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Low density lipoprotein oxidized under lysosomal conditions decreases arterial vasodilatation

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Alboaklah, H. K. M. ORCID: https://orcid.org/0000-0003-0272-5040, McNeish, A. J. ORCID: https://orcid.org/0000-0002-3466-8442 and Leake, D. S. ORCID: https://orcid.org/0000-0002-1742-6134 (2024) Low density lipoprotein oxidized under lysosomal conditions decreases arterial vasodilatation. Free Radical Research, 58 (8). pp. 509-516. ISSN 1029-2470 doi: 10.1080/10715762.2024.2403038 Available at https://centaur.reading.ac.uk/118582/

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To link to this article DOI: http://dx.doi.org/10.1080/10715762.2024.2403038

Publisher: Taylor and Francis

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To cite this article: Hadeel K. M. Alboaklah, Alister J. McNeish & David S. Leake (13 Sep 2024): Low density lipoprotein oxidized under lysosomal conditions decreases arterial vasodilatation, Free Radical Research, DOI: 10.1080/10715762.2024.2403038

To link to this article: https://doi.org/10.1080/10715762.2024.2403038

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RESEARCH ARTICLE

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Low density lipoprotein oxidized under lysosomal conditions decreases arterial vasodilatation

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ABSTRACT

Endothelial dysfunction is a risk factor for atherosclerosis and includes impaired endothelium-dependent vasodilatation. We have shown previously that low density lipoprotein (LDL) can be oxidized by iron in the lysosomes of macrophages. Macrophage lysis in atherosclerotic lesions might expose endothelial cells to this oxidized LDL and adversely affect their function. LDL was oxidized by ferrous sulfate (5 µM) for 24 h at pH 4.5 at 37 °C. Aortas from male Wistar rats were cut into rings and subjected to wire myography for isometric tension recording. The rings were incubated with or without oxidized LDL (50 µg protein/ml) for one hour, constricted with 100 nM phenylephrine and relaxation to acetylcholine (1 nM – 3 µM) was measured. There was about 50% less relaxation in the presence of this oxidized LDL. Endothelial-independent vasodilatation induced by sodium nitroprusside was less affected by oxidized LDL. Oxidized LDL increased the formation of reactive oxygen species by the aortic rings and by cultured human aortic and dermal microvascular endothelial cells, which might have inactivated nitric oxide. Acetylcholine increased the activatory phosphorylation of eNOS (ser-1177), but oxidized LDL had little effect on this activation in cultured human aortic endothelial cells. These findings raise the possibility that LDL oxidized in lysosomes and released from lysed macrophages might decrease vasodilatation in atherosclerotic arteries.

ARTICLE HISTORY
Received 26 April 2024
Revised 28 August 2024
Accepted 1 September 2024

KEYWORDS
Atherosclerosis; low density lipoprotein; lysosomes; oxidized low density lipoprotein; vasodilatation

Introduction

Impaired arterial vasodilatation is a risk factor for cardiovascular disease, a major cause of death in the world [1]. Endothelial cells dilate arteries by generating nitric oxide, endothelium-derived hyperpolarisation and prostacyclin [2–4]. Nitric oxide is produced by endothelial nitric oxide synthase (eNOS), which has a complex mechanism of activation [5]. As well as mediating vasodilatation and regulating blood pressure, nitric oxide affects many other processes, such as, inflammation and platelet activity [5]. Vasodilatation is well known to be decreased by low density lipoprotein (LDL) oxidized by copper ions [6–12] or by endothelial cells [11,13].

There has been a great deal of interest in the oxidation of low density lipoprotein (LDL) [14], as oxidized LDL has many pro-atherogenic activities [14,15], in addition to decreasing vasodilatation. It is widely assumed that LDL is oxidized in the extracellular space of the intima of the arterial wall, but we have discovered that it can be oxidized by redox-active iron in the lysosomes of macrophages, a prominent cell type present in atherosclerotic lesions [16–18]. The mechanisms of LDL oxidation at lysosomal pH (about 4.5) might well be different to those of LDL oxidation by copper or cultured cells at pH 7.4 [19] and this might alter the effects of the oxidized LDL on cells. Very little is known about how the pH of oxidation affects the oxidation products in LDL.

As macrophage death is a prominent feature of advanced atherosclerotic lesions [20] and oxidized cholesteryl esters can cause the exocytosis of the contents of lysosomes in macrophages [21], it is possible that LDL which was oxidized in lysosomes might be released into the extracellular space of atherosclerotic lesions and interact with cells present in these lesions, including the endothelium. We have therefore investigated if

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LDL oxidized under lysosomal conditions, that is by iron at lysosomal pH (pH 4.5), can affect the vasodilatation of arteries. We show here that LDL oxidized under these conditions can indeed decrease vasodilatation of rat aortas and present data regarding the mechanisms that might be involved.

Materials and methods

Myography

Male healthy Wistar rats (12-14 week-old, body weight 250-350 g, $n=10$) were anesthetized by isoflurane and killed by cervical dislocation. The thoracic aorta was immediately and carefully removed and placed in ice cold Krebs' solution (118 mM NaCl, 3.6 mM KCl, 1.2 mM MgSO4, 1.2 mM KH2PO4, 2.5 mM CaCl2, 11 mM glucose and 24 mM NaHCO3). The aortas were cleaned of adipose and connective tissue, cut into rings (2 mm width) and mounted on a wire myograph (Danish Myo Technology, 620 M) connected to a force transducer (PowerLab ML846, ADInstruments) and the LabChart 7 Software suite (ADInstruments) for isometric tension recording, as previously described [22]. Briefly, the organ bath was filled with Krebs' solution heated at 37 °C and bubbled with carbogen (95% O2 and 5% CO2). The aortic rings were subjected to zero tension followed by equilibration for 30 min and then stretched to a standardized tension of 10 mN [23]. The rings were pre-constricted with the α_1-adrenoceptor agonist phenylephrine (100 nM) and only arteries that were able to relax by ≥75% to the muscarinic agonist acetylcholine (1 µM) were deemed to have functional endothelium and used for further study. In arteries preconstructed to about 80% of maximal phenylephrine-induced tone (50 – 300 nM phenylephrine), concentration-response curves to acetylcholine (1 nM-3 µM) were obtained to confirm that the aortic rings were responding as expected. Rings were then washed several times with Krebs' solution and incubated with oxidized LDL (50 µg protein/ml) for 60 min before being contracted with phenylephrine and relaxed with acetylcholine (1 nM – 3 µM). Aortic rings were also treated with sodium nitroprusside (1 nM – 3 µM) to assess endothelium-independent vasodilatation.

Oxidized LDL

LDL was isolated from healthy volunteers by sequential density ultracentrifugation (1.019 – 1.063 g/ml), as described previously [24]. It was oxidized by $FeSO_4$ (5 µM) for 24 h at 37 °C in 150 mM/10 mM sodium acetate buffer of pH 4.5 [25].

Culture of endothelial cells

SV40 large T antigen-transformed human dermal microvascular endothelial cells (HMEC-1) were obtained from the Center For Disease Control and Prevention (Atlanta, Georgia) and were grown in MCDB 131 supplemented with 10% (v/v) heat-inactivated FBS, L-glutamine (1.461 g/l), hydrocortisone acetate (1 mM) and human epidermal growth factor (10 ng/ml).

Human aortic endothelial cells (Lonza) were cultured in EBM™-2 Endothelial Cell Growth Basal Medium-2 containing fetal bovine serum, human epidermal growth factor, vascular endothelial growth factor, R3-insulin-like growth factor-1, ascorbic acid, hydrocortisone, human fibroblast growth factor-β, heparin, gentamicin and amphotericin-B (Lonza).

Measurement of reactive oxygen species in aortic rings and cultured endothelial cells

After the wire myography experiments, the rat aorta was frozen in liquid nitrogen-cooled isopentane and mounted in Optimal Cutting Temperature compound cooled by dry ice/ethanol and cryosections (10 µm) were prepared.

Superoxide production by the aortic sections or cultures of endothelial cells was detected by a dihydroethidium

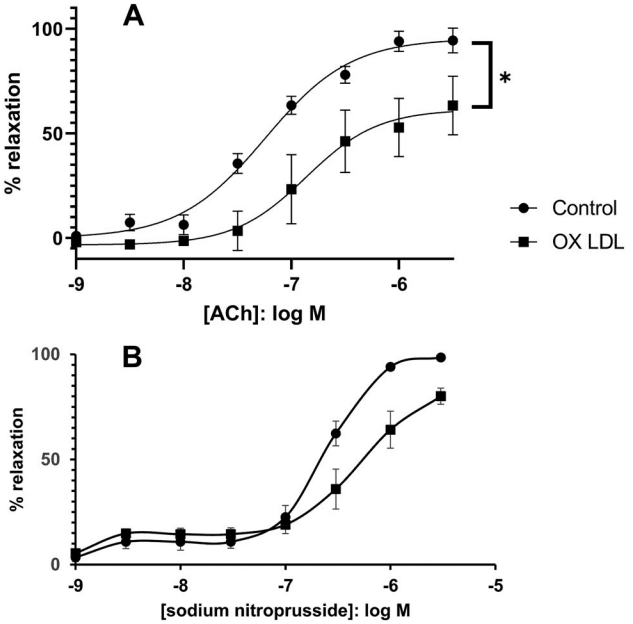

Figure 1. Effect of LDL oxidized by iron at pH 4.5 on vasodilatation. Rat aortic rings were incubated for 60 min with LDL oxidized by $FeSO_4$ at pH 4.5 (50 µg protein/ml), contracted by phenylephrine (100 nM) and the relaxations by increasing concentrations of (A) acetylcholine or (B) sodium nitroprusside were measured in a wire myograph. The means ± SEM of 5 independent experiments were compared by a t-test. * indicates $p<0.05$ for the EC_{50} compared to the controls.

assay, according to the manufacturer's instructions. In brief, aortic ring sections or cells were incubated with 10 µM dihydroethidium in PBS for 30 min, fluorescence was measured with a fluorescence microscope with an excitation wavelength of 488 nm and quantified using ImageJ analysis software.

Western blots

Proteins from human aortic endothelial cells were extracted using RIPA buffer (Sigma-Aldrich) and Halt™ protease and phosphatase inhibitor (ThermoFisher Scientific). Western blotting was performed [26] for total and phosphorylated eNOS. Proteins in the lysates were separated by sodium dodecyl sulfate-polyacylamide gel electrophoresis (SDS-PAGE) on 10-12% gels and transferred to polyvinylidene difluoride membranes (Millipore). The membranes were blocked by incubation in 10 mM Tris-buffered 100 mM NaCl, pH 7.5 containing 0.1% (v/v) Tween 20 and 5% (v/v) nonfat dry milk for 1 h to decrease nonspecific binding, followed by a 24 h incubation at 4 °C with rabbit polyclonal antibodies to eNOS and phosphorylated-eNOS antibody (ser-1177) (1:1,000 dilution) (Cell Signaling Technology). β-Actin (mouse monoclonal, Developmental Studies Hybridoma Bank) was used as a loading control. The membranes were washed in Tris-buffered saline containing 0.1% (v/v) Tween 20 before incubation for 1 h at room temperature with a horseradish peroxidase-conjugated secondary antibody (Invitrogen). The membranes were then washed and developed using ECL substrate (ThermoFisher Scientific). Band intensities were measured using Image J.

Immunoflurorescence measurement of phosphorylated eNOS

Human aortic endothelial cells grown on glass coverslips were washed once with PBS and then incubated

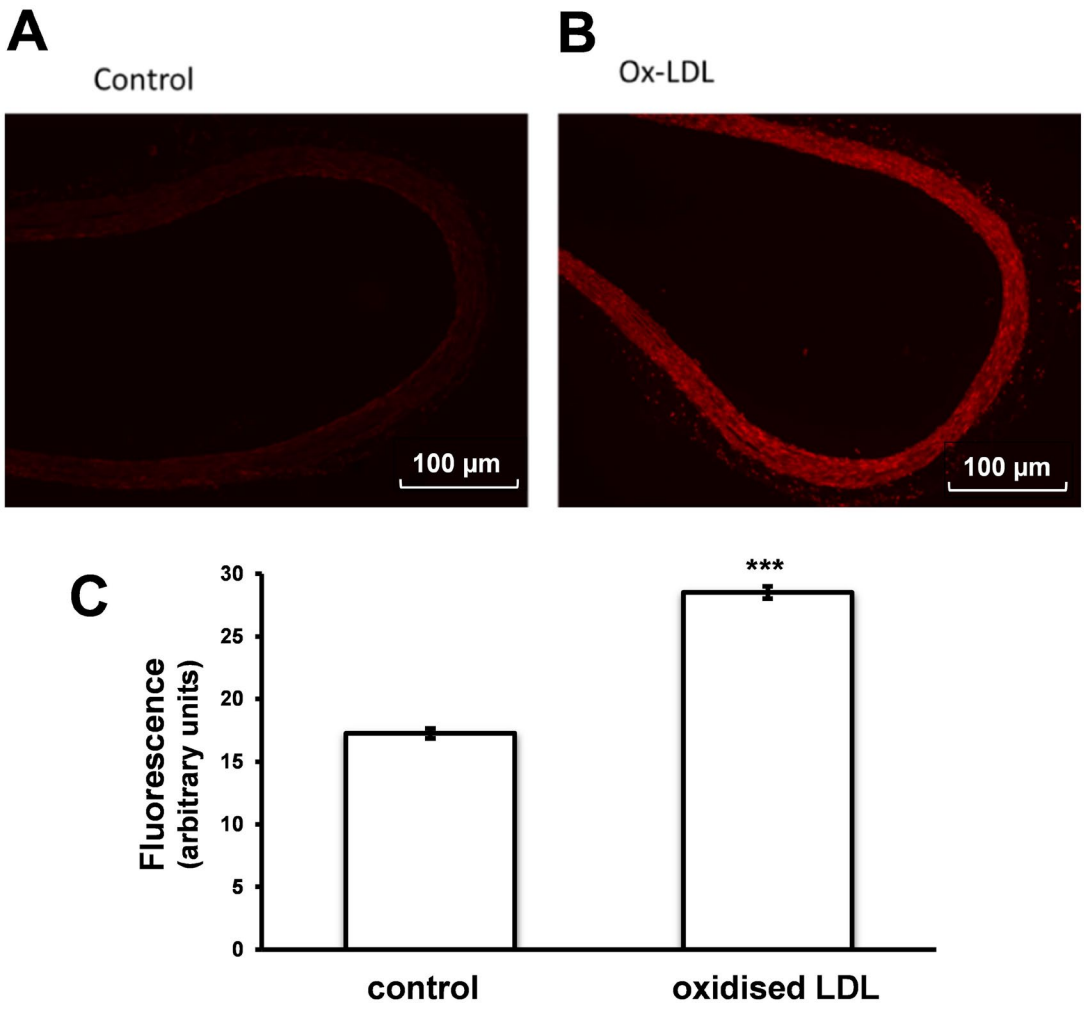

Figure 2. Effect of oxidized LDL on reactive oxygen species formation by sections of rat aorta. Representative dihydroethidium fluorescence image of the control (A) and oxidized LDL-treated (B) aortic tissue. Quantification of dihydroethidium fluorescence (C). The mean ± SEM of five experiments is shown. Paired t-test. ***$p < 0.001$.

at 37 °C with oxidized LDL (50 µg protein/ml) for 60 min, followed by acetylcholine (3 µM) for 8 min. Control and oxidized LDL treated cells were then washed by phosphate buffer and fixed for 10 min with 4% (w/v) paraformaldehyde in phosphate buffer. After fixation, the cells were rinsed three times with phosphate buffer, permeabilised with 0.1% Triton X-100 for 2 min and rinsed three times in phosphate buffer. Cells were then incubated for 24 h at 4 °C with a monoclonal antibody to phosphorylated eNOS (ser1177) (20 µg/ml) in phosphate buffer plus 1% (w/v) bovine serum albumin. Cells were washed three times with phosphate buffer and then incubated for 60 min at room temperature with a goat anti-rabbit secondary antibody (20 µg/ml) in phosphate buffer containing 1% (w/v) bovine serum albumin. Cells were washed three times with phosphate buffer and once in de-ionised water, mounted in Fluorescence Mounting medium (Dako) and examined using a Zeiss Axioskop epifluorescence microscope.

Statistics

The mean ± SEM of the given number (*n*) of independent experiments is shown. In isolated arteries *n* corresponds to the number of animals used. A paired t-test or one- or two-way ANOVA and a Bonferroni or Tukey's post-hoc test were used to compare treatments as appropriate using GraphPad Prism 4software (Ja Jolla, CA, USA). A p value of < 0.05 was considered statistically significant.

Results

Effects of oxidized LDL on relaxation of aortic rings

We investigated the effects of LDL oxidized under lysosomal conditions on arterial vasodilatation. Rat aortic rings pre-incubated with LDL oxidized by ferrous ions at lysosomal pH (pH 4.5) showed less relaxation induced by acetylcholine than did the control aortic rings and

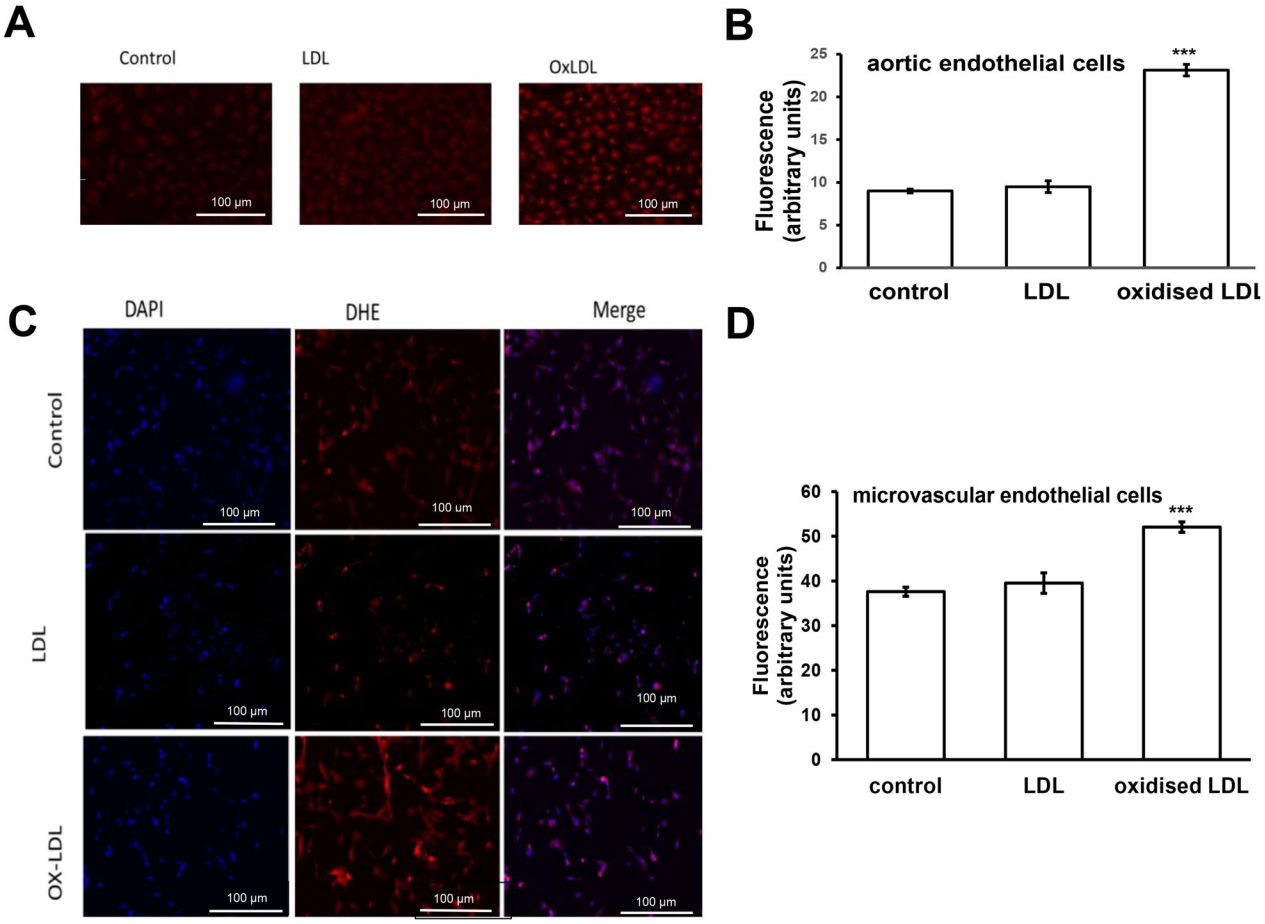

Figure 3. Effect of oxidized LDL on reactive oxygen generation by cultured endothelial cells. Human aortic endothelial cells (A, B) or human dermal microvascular endothelial cells (C, D) were treated for 1 h with control LDL or LDL that had previously been oxidized with 5 µM $FeSO_4$ for 24 h at pH 4.5 (both at 50 µg protein/ml). Reactive oxygen species were measured using dihydroethidium. DAPI was used to stain the nuclei in the microvascular cells. The mean ± SEM of five independent experiments is shown. ***$p < 0.001$ (ANOVA, followed by tukey's post hoc test).

the EC_{50} was significantly increased (Figure 1A). This was due mainly to an effect on the endothelial cells, rather than the smooth muscle cells, as there was less effect of oxidized LDL on endothelium-independent relaxation induced by sodium nitroprusside, especially at low sodium nitroprusside concentrations (Figure 1B).

Effect of oxidized LDL on reactive oxygen species in aortic rings and cultured endothelial cells

We next investigated the mechanisms responsible for this decreased vasodilatation. One possibility is that the oxidized LDL increased the formation of superoxide, as superoxide can inactivate nitric oxide. Oxidized LDL increased the generation of reactive oxygen species, mainly superoxide, by rat aortic sections, as measured using dihydroethidium (Figure 2). To investigate the mechanisms responsible for the decreased vasodilatation in more detail, we used cultured endothelial cells. In agreement with its effect on rat aortic rings, LDL oxidized under lysosomal conditions increased the generation of reactive oxygen species by cultured endothelial cells from both large and small blood vessels, namely human aortic endothelial cells and human dermal microvascular endothelial cells (Figure 3). Control LDL had no effect.

Effect of oxidized LDL on eNOS phosphorylation

As eNOS is activated by phosphorylation, we investigated the effect of the oxidized LDL on the phosphorylation of this enzyme using western blots. Incubation of human aortic endothelial cells in culture with acetylcholine (3 µM) for 8 min increased the levels of phosphorylated eNOS (Figure 4A and B). Pre-incubation with control LDL or oxidized LDL had no effect on the phosphorylation of eNOS (Figure 4A and B). We explored this further using immunofluorescence with the cultured endothelial cells. This confirmed that acetylcholine alone or in cells pretreated with native LDL

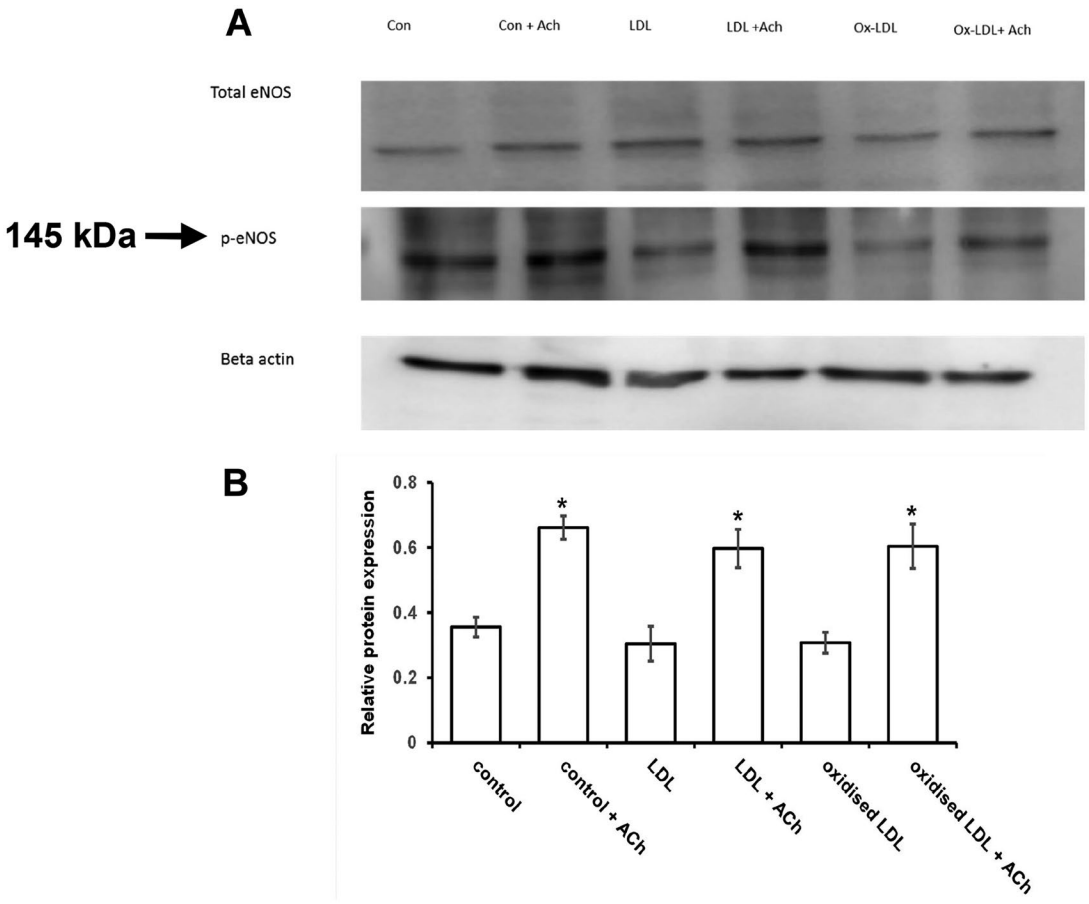

Figure 4. Phosphorylated and total eNOS in cultured human aortic endothelial cells measured by Western blotting. Endothelial cells (A, B) were incubated for 1 h with control LDL or LDL that had been oxidized by 5 µM $FeSO_4$ for 24 h at pH 4.5 (both at 50 µg protein/ml). Acetylcholine (3 µM) was added for 8 min and the cells were then lysed and total and phosphorylated eNOS (ser-1177) measured by Western blotting. Protein levels were normalized to beta-actin. Illustrative western blots are shown. The mean ± SEM of three independent experiments is shown. *indicates $p < 0.05$.

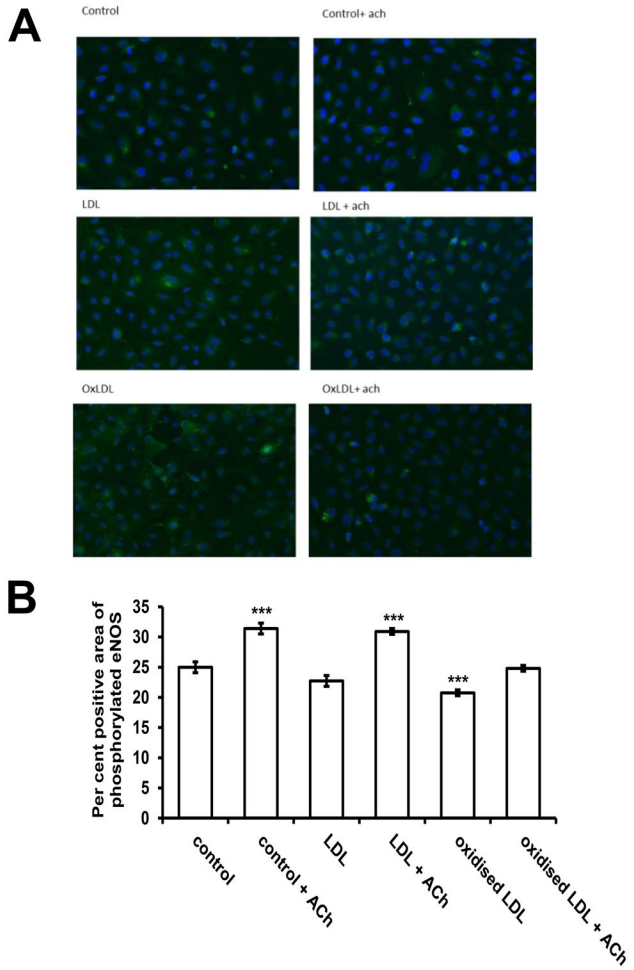

Figure 5. Phosphorylated eNOS in endothelial cells measured by immunofluorescence microscopy. Human aortic endothelial cells were incubated for 1 h with control LDL or LDL that had been oxidized by 5 μM $FeSO_4$ for 24 h at pH 4.5 (both at 50 μg protein/ml) and then with acetylcholine (3 μM) for 8 min. Phosphorylated eNOS (ser-1177) was measured by immunofluorescence microscopy, with DAPI used to stain the nuclei. The mean ± SEM of three independent experiments is shown. *** indicates $p < 0.001$ test compared to the control (two-way ANOVA, followed by Tukey's post hoc test).

increased eNOS phosphorylation, but this phosphorylation was not statistically significantly increased compared to the control cells in the cells pre-incubated with oxidized LDL (Figure 5A and B).

Discussion

Oxidized LDL is well known to decrease vasodilatation mediated by nitric oxide but there are many forms of oxidized LDL. LDL is often oxidized by copper at pH 7.4 but this type of oxidation is unlikely to take place *in vivo*. We have shown that LDL can be oxidized by iron in the lysosomes of macrophages [16]. Macrophage death occurs in atherosclerosis, especially in advanced atherosclerotic lesions [20], and oxidized LDL might be released from their lysosomes to the interstitial fluid of the arterial wall when the cells lyse. Also oxidized cholesteryl esters have been shown to cause the exocytosis of the contents of lysosomes in macrophages [21]. The extracellular oxidized LDL might adversely affect the function of endothelial cells.

We have shown here that the relaxation of rat aortic rings by acetylcholine was decreased by LDL oxidized under lysosomal conditions, that is by iron at low pH (Figure 1A). The relaxation by sodium nitroprusside, which is not dependent on the endothelium, was not decreased significantly by oxidized LDL (Figure 1B), suggesting that the main effect of the oxidized LDL was on the endothelial cells.

The generation of reactive oxygen species by the aortic rings was increased by oxidized LDL (Figure 2). This raises the possibility that at least some of the decrease in vasodilatation might have been due to reactive oxygen species, mainly superoxide, inactivating nitric oxide, which is itself a free radical. To explore this in more detail, we incubated two types of cultured endothelial cells, human aortic and human dermal microvascular endothelial cells, with oxidized LDL. We found that oxidized LDL, but not control LDL, increased reactive oxygen species generation in both types of cells (Figure 3). It has previously been shown that LDL oxidized by copper at pH 7.4 bound to LOX-1 on bovine aortic endothelial cells and increased superoxide formation by the cells, which inactivated nitric oxide and decreased its levels inside the cells [27].

We then investigated the activation by phosphorylation of eNOS in cultured human aortic endothelial cells. eNOS is activated by phosphorylation at ser-1177 (in the human enzyme) by Akt [28,29]. Western blotting and immunofluorescence microscopy showed that acetylcholine increased the phosphorylation of eNOS, but oxidized LDL had no major effect on this activation (Figures 4 and 5).

In conclusion, LDL oxidized under lysosomal conditions might have inhibited endothelium-dependent vasodilatation by inactivating nitric oxide with superoxide radicals. These results raise the possibility that LDL oxidized in lysosomes of macrophages might decrease vasodilatation of arteries when the macrophages die and lyse releasing their oxidized LDL into the interstitial space of atherosclerotic lesions. This adds to the list of potentially atherogenic effects caused by the oxidation of LDL in the lysosomes of macrophages, namely the increased secretion of pro-inflammatory cytokines and the increase in pH of the lysosomes [30].

Acknowledgements

We thank our colleagues who donated and took blood for LDL isolation,

Author contributions

H.K.M.A. and A.J.M. carried out the experiments and H.K.M.A., A.J.M. and D.S.L. wrote the paper.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

The study was funded by the Ministry of Higher Education and Scientific Research, Iraq, the College of Pharmacy, University of Kerbala, Iraq. (H.K.M.A) and a grant from the British Heart Foundation (PG/19/57/34568) (A.J.M.).

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