oxLDL induces injury and defenestration of human liver sinusoidal endothelial cells via LOX1

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Abstract

Non-alcoholic fatty liver disease is associated with hepatic microangiopathy and liver inflammation caused by type 2 diabetes mellitus. Oxidised LDL (oxLDL) is involved in proinflammatory and cytotoxic events in various microcirculatory systems. The lectin-like oxLDL receptor 1 (LOX1) plays a crucial role in oxLDL-induced pathological transformation. However, the underlying mechanism of oxLDL's effects on liver microcirculation disturbances remains unclear. In this study, we investigated the effects of oxLDL on LOX1 (OLR1) expression and function, as well as on the fenestration features of human liver sinusoidal endothelial cells (HLSECs) *in vitro*. Primary HLSECs were obtained and cultured. The cells were treated with various concentrations of oxLDL (25, 50, 100 and 200 µg/ml), and the cytotoxicity and expression of LOX1 were examined. Furthermore, *LOX1* knockdown was performed using siRNA technology, and the changes in intracellular reactive oxygen species (ROS), NFκB, p65, (p65), endothelin 1 (ET1 (EDN1)), eNOS (NOS3) and caveolin 1 (CAV1) levels were measured. Cells were treated with 100 µg/ml oxLDL, and the fenestra morphology was visualised using scanning electron microscopy. oxLDL significantly increased LOX1 expression at both the mRNA and protein levels in HLSECs in a dose- and time-dependent manner. oxLDL stimulation increased ROS generation and NFκB activation, upregulated ET1 and caveolin 1 expression, down-regulated eNOS expression and reduced the fenestra diameter and porosity. All of these oxLDL-mediated effects were inhibited after *LOX1* knockdown. These results reveal a mechanism by which oxLDL stimulates the production of LOX1 through the ROS/NFκB signalling pathway and by which LOX1 mediates oxLDL-induced endothelial injury and the defenestration of HLSECs.

Key Words

- ▶ oxidised LDL
- ▶ lectin-like oxLDL receptor 1
- ▶ endothelial injury
- ▶ defenestration
- ▶ human liver sinusoidal endothelial cells

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Introduction

Type 2 diabetes mellitus (T2DM) is potentially complicated by hepatic microangiopathy and liver inflammation (Hudacko *et al.* 2009) and is an independent risk factor for the formation and development of non-alcoholic fatty liver disease (NAFLD; Krishnan *et al.* 2013). Metabolic

syndrome plays a major role in the pathogenesis of both NAFLD and T2DM (El-Serag *et al.* 2004). NAFLD is commonly observed in adults and adolescents with T2DM (Lv *et al.* 2013). Cross-sectional studies have revealed that the prevalence of NAFLD in patients with

T2DM ranges from 42.1 to 75.2% in China (Zhou *et al.* 2007). However, the cause–effect relationship between these conditions is complex and difficult to decipher.

NAFLD is associated with hepatic sinusoid microcirculation (Farrell *et al.* 2008). Liver sinusoidal endothelial cells (LSECs) play important roles in regulating hepatic sinus function. LSECs line the capillaries of the microvasculature and possess fenestrae that facilitate filtration between the liver parenchyma and sinusoids by serving as a selectively permeable barrier (Braet *et al.* 1995, Braet & Wisse 2002). Results have indicated that NAFLD is associated with dysfunction of LSECs (Braet *et al.* 2002). Sinusoidal endothelial fenestrae, which represent complete pores in LSECs, play important roles in liver physiology and disease. Alterations in LSEC fenestration have implications for liver function, including lipoprotein metabolism (Le Couteur *et al.* 2002). Preclinical studies have demonstrated that LSECs undergo defenestration as an early event that precedes liver fibrosis (Pasarín *et al.* 2012), and endothelin 1 (ET1) and caveolin 1 (Cav1) induce defenestration in LSECs (Yokomori *et al.* 2006, 2009).

Oxidised LDL (oxLDL) is formed in large quantities in the circulating blood of diabetic patients (Holvoet *et al.* 2008) and is a crucial risk factor for atherosclerosis (AS) and diabetic microangiopathy (Lopes-Virella *et al.* 1999, Witztum & Steinberg 2001, Pirillo *et al.* 2013). oxLDL can lead to oxidative stress and an inflammatory response (Zmijewski *et al.* 2005). oxLDL has also been reported to facilitate the formation of more oxLDL and other pro-inflammatory cytokines through a vicious cycle mediated by a specific receptor known as the lectin-like oxidised LDL receptor 1 (LOX1; Mehta *et al.* 2006). LOX1 is one of the major scavenger receptors (SRs) responsible for binding, internalising and degrading oxLDL (Sawamura *et al.* 1997). LOX1 activation is associated with many pathophysiological events, including endothelial cell (EC) and vascular smooth muscle cell proliferation, alterations in cell cycle signals, apoptosis and autophagy (Zabirnyk *et al.* 2010, Yoshimoto *et al.* 2011). Accumulating evidence indicates that oxLDL binding to LOX1 increases intracellular reactive oxygen species (ROS), activates the NFκB signaling pathway, decreases intracellular nitric oxide (NO) and increases endothelin 1 (ET1), which could lead to endothelial dysfunction (Cominacini *et al.* 2000, Morawietz *et al.* 2002, Fleming *et al.* 2005).

oxLDL is involved in liver microcirculation disturbance and NAFLD (Oteiza *et al.* 2011, Walenbergh *et al.* 2013). oxLDL can activate hepatic Kupffer and stellate cells, trigger inflammation and fibrogenesis and induce sinusoidal endothelial dysfunction (Dorman *et al.* 2006,

Li *et al.* 2011). Atherogenic blood-borne oxLDL is primarily removed by cells lining the liver sinusoids (McCuskey 2006). LSECs play an important role in the blood clearance of oxLDL (Li *et al.* 2011). Various SRs can bind to and/or mediate endocytosis of oxLDL in LSECs (Steinberg 1997). We have previously demonstrated that LOX1 is present in LSECs. However, the role of LOX1 in oxLDL-mediated LSEC dysfunction has not been specifically investigated. In addition, whether changes in the fenestration of LSECs are the result of oxLDL binding to LOX1 is largely unknown.

In this study, we explored models of oxLDL-induced injury and *LOX1* (*OLR1*) gene knockdown in human LSECs (HLSECs) to investigate the effects of oxLDL on endothelium function and the fenestration characteristics of HLSECs *in vitro*.

Materials and methods

Reagents

DMEM and foetal bovine serum (FBS) were purchased from Gibco-BRL (Gibco). The NO assay kit was purchased from Nanjing Jiancheng Bioengineering Institute (China). The primer pairs for *GAPDH*, *LOX1*, *ET1* (*EDN1*), endothelial nitric oxide synthase (*eNOS* (*NOS3*)) and *CAV1* were synthesised by TaKaRa Biotechnology Co., Ltd. (Dalian, China). TRIzol reagent, RNase inhibitor, *LOX1* siRNA vector, Xfect transfection reagent and the RT-PCR kit were purchased from TaKaRa Bio, Inc. Rabbit antibodies to LOX1, CD31, von Willebrand factor (vWF), ET1, eNOS, caveolin 1, NFκB and GAPDH, and goat anti-rabbit HRP-conjugated secondary antibodies were purchased from Abcam (Xinjie, Hong Kong, China) Ltd; 2',7'-dichloro-fluorescein diacetate (DCFH-DA) was obtained from Invitrogen. OxLDL was purchased from Peking Union-Biology Co., Ltd (Beijing, China). All other chemicals of analytical grade were purchased from commercial suppliers.

Primary human LSEC isolation, culture and identification

Human liver tissue was obtained from a single 53-year-old Chinese woman undergoing resection for liver cysts under sterile conditions. Informed consent was obtained from the patient and this study was approved by the ethics research committee of The First Clinical College of Lanzhou University. HLSECs were isolated from 25 g of human liver tissue as described previously (Danecker *et al.* 1998). HLSECs were cultured in DMEM supplemented with 10% (v/v) FBS (Gibco), penicillin, streptomycin and glutamine (100 U/ml,

100 and 292 mg/ml respectively), as well as hepatocyte growth factor and vascular endothelial growth factor (both 10 ng/ml) under standard cell culture conditions (humidified atmosphere with 5% CO₂ at 37 °C). Cells were identified using an anti-CD31 antibody via immunocytochemistry and characteristic morphology in culture. Cells were cultured to confluence and were used in all experiments.

Experimental protocol

First, to investigate the effects of oxLDL on LOX1 expression in HLSECs, the cells were treated with oxLDL (25, 50, 100 or 200 µg/ml) for 12, 24 or 48 h. Secondly, to further examine the role of LOX1 in oxLDL-induced functional injury of HLSECs, LOX1 expression was silenced. Cells were successfully transfected with LOX1 siRNA or pretreated with 250 µg/ml polyinosinic-cytidylic acid (PCA, a LOX1 inhibitor). The cells were divided into four experimental groups (the control, LOX1 siRNA, scrambled siRNA (control siRNA) and LOX1 inhibitor groups) and exposed to oxLDL (100 µg/ml) for 24 h. The levels of LOX1, ROS, p65, eNOS, ET1, CAV1 and GAPDH were then determined; the characteristics of fenestration were simultaneously detected.

Cell viability measurement

Cells were seeded at a density of 5×10^4 cells/ml in 96-well plates with various concentrations (25, 50, 100 and 200 µg/ml) of oxLDL for 6, 12, 24 or 48 h, and the cell viability was measured using the MTT assay. Briefly, the culture supernatant was removed at the indicated time points after the treatment, and the cells were washed with PBS and incubated with MTT (5 mg/ml) in the culture medium at 37 °C for an additional 3 h. After MTT removal, the coloured formazan was dissolved in 100 µl DMSO. The absorption values were measured at a wavelength of 490 nm using a Thermo Scientific Microplate Reader (Multiskan Spectrum, Thermo LabSystems, Philadelphia, PA, USA). The viability of HLSECs in each well is presented as a percentage of that of control cells.

Designing the LOX1 siRNA sequence and constructing the LOX1 silencing plasmid

For LOX1 knockdown, LOX1 siRNAs were designed based on human LOX1 cDNA. The mRNA sequences of human LOX1 were retrieved from GenBank (ID: NM_002543.3). The interference sequences were designed using the siRNA Design Support System R5: target sense sequence,

Table 1 Designed shRNA oligonucleotide sequence for LOX1 siRNA

	shRNA oligonucleotide sequence (5'-3')
Top strand	GATCCCCCTTGCTCGGAAGCTGAATGAGAATTCAA-GAGATTCTCA TTCAGTTCCGAGCAAGGGTTTTTG
Bottom strand	AATTCAAAAAACCCCTTGCTCGGAAGCTGAATGA-GAATCTCTTGAA TTCTATTAGCTTCCGAG-CAAGGGG

5'-CCCTTGCTCGGAAGCTGAATGAGAA-3'; target anti-sense sequence, 5'-TTCTCATTCAGCTTCCGAGCAAGGG-3'. Two complementary oligonucleotides (a top strand and a bottom strand) were synthesised (Table 1). Each oligonucleotide was composed of a target sequence, the hairpin loop TTCAAGAGA, the terminator sequence TTTTTT and restriction sites (BamHI/EcoRI) added at both ends of the sequences. The complementary oligonucleotides were annealed, and the pSIREN-RetroQ-DsRed-Express vector was linearised by BamHI/EcoRI enzyme cleavage. The annealed oligonucleotide was ligated into pSIREN-RetroQ-DsRed-Express using T₄ DNA ligase. The connected product (pSIREN-RetroQ-DsRed-Express-LOX1 shRNA) was transformed into JM109 competent cells (code no. D9052) using the heat shock method. Subsequently, positive clones were screened, and plasmids were extracted for PCR identification and sequencing identification. Similarly, a control plasmid was constructed by randomising the shRNA sequence (5'-GGA-GAGCATCTCATTCGATGCAGAC-3').

Cell transfection

Cells were seeded into 6-well plates. When confluent, cells were transfected with pSIREN-RetroQ-DsRed-Express-LOX1 shRNA (7.5 µg) or control plasmid (scrambled siRNA) using Xfect transfection reagent (TaKaRa) according to the manufacturer's instructions. The transfection efficiency was monitored by fluorescence intensity and LOX1 expression levels. A transfection rate above 70% was used for the other experiments. The cell phenotype and characteristics were evaluated after successful transfection.

Immunofluorescence

The cells were grown in 4-well chamber slides, fixed, quenched, blocked and incubated with an antibody against vWF (1:500, Abcam), or CD31 (1:500, Abcam) overnight at 4 °C. Fluorescently tagged secondary antibodies were used at 1:1000 dilution. Nuclear counterstaining was performed

with DAPI. Cells were mounted and imaged by confocal microscopy (LEXT OLS4000).

Measurement of intracellular ROS

Intracellular ROS was measured using the fluorescent signal H_2DCF -DA. Cells cultured in 6-well plates were incubated with 100 μ g/ml oxLDL. Then, the cells were harvested after the target time had been reached and incubated with 10 μ mol/l DCFH-DA for 30 min at 37 °C. DCFH-DA is converted by intracellular esterases to DCFH, which is oxidised into the high-fluorescence dichlorofluorescein (DCF) in the presence of a proper oxidant. HLSECs were washed with ice-cold PBS three times and placed in the dark. Subsequently, the DCF fluorescence was measured by flow cytometry at 488 and 525 nm. The results were analysed using the Cell Quest software.

Quantitative RT-PCR

Total RNA was extracted with TRIzol following the manufacturer's instructions. RNA concentrations were determined using a spectrophotometer (Beckman Instruments, Fullerton, CA, USA). Approximately 2 μ g RNA was reverse transcribed to cDNA using the Prime Script RT reagent kit. Quantitative RT-PCR (qRT-PCR) assays were performed using the LightCycler Real-Time PCR System (Roche 480). Samples were denatured at 95 °C for 30 s followed by 40 PCR cycles of 95 °C for 5 s, 60 °C for 30 s and 72 °C for 60 s. A melting curve was used to confirm the formation of the intended PCR products. The results are expressed as the fold difference relative to the level of GAPDH using the $2^{-\Delta\Delta CT}$ method. Each reaction was performed in triplicate. The *LOX1* (NM_002543.3) primers were as follows: forward, 5'-AGCAAATGGAACCTCACCAC-CAG-3'; reverse, 5'-CTTGGCATCCAAAGACAAGCAC-3'. *GAPDH* (NM_002046.2) was used as an endogenous control with the following primers: forward, 5'-CCACC-CATGGCAAATTCATGGCA-3'; reverse, 5'-TCTAGACGG-CAGGTCAGGTCCACC-3'.

Western blot analysis

Cells were lysed for 30 min at 4 °C in lysis buffer. The extracted total protein concentrations were measured using the bicinchoninic acid reagent. Equal amounts of lysate proteins were loaded and separated by SDS-PAGE and transferred onto polyvinylidene fluoride membranes. After incubation in a blocking solution (5% non-fat milk, Sigma),

the membranes were immunoblotted with primary anti-NF κ B (phos-p65) (1:1500), anti-LOX1 (1:2000, Abcam), anti-eNOS (1:2000), anti-ET1 (1:2000), anti-caveolin 1 (1:2000), or anti-GAPDH (1:2500) overnight at 4 °C. Membranes were washed and then incubated in a 1:3000 dilution of the specific secondary antibodies for 1.5 h at room temperature, and the membranes were washed. Following enhanced chemiluminescence development, the exposed film was later developed. The films were scanned using Image Quant 350 (GE Healthcare, Little Chalfont, Buckinghamshire UK), and the relative densities of protein bands were analysed using the Image J software (National Institutes of Health, Bethesda, MD, USA). The density of each protein band was normalised to that of GAPDH.

Scanning electron microscopy

LSECs cultured on glass coverslips were fixed in 1.2% glutaraldehyde buffered with 1 M cacodylate buffer (pH 7.4) for 1 h at 4 °C and post-fixed with 1% osmium tetroxide in cacodylate buffer (pH 7.4) for 1 h at 4 °C. After dehydration in a graded series of ethanol solutions, the cultured cells were dried in a critical point apparatus (HCR-2, Hitachi) and coated with gold in a vacuum coating unit. The fenestrae were observed for each experimental variable under a scanning electron microscope (SU8010, Hitachi) at a 15-kV acceleration voltage and $\times 20\,000$ magnification.

Statistical analysis

All data are expressed as mean $\pm$ s.d. from at least three independent experiments each performed in triplicate. All data were analysed by one-way ANOVA followed by the Bonferroni comparison using SPSS 17.0. A *P* value of <0.05 was considered statistically significant.

Results

Isolated HLSECs express CD31 in culture

We observed that cultured primary HLSECs were polygonal or fusiform with a cobblestone appearance, as demonstrated by inverted microscopy after 2–4 days in culture (Fig. 1A). To confirm the identity of HLSECs, we assessed the expression of CD31 in HLSECs, after culturing the cells for 48 h, using immunofluorescence (IF) staining and nuclear counterstaining. We found that the cells expressed CD31, as indicated by green and blue fluorescence under an inverted fluorescence microscope (Fig. 1B).

Figure 1

Characterisation and identification of isolated human liver sinusoidal endothelial cells (HLSECs) *in vitro*. (A) HLSECs were isolated from human liver and cultured. HLSEC morphology was assessed on day 2 or 4 of culture by inverted microscopy. The cells grew by static adherence, and the shape of the cells was fusiform or polygon, resembling cobblestones. (B) HLSECs isolated from human liver were cultured for 2 days and stained with anti-CD31 antibodies. Fluorescence images were acquired at an original magnification of 20 $\times$ (green, CD31 expression; blue, nuclear counterstaining with DAPI).

oxLDL exhibits cytotoxic effects on HLSECs *in vitro*

To investigate whether oxLDL is cytotoxic to HLSECs, cells were treated with 25, 50, 100 or 200 $\mu\text{g}/\text{ml}$ oxLDL for 6, 12, 24 or 48 h and then assessed using the MTT assay. We observed that the cell viability decreased in a dose- and time-dependent manner ($P < 0.05$; Fig. 2A), whereas the native LDL had no effect. oxLDL concentrations $> 100 \mu\text{g}/\text{ml}$ had noticeable cytotoxic effects. Furthermore, exposure to 200 $\mu\text{g}/\text{ml}$ oxLDL for 12 h caused a significant (23.3%) decrease in cell viability; increased exposure for 48 h decreased the viability of HLSECs (by 42% total from time 0).

oxLDL increased LOX1 expression in HLSECs

To determine whether LOX1 is involved in oxLDL-induced inflammatory responses in HLSECs, we examined the effect of oxLDL on LOX1 expression. HLSECs were treated with 25, 50, 100 and 200 $\mu\text{g}/\text{ml}$ oxLDL for 6, 12, 24 or 48 h. Both qRT-PCR (Fig. 2B) and western blotting (Fig. 2C) results revealed that LOX1 mRNA and protein expression gradually increased in response to oxLDL. Hence, oxLDL induced LOX1 expression in a time- and dose-dependent manner. LOX1 expression peaked at 100 $\mu\text{g}/\text{ml}$ and 24 h (3.1-fold vs control, $P < 0.05$);

therefore, this concentration was used for all subsequent experiments.

The transfected HLSECs maintain specific markers and limited fenestrae

To confirm endothelial phenotype and characterisation of transfected HLSECs, the classic EC markers, vWF and CD31 were analysed using IF staining and nuclear counterstaining, and fenestrae were visualised using scanning electron microscopy (SEM). Compared with the isolated HLSECs, we found that transfected cells maintained similar expression of vWF and CD31 (Fig. 3A and B) and maintained limited transcytoplasmic fenestrations (Fig. 3C, arrows).

LOX1 siRNA successfully suppressed LOX1 mRNA and protein expressions

To ensure the efficiency of LOX1 gene silencing, we designed three pairs of siRNA for LOX1 cDNA. After transfection, we screened for the most efficacious siRNA for LOX1 silencing using RT-PCR and western blotting. As shown in Fig. 3D, red fluorescence intensity was clearly increased relative to the control, indicating that the HLSECs had successfully been transfected with the LOX1 siRNA. The LOX1 mRNA and protein expressions were significantly decreased compared with the positive control (100 $\mu\text{g}/\text{ml}$ oxLDL, $P < 0.05$), and the control plasmid had no effect. The inhibition efficiencies of LOX1 siRNA on mRNA and protein expressions were 77 and 74% respectively (Fig. 3E and F). Moreover, PCA, which is a LOX1 inhibitor, imparted a weak reduction in LOX1 expression compared with LOX1 siRNA treatment; the inhibition efficiencies of PCA on LOX1 mRNA and protein expressions were 21 and 32% respectively (Fig. 3E and F).

oxLDL stimulated ROS formation and NF κ B activation in HLSECs through LOX1

To confirm that LOX1 is involved in oxLDL-induced ROS/NF κ B activation, we investigated the influence of oxLDL (100 $\mu\text{g}/\text{ml}$) on ROS formation and NF κ B activation in HLSECs under control conditions, LOX1 siRNA transfection and LOX1 inhibitor treatment for 24 h. As shown in Fig. 4A, oxLDL markedly enhanced ROS formation and activated p65 in HLSECs (Fig. 4B and C). Interestingly, LOX1 silencing significantly attenuated oxLDL-induced ROS formation (Fig. 4A) and inhibited p65 expression and NF κ B activation (Fig. 4B and C).

Figure 2

Effect of oxidised LDL (ox-LDL) on cytotoxicity and the expression of lectin-like oxidised LDL receptor 1 (LOX1) in cultured HLSECs. HLSECs were treated with ox-LDL (25, 50, 100 and 200 $\mu\text{g/ml}$) for 6, 12, 24 or 48 h. (A) The effect of oxLDL on HLSEC viability (control is native LDL). (B) Quantitative

real-time PCR analysis of *LOX1* mRNA. The results were normalised to *GAPDH* mRNA levels. (c) Western blotting for *LOX1* protein expression in HLSECs normalised to *GAPDH* (37 kDa) protein levels. All data are expressed as mean $\pm$ s.d. from three independent experiments. * $P < 0.05$ vs control.

in HLSECs. PCA had similar but milder effects (Fig. 4A, B and C). These results indicate that oxLDL induced marked oxidative stress and that LOX1 mediated the activation of the ROS/NF κ B pathway in oxLDL-treated HLSECs.

oxLDL stimulation reciprocally decreases eNOS via LOX1

We investigated the influence of oxLDL on HLSEC eNOS expression using western blotting. As shown in Fig. 4D, eNOS expression at 24 h was decreased by 0.55-fold after exposure to 100 $\mu\text{g/ml}$ oxLDL in HLSECs compared with controls. Similarly, *LOX1* silencing and *LOX1* inhibition elevated oxLDL-induced eNOS expression by 45.5 and 16% respectively (Fig. 4D). Taken together, these results indicate that oxLDL downregulates eNOS expression via *LOX1* in cultured HLSECs.

oxLDL upregulates ET1 and caveolin 1 via LOX1 in HLSECs

To ascertain whether *LOX1* is involved in the effect of oxLDL on ET1 and caveolin 1 (CAV1), we examined the effects of oxLDL on ET1 and CAV1 expression in HLSECs. Cells were pretreated with oxLDL (100 $\mu\text{g/ml}$) for 24 h. oxLDL exposure notably increased ET1 and CAV1 levels in HLSECs compared with controls ($P < 0.05$): ET1 and CAV1 were overexpressed by 3.0- and 2.7-fold respectively (Fig. 4E and

F). The treatment of cells with *LOX1* siRNA markedly decreased ET1 and CAV1 levels to 65 and 48% respectively; however, PCA inhibition resulted in milder effects (Fig. 4E and F). These results demonstrate that oxLDL promotes the increase in ET1 and CAV1 expression observed with oxLDL treatment and further confirm that *LOX1* plays a critical role in mediating the ET1 and Cav1 response to oxLDL in HLSECs.

oxLDL stimulated fenestra contraction and defenestration via LOX1

We examined whether oxLDL interacts with the fenestrae of HLSECs. We used SEM at 5000 $\times$ and 20 000 $\times$ magnification to observe the porosity and number of fenestrae in oxLDL (100 $\mu\text{g/ml}$)-treated HLSECs for 24 h. As shown in Fig. 5 and Table 2, we observed that oxLDL caused a significant decrease in the porosity, diameter and number of fenestrae compared with the control group (Fig. 5A and B, a and b, arrows), *LOX1* silencing and *LOX1* inhibitor treatment maintained a limited number of fenestrae and prevented fenestra contraction when HLSECs were treated with oxLDL (Fig. 5A and B, c, d, e, f, g and h, arrows), indicating that oxLDL induced both the contraction of fenestrae and defenestration in HLSECs and that *LOX1* plays an important role in this pathway.

Figure 3

Characterisation of transfected HLSECs and transfection efficiency of *LOX1* siRNA. HLSECs were transfected with a retrovirus *LOX1* siRNA plasmid (Re-siRNA) or with a scrambled siRNA control plasmid (Re-scr-siRNA), then the specific phenotype markers and fenestrae of cells were visualised. In addition, HLSECs were treated previously with a *LOX1* inhibitor (PCA 250 μ g/ml). All samples were treated with 100 μ g/ml oxLDL for 24 h. Untreated controls were run in parallel. (A) Immunofluorescent (IF) staining confirms homogeneous expression and demonstrates a cytoplasmic and plasma membrane distribution of vWF (green) with nuclear counterstaining (blue). (B) IF staining confirms homogeneous expression

and demonstrates a cytoplasmic and plasma membrane distribution of CD31 (green). (C) Scanning electron microscopy (SEM) demonstrates that transfected HLSECs maintain a limited number of fenestrae (white arrows). (D) Fluorescence intensity analysis of Re-*LOX1* siRNA and Re-scr-siRNA transfection. (E) Quantitative RT-PCR analysis of *LOX1* mRNA in various samples normalised to *GAPDH* mRNA levels. (F) Western blotting was performed to assess *LOX1* protein expression in various samples. *GAPDH* served as a loading control. (E and F) Data expressed as mean $\pm$ s.d. from three independent experiments, * $P < 0.05$ vs control.

Figure 4

Role of LOX1 in oxLDL-mediated injury in cultured HLSECs. Cells were transfected with LOX1 siRNA and blocked with a LOX1 inhibitor (PCA 250 µg/ml). All samples were treated with 100 µg/ml oxLDL for 24 h. Untreated intact samples were run as a control in each experiment. (A) Relative ROS formation was measured as fluorescence intensity. (B) Western blotting results showing relative p65 expression. (C) Western blotting results showing

relative phos-p65 expression of NFκB activation. (D) Western blotting results showing relative eNOS expression. (E) Western blotting results showing relative ET1 expression. (F) Western blotting results showing relative Cav1 expression. (A, B, C, D, E and F) All results are expressed as mean ± s.d. from three independent experiments, * $P < 0.05$ vs control (NC), ** $P < 0.05$ vs NC + oxLDL. (B, C, D, E, and F) GAPDH (37 kDa) represents the loading control.

Figure 5

The role of LOX1 in oxLDL-mediated defenestration in cultured HLSECs. Cells were transfected with LOX1 siRNA and blocked with a LOX1 inhibitor (PCA 250 µg/ml). All samples were treated with 100 µg/ml oxLDL for 24 h. Untreated intact samples were used as controls. Scanning electron microscopy (SEM) images at 5000 $\times$ (A) and 20 000 $\times$ (B) magnification of LSECs demonstrate the relative diameter and porosity of the fenestrae. (a) Fenestrae or clusters of fenestrae (sieve plates) in normal cultured HLSECs (white arrow). (b) Fenestra contraction, reduction and defenestration in 100 µg/ml oxLDL-treated HLSECs (white arrow). (c) Fenestrae or clusters of fenestrae (sieve plates) in HLSECs transfected with Re-LOX1 siRNA (white arrow). (d) Fenestra contraction and dispersion in 100 µg/ml oxLDL-treated HLSECs transfected with Re-LOX1 siRNA (white arrow). (e) Fenestrae or clusters of fenestrae (sieve plates) in HLSECs transfected with Re-scrambled siRNA (white arrow). (f) Fenestra contraction, reduction and defenestration in 100 µg/ml oxLDL-treated HLSECs transfected with Re-scrambled siRNA (white arrow). (g) Fenestrae or clusters of fenestrae (sieve plates) in HLSECs inhibited with PCA (white arrow). (h) Fenestra contraction and reduction in 100 µg/ml oxLDL-treated HLSECs inhibited with PCA (white arrow).

Discussion

Endothelial dysfunction is a major cause of diabetic microangiopathy (Cameron & Cruickshank 2007, Renier *et al.* 2007), and oxidative damage is a critical step in this process (Hink *et al.* 2001, Hamilton & Watts 2013). Hepatic sinusoid endothelial dysfunction is an early event implicated in the progression to NAFLD (Pasarín *et al.* 2012). This dysfunction is known to impair endothelium-dependent relaxation in the liver microcirculation and contribute to increased intrahepatic vascular resistance (Yokomori *et al.* 2012). oxLDL has been implicated as a causal factor in the pathogenesis of AS and endothelial dysfunction (Lopes-Virella *et al.* 1999, Galle *et al.* 2000). Evidence increasingly indicates that oxLDL has numerous deleterious effects through inducing the expression of certain surface molecules on ECs (Mertens & Holvoet 2011), stimulating EC apoptosis, inducing ROS formation, uncoupling eNOS and impairing endothelial NO formation (Maziere *et al.* 1996, Cominacini *et al.* 2000, Fleming *et al.* 2005).

Responses to oxLDL in the endothelium are mediated by LOX1 (Cominacini *et al.* 2001).

The focus of the present article is the effect of oxLDL on LOX1 expression, tissue injury and the fenestration characteristics of HLSECs. Our results indicated for the first time, to our knowledge, that oxLDL increases LOX1 expression and induces injury through ROS formation, NF κ B activation, eNOS downregulation, ET1 and CAV1 upregulation and defenestration in HLSECs. These processes are mediated via LOX1 and blocked by a LOX1-directed siRNA or a LOX1 inhibitor.

LOX1 not only internalises modified lipids but also causes endothelial dysfunction, apoptosis, inflammation and smooth muscle cell proliferation, contributing to the process of atheroma formation at multiple levels (Xu *et al.* 2013). LOX1 has been identified as the main endothelial receptor for oxLDL (Sawamura *et al.* 1997). Evidence increasingly indicates that LOX1 activity promotes vascular dysfunction and AS (Pirillo *et al.* 2013). Through interactions with LOX1, oxLDL activates ECs and induces endothelial dysfunction and apoptosis (Mehta *et al.* 2006). In this study, we found that LOX1 is highly expressed in primary HLSECs upon oxLDL stimulation, and the effect of oxLDL is dose- and time-dependent in vascular ECs (Li & Mehta 2000, Ishino *et al.* 2007). Results from a previous study have indicated that LOX1 expression is increased in atherosclerotic plaques when compared with normal endothelial tissue (Ishino *et al.* 2007), and *Lox1* knockout mice exhibit markedly smaller atherosclerotic lesions (Mehta *et al.* 2007), indicating that LOX1 plays an important role in HLSEC dysfunction.

oxLDL is a proatherogenic lipoprotein that has been identified as a potent stimulus for vascular ROS and has been suggested to modulate gene expression in vascular cells by binding to the receptor LOX1 (Mehta *et al.* 2006). High levels of ROS are produced in several disease conditions, including AS, and contribute to endothelial dysfunction (Ballinger 2005, Doughan *et al.* 2008). ROS are involved in LDL oxidation and mediate many of its biological effects by generating more intracellular ROS through binding to LOX1. LOX1 activation can stimulate ROS generation, indicative of a positive feedback loop between ROS and LOX1. Indeed, ROS enhances LOX1 expression, and LOX1 enhances ROS production (Galle *et al.* 2006, Chen *et al.* 2009). Our results are consistent with these findings, demonstrating that oxLDL markedly increases ROS formation. Furthermore, a LOX1-directed siRNA and PCA both inhibited oxLDL-induced ROS formation, indicating that reducing LOX1 expression might suppress ROS generation and inhibit the

Table 2 The effects of oxLDL on the fenestrae of HLSECs determined by analysis of images obtained by electron microscopy. Results are expressed as mean $\pm$ s.d. of fields from each experiment ($n=3$). Cells were transfected with *LOX1* siRNA and blocked with a LOX1 inhibitor (PCA 250 μ g/ml). All samples were treated with 100 μ g/ml oxLDL for 24 h. Untreated intact samples were used as controls. The diameter, frequency and porosity of the fenestrae were examined by scanning electron microscopy (SEM) at $\times 5000$ or $\times 20\,000$ magnification. Data are expressed as mean $\pm$ s.d. from analysis of images from each experiment ($n=3$ for each group). The eight groups were compared by ANOVA

	Normal		Re-siRNA		Re-scr-siRNA		PCA	
	–	oxLDL	–	oxLDL	–	oxLDL	–	oxLDL
Fenestration frequency (number/ μ m ²)	6.5 $\pm$ 0.5	4.0 $\pm$ 0.5*	6.3 $\pm$ 0.5	5.7 $\pm$ 0.6* [†]	6.2 $\pm$ 0.5	4.1 $\pm$ 0.5*	6.3 $\pm$ 0.5	4.7 $\pm$ 0.5* [†]
Porosity (%)	4.6 $\pm$ 0.4	2.7 $\pm$ 0.3*	4.7 $\pm$ 0.4	4.4 $\pm$ 0.3* [†]	4.4 $\pm$ 0.4*	2.6 $\pm$ 0.4*	4.5 $\pm$ 0.3	3.4 $\pm$ 0.3* [†]
Fenestration diameter (nm)	127 $\pm$ 4	49 $\pm$ 2*	115 $\pm$ 3 [†]	101 $\pm$ 4* [†]	117 $\pm$ 4 [†]	51 $\pm$ 2*	122 $\pm$ 3	76 $\pm$ 3* [†]

* $P<0.05$ vs control (–), [†] $P<0.05$ vs normal (–), [‡] $P<0.05$ vs normal + oxLDL.

positive feedback loop. NF κ B is a relevant transcription factor for a variety of inflammatory mediators (Mohamed & McFadden 2009). Recent evidence has indicated that the binding of oxLDL to LOX1 activates the NF κ B signalling pathway and causes cell injury (Cominacini *et al.* 2000). Our findings indicate that NF κ B is activated by oxLDL and might serve as an intermediate link in the inflammatory response that occurs during HLSEC dysfunction. Inhibition of LOX1 expression with an siRNA or an inhibitor decreased oxLDL-induced ROS formation and NF κ B activation. Our results indicate that oxLDL-induced NF κ B activation is at least partially mediated via LOX1 in HLSECs.

NO, a potent vasodilatory substance, is generated from L-arginine by eNOS (Channon 2004). ET1 is a potent vasoconstrictor peptide, and both ET1 and NO play pivotal roles in vascular homeostasis (Kauser *et al.* 2000). ET1 and NO work as negative feedback signals for each other (Li *et al.* 2003, Oishi *et al.* 2006, Kohan *et al.* 2011). The reduced activation of eNOS is a characteristic of vascular endothelial dysfunction (Wohlfart *et al.* 2008), and ET1 could promote early events leading to endothelial dysfunction and AS (Rautureau & Schiffrin 2012). Diminished production of NO and aggravated release of ET1 are thought to be key initiators of endothelial injury (Little *et al.* 2008, Li *et al.* 2011). oxLDL causes abnormalities in endothelial function by decreasing eNOS activity in ECs (Cominacini *et al.* 2001, Zhou *et al.* 2013) and further promotes a proatherosclerotic 'vicious circle' by augmenting ET1 release and superoxide anion formation in the vessel wall (Morawietz *et al.* 2002, Kalani 2008). It is therefore possible that ET1 contributes to endothelial dysfunction both directly, through its vasoconstrictor effects, and indirectly, through inhibition of NO production. Liver sinusoidal endothelial dysfunction with

decreased intrahepatic NO production has long been considered a relevant pathogenic factor in the progression of liver cirrhosis (Fraser *et al.* 2012). We demonstrated that oxLDL increases ET1 expression and decreases eNOS expression in HLSECs and thereby impairs endothelial dysfunction. Similarly, our findings indicate that *LOX1* gene silencing suppresses ET1 expression and increases eNOS expression in cultured HLSECs. These findings further support the importance of LOX1 in oxLDL-stimulated dysfunction in HLSECs.

CAV1 is a membrane protein involved in the maintenance of fenestrae (Braet *et al.* 2003) and has been observed in the fenestrae of isolated LSECs (Ogi *et al.* 2003). CAV1 potentially plays an important positive role in the regulation of LSEC tubular formation (Yokomori *et al.* 2009). Recent studies demonstrate that CAV1 expression increases oxLDL uptake by hepatic cells and mediates oxLDL endocytosis and transcytosis by ECs (Truong *et al.* 2009, Sun *et al.* 2010). Furthermore, oxLDL upregulates CAV1 expression and downregulates eNOS expression at the mRNA and protein levels in HUVECs (Ma *et al.* 2012). oxLDL upregulates CAV1 expression in macrophages via the MAPK/NF κ B pathway (Wu *et al.* 2009). Consistent with these reports, we found that oxLDL upregulated CAV1 expression in HLSECs; this effect was inhibited by blocking LOX1 using an siRNA or PCA. Thus, our results indicated that LOX1 is involved in oxLDL-induced CAV1 expression in HLSECs.

Defenestration of LSECs influences the hepatic clearance of medications owing to the impaired transfer of protein-bound substrates or dissolved substrates through the fenestrations (Braet *et al.* 2003). LSEC defenestration occurs in models of pathological liver states, such as cirrhosis and type 1 diabetic liver (Fraser *et al.* 2012).

ET1, LPS and ethanol induce defenestration in LSECs (Dobbs *et al.* 1994, Wang *et al.* 2005, Yokomori *et al.* 2006). Thrombospondin 1 can cause defenestration of LSECs through the Rho–Rho kinase–myosin pathway (Venkatraman & Tucker-Kellogg 2013), and Cav1 regulates endothelial capillary-like tubular formation and fenestra contraction in sinusoidal ECs (Yokomori *et al.* 2009). Our findings indicate that expression of both ET1 and CAV1 is upregulated in oxLDL-treated HLSECs, which may be the cause of fenestra contraction and defenestration. Analysis of our scanning EM results indicated that the diameter, number and porosity of the fenestrae in oxLDL-treated HLSECs were reduced or absent. Furthermore, inhibiting LOX1 using an siRNA or an inhibitor suppressed defenestration. These associations support the conclusion that LOX1 is involved in oxLDL-induced defenestration of HLSECs. However, the mechanism by which LOX1, ET1 and caveolin 1 influence each other is unknown and requires further investigation.

In conclusion, our results demonstrate that oxLDL induces dysfunction and defenestration of HLSECs through LOX1 activation. In contrast, gene silencing or blockade of LOX1 significantly reduces those effects. These findings indicate that LOX1 mediates oxLDL-induced injury and defenestration of HLSECs. Furthermore, these results could provide us with insights into NAFLD, which is associated with LOX1 upregulation in HLSECs in T2DM.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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