial smooth muscle cells (35), in contrast to the present findings with macrophages. This may have been because the smooth muscle cells contained few, if any, scavenger receptors for OxLDL and therefore did not take up highly OxLDL as rapidly as did the macrophages.

Decreasing the pH from 7.4 to 7.0 reduced the uptake of native LDL and both types of OxLDL (Fig. 5). Additionally, the cell surface binding of oxysterol-rich LDL was reduced at pH 7.0 (Fig. 6). Hydroperoxide-rich LDL did not show much cell surface binding at either pH (Fig. 6A), possibly because the extensive washing procedure removed most of it, if its binding affinity was lower than that of oxysterol-rich LDL. The observation that uptake was reduced at low pH is in keeping with previous studies showing that OxLDL dissociates from scavenger receptor A at low pH in endosomes after uptake by cells (36). If a low extracellular pH increases LDL oxidation in the interstitial fluid of the arterial wall (2–4) and decreases the uptake of OxLDL by cells, it may increase the concentration of OxLDL in the interstitial fluid. The reduced endocytosis of OxLDL at low pH may explain why apoptosis induced by OxLDL was less at low pH. This explanation is consistent with our finding that a low pH reduced the toxicity of OxLDL to human monocyte-derived macrophages but not monocytes (Fig. 4). The expression of scavenger receptor AI increases greatly when human monocytes differentiate into macrophages (28). CD36 increases transiently upon monocyte differentiation but returns to a low baseline level by about day 7 (29). The effect of pH on the uptake of OxLDL in our experiments, therefore, probably is attributable mainly to an effect on scavenger receptor AI.

The data showing that OxLDL caused substantial apoptosis of monocytes (Fig. 4A) suggest that there may be endocytosis-independent mechanisms of toxicity, such as lipid hydroperoxides or additional cholesterol oxidation

products affecting membrane structure or function directly (37).

OxLDL was not degraded as completely as was native LDL, as described previously (30, 31). OxLDL is resistant to degradation by lysosomal cathepsins (30, 31, 38). The efficiency of proteolysis of ¹²⁵I-labeled LDL in cells was similar for hydroperoxide-rich and oxysterol-rich LDL (Fig. 5B). OxLDL, when present in lysosomes, not only affects their contents but also damages their membranes, resulting in leakage to the cytosol of hydrolytic enzymes (39). Interestingly, low pH inhibited the ability of J774 cells to degrade native LDL but not OxLDL. Hence, over an extended period of time, macrophages situated at regions of low extracellular pH in atherosclerotic lesions might tend to accumulate native LDL or its components.

A low extracellular pH reduced the uptake of OxLDL by macrophages, and this may help to decrease the extent of lipid accumulation within foam cells in atherosclerotic lesions. It should be borne in mind, however, that atherosclerosis is a chronic inflammatory disease. The resolution of this inflammation may be helped by apoptosis of macrophages if their subsequent clearance by phagocytosis is efficient (17). We have demonstrated that a modestly low extracellular pH considerably inhibits OxLDL-induced apoptosis, at least in part, by an inhibition of receptor-mediated endocytosis of OxLDL. This may possibly increase the level of inflammation attributable not only to the increase in the number of macrophages present but also to the eventual increase in necrotic cell death that may occur, which would have an inflammatory effect. ■

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