Effects of oxidized low-density lipoproteins on the hepatic microvasculature

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Oxidized low-density lipoproteins (oxLDLs) are involved in proinflammatory and cytotoxic events in different microcirculatory systems. The liver is an important scavenger organ for circulating oxLDLs. However, the interaction of oxLDL with the hepatic microcirculation has been poorly investigated. The present study was conducted to examine the effects of differently modified oxLDLs on the hepatic microvasculature. C57Bl/6J mice were injected intravenously with low-density lipoprotein (LDL), or LDL oxidized for 3 h (oxLDL3) or 24 h (oxLDL24), at doses resembling oxLDL plasma levels in cardiovascular disease patients. Radiolabeled ligands were used to measure blood decay and organ distribution, and nonlabeled ligands to evaluate microcirculatory responses, examined by in vivo microscopy 30–60 min after ligand injection, immunohistochemistry, and scanning and transmission electron microscopy. Mildly oxLDL (oxLDL3) was cleared from blood at a markedly slower rate than heavily oxLDL (oxLDL24), but significantly faster than LDL (P < 0.01). Injected oxLDLs distributed to liver. OxLDL effects were most pronounced in central areas of the liver lobules where oxLDL3 elicited a significant (P < 0.05) reduction in perfused sinusoids, and both oxLDL3 and oxLDL24 significantly increased the numbers of swollen endothelial cells and adherent leukocytes compared with LDL (P < 0.05). OxLDL-treated livers also exhibited increased intercellular adhesion molecule (ICAM)-1 staining. Electron microscopy showed a 30% increased thickness of the liver sinusoidal endothelium in the oxLDL3 group (P < 0.05) and a reduced sinusoidal fenestration in central areas with increased oxidation of LDL (P for linear trend <0.05). In conclusion, OxLDL induced several acute changes in the liver microvasculature, which may lead to sinusoidal endothelial dysfunction.

The term oxLDL is used to describe a broad spectrum of heterogeneously modified lipoprotein particles (17), and clearance mechanism and affinity to scavenger receptors may vary with aggregation and degree of modification (7, 32). Heavily modified oxLDL, which is mainly present in atherosclerotic plaques (63), is effectively cleared from the circulation mainly in KCs with a significant uptake also in LSECs (35, 57), whereas in vitro studies in freshly isolated rat LSECs and KCs showed that mildly oxLDL, which is the major form of oxLDL found in plasma (6, 13, 14), was taken up in LSECs only (32). Together, these two cell populations constitute the most powerful scavenger cell system in mammals, with KCs removing particulate matter (>200 nm) by phagocytosis and LSECs removing colloids and soluble macromolecules by receptor-mediated endocytosis (47, 51).

Several authors have suggested a connection between the high levels of circulating oxLDL and impaired function of the liver cell clearance system in cardiovascular disease (13, 18, 51). Intravital microscopy studies in rodents have shown that oxLDL triggers an inflammatory response in different microcirculatory systems (i.e., mesentry and striated skin muscle), inducing rolling and recruitment of leukocytes (29–31, 33, 34). Other effects include oxLDL-stimulated expression of adhesion molecules such as P-selectin, vascular cell adhesion molecule-1 (VCAM-1), and intercellular adhesion molecule-1 (ICAM-1) (24, 55, 59). OxLDL also impairs the endothelium nitric oxide-dependent vasodilatation responses, which may lead to vascular endothelial dysfunction (33, 49). However, there are no reports about the in vivo effects of oxLDLs on the hepatic microvascular system, which harbors the most important scavenger cells for these ligands.

In addition to its important scavenger function, the liver sinusoidal endothelium is characterized by numerous open holes or fenestrae of ~100–150 nm, which allow the exchange of fluid, soluble molecules, and lipoproteins between blood and hepatocytes (3, 9). The LSEC fenestrae are dynamic structures, and the sinusoidal porosity may vary in response to diet, drugs, or diseases (3). Reduced LSEC fenestration, as seen in ageing livers (28), has been shown to contribute to an impairment of hepatocyte uptake of circulating chylomicron remnants, which may increase the risk of atherosclerosis (9, 11, 27).

On this background, we aimed to study the in vivo effects of oxLDL on the sinusoidal microvasculature of mouse livers by intravital and electron microscopic methods, after intravenous injections of oxLDL at doses resembling plasma values reported in human patients with cardiovascular disease (14).

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Because oxLDL is heterogeneously modified and different types of circulating oxLDL can be present in vivo (1, 6, 17), both heavily and mildly modified oxLDLs were used in the study.

MATERIALS AND METHODS

Experimental Animals

Male C57Bl/6j mice were purchased from Charles River Laboratory. The animals were kept in the rodent facility at the Animal Research Unit of the University of Tromsø. Animals were fed a standard soya bean-based diet (Rat and Mouse no.1 Maintenance) manufactured by Special Diet Service (SDS, Essex, UK). The diet has a low content of fat and protein (2.71% crude oil, 14.38% crude protein).

The in vivo microscopy procedure, blood clearance, and organ distribution studies were performed in mice anesthetized with a mixture of tiletamin/zolezepam (Zoletil forte vet; Virbac, Carros Cedex, France) and xylazine (Narcoxyl vet; Veterinaria, Zurich, Switzerland), given intraperitoneally to provide surgical anesthesia, followed by a subcutaneous injection of buprenorphine hydrochloride (Temgesic; Schering-Plough, Kenilworth, NJ), according to protocols approved by the Norwegian Animal Research Authority in accordance with the Norwegian Animal Experimental and Scientific Purposes Act of 1986.

Preparation of oxLDLs

LDL (density = 1.019–1.063 g/ml) was isolated from freshly prepared human plasma by NaCl density gradient ultracentrifugation, as described (45), and oxidized by incubating LDL (0.2 mg/ml) with copper sulfate (CuSO₄, 10 µM) at 37°C for 3 h (oxLDL₃) or 24 h (oxLDL₂₄). Excess amounts of EDTA (305 µM) were added to stop the reaction. Control LDL (cLDL) was produced by incubating LDL at 37°C for 24 h without CuSO₄. CuSO₄ and EDTA were removed by extensive dialysis against PBS at 4°C. Relative electrophoretic mobility (REM) of differently oxidized LDLs (42, 52) was measured by agarose gel electrophoresis in 0.75% agarose/barbitral gels. REM values of cLDL, oxLDL₃, and oxLDL₂₄ compared with non-copper-treated and nonincubated LDL (nLDL) were 0.99 ± 0.10 (n = 2), 1.46 ± 0.17 (n = 12), and 2.16 ± 0.23 (n = 7) (mean ± SD), respectively. LDLs and oxLDLs tested negatively for endotoxin by the Limulus Amebocyte Lysate pyrochrome test (Cape Cod, East Falmouth, MA).

In Vivo Microscopy

Nonradiolabeled LDLs, oxLDL₃, and oxLDL₂₄ were injected in the tail vein of anesthetized mice in similar doses as in the blood clearance studies, and the liver microcirculation was examined using established high-resolution in vivo microscopic methods (19, 41). Briefly, a compound binocular microscope (Leitz, Wetzlar, Germany) adapted for in vivo microscopy was equipped to provide transillumination as well as video microscopy. Following laparatomy, the left liver lobe of the mouse was transilluminated using a ×8/ numeric aperture 1.00 Leitz water immersion objective and ×10 oculars. This system allows the differentiation of the microvasculature into arterioles, sinusoids, and venules.

Analysis of microvascular events started at 30 min after ligand administration, and the procedure in each animal was finished ~1 h after injection. In each animal, 10 periporal and 10 centrilobular areas (microscopic fields) were observed. To examine the interaction of leukocytes with the sinusoidal wall, the number of leukocytes adhering to the endothelial lining was counted in each microscopic field. A leukocyte was defined as adherent if it remained stationary for at least 30 s. Endothelial swelling, which is thought to be a sign of endothelial activation and/or injury, was evaluated by counting the number of endothelial cells in which nuclear regions protrude across one-third or more of the lumen in the same microscopic field (19, 37).

Electron Microscopy

At the end of the in vivo microscopy procedure, the animals were killed by cervical dislocation and perfused via the left cardiac ventricle with cold PBS to remove free tracer from the vasculature. Internal organs were collected and analyzed for radioactivity. Recovery of tracer was taken as the sum of radioactivity in individual organs, rest of carcass (also containing the brain and gonads), and blood at 60 min after injection. Blood volume was calculated as described (62).

Blood Clearance and Organ Distribution of Radiolabeled Lipoproteins

Blood clearance and organ distribution of intravenously administered lipoproteins in anesthetized mice were performed as described (36). ¹²⁵I-nLDL, ¹²⁵I-cLDL, ¹²⁵I-oxLDL₃, or ¹²⁵I-oxLDL₂₄ were mixed with their respective nonradioiodinated homologous ligand (dose: 3.6 µg/g body wt; 2×10⁶ cpm/animal) and injected in the tail vein. Assuming that the mouse plasma volume is 55% of the estimated blood volume [0.08 ml/g body wt (62)], the injected dose was calculated to achieve an initial plasma concentration of radiolabeled lipoproteins of 8 µg/dl, resembling the oxLDL concentrations (1–6 µg/dl) reported in plasma of cardiovascular disease patients (14).

Blood samples (5 µl) were collected from the tail tip at short intervals for 1 h and analyzed for degraded (acid-soluble) and non-degraded (acid-precipitable) ligand (36). The acid precipitation was carried out in 20% trichloroacetic acid, which precipitates intact macromolecules and degradation products of high molecular mass, whereas free ¹²⁵I and small degradation products are recovered in the acid-soluble fractions.

The anesthetized animals were killed in 100% CO₂ 1 h after injection and perfused via the left cardiac ventricle with cold PBS to remove free tracer from the vasculature. Internal organs were collected and analyzed for radioactivity. Recovery of tracer was taken as the sum of radioactivity in individual organs, rest of carcass (also containing the brain and gonads), and blood at 60 min after injection. Blood volume was calculated as described (62).

Transmission EM. Two liver blocks from each animal were selected at random, and ultrathin sections (70–90 nm) were photographed. At the end of the in vivo microscopy procedure, the animals were killed by cervical dislocation and perfused via the left cardiac ventricle with PBS to remove blood before perfusion fixation with 4% paraformaldehyde (PFA), pH 7.4. Cut samples from the median liver lobe were further fixed in 4% PFA, dehydrated, and embedded in paraffin for light microscopy, or fixed in McDowell’s fixative (39) and processed for scanning and transmission electron microscopy (EM) (26).

Scanning EM. Specimens were postfixed in osmium tetroxide, dehydrated in ethanol, fractured in liquid nitrogen, dried in hexamethydisilazane (Sigma, St. Louis, MO), and sputter coated with 10 nm gold/palladium alloy. Two specimens per liver were chosen at random and examined in a JEOL JSM-6300 microscope; two periporal and two centrilobular areas were further chosen at random from these two blocks, and five sinusoids per area were photographed at ×8,000 magnification and analyzed for numbers of fenestrae per 10 µm².
Immunohistochemistry

Paraffin-embedded sections (5 μm) of the median liver lobe of mice used in the in vivo microscopy study were immune labeled for ICAM-1 expression. Antigen was demasked by boiling the dewaxed, rehydrated sections in 10 mM sodium citrate, 0.05% Tween 20, pH 6.0, in a microwave oven four times for 5 min. Endogenous peroxidase was quenched in 3% H2O2 in water. After a blocking step (1% BSA in PBS), sections were incubated with primary anti-ICAM-1 antibody (hamster anti-mouse CD54; 1.25 μg/ml; BD Pharmingen, San Diego, CA) in 1% BSA in PBS at 4°C overnight. Isotype-matched hamster IgG (1.25 μg/ml; BD Pharmingen) was used as control. After being rinsed, sections were incubated for 30 min at room temperature with secondary biotinylated antibody (Biotin-SP-conjugated AffiniPure Goat Anti-Armenian Hamster IgG, 1 μg/ml; eBioscience, Hatfield, UK) and thereafter with streptavidin-peroxidase complex (BD Pharmingen). Reaction products were visualized using a diaminobenzidine substrate chromogen system (BD Pharmingen). Sections were counterstained with hematoxylin, dehydrated, and mounted in a xylene-based mounting medium. Evaluation of the immune staining in periportal and centrilobular areas of the liver lobule was carried out in a blind mode giving punctuation from zero to five by three researchers individually, where zero is negative and five is strong staining. The evaluation of ICAM-1 staining took into account both the staining intensity and the extension of the stained area.

Serum Analysis of Alanine Aminotransferase

Blood samples were collected from one-half of the animals used in the in vivo microscopy study, by puncture of the right cardiac

Fig. 1. Blood clearance of low-density lipoprotein (LDL) and oxidized low-density lipoproteins (oxLDLs). Mice were injected iv with 3.6 μg/g body wt of 125I-labeled non-copper-treated and nonincubated LDL (nLDL) (A, n = 3), 125I-labeled control LDL (cLDL) (B, n = 3), 125I-labeled LDL oxidized for 3 h (oxLDL3) (C, n = 3), or 125I-labeled LDL oxidized for 24 h (oxLDL24) (D, n = 3), and blood samples were collected at different time points up to 60 min. Blood sample radioactivity was plotted against time after injection, and radioactivity of the 1-min sample was set as 100%. Degradation of ligand (data not shown) was negligible. Data from each individual mouse are indicated by circles, triangles, or squares. Semilogarithmic plots (data not shown) revealed a monophasic pattern of clearance for nLDL and cLDL and a biphasic clearance pattern for the two oxLDLs; 77.3 ± 12.0% of injected oxLDL24 was eliminated from blood during an initial rapid clearance α-phase with a t1/2(α) of 0.43 ± 0.12 min, whereas the remainder was eliminated with a t1/2(β) of 5.0 ± 0.8 min. For oxLDL3, 20 ± 7.8% was removed during the α-phase with a t1/2(α) of 4.2 ± 2.7 min, whereas the remainder was eliminated with a t1/2(β) of 91 ± 44 min. The α- and β-phases were calculated according to Ref. 10.

Fig. 2. Organ distribution of radiolabeled LDLs and oxLDLs. The animals used in the blood clearance studies (Fig. 1) were analyzed for organ distribution of LDLs and oxLDLs 60 min after iv injection of 125I-labeled LDL, 125I-cLDL, 125I-oxLDL3, or 125I-oxLDL24. The sum of radioactivity in the listed tissues and organs was considered as 100%. Results are presented as percentages of the total recovered radioactivity in collected organs and tissues ± SE (n = 3). The term “Rest of carcass” refers to body without the tissues and organs listed in the x-axis. *P < 0.05 compared with nLDL and cLDL. GI, gastrointestinal.
ventricle before PBS/PFA perfusion fixation for light microscopy and EM. Serum samples were analyzed for alanine aminotransferase (ALT) activity by an ultraviolet test with pyridoxal phosphate activation in a Roche automated clinical chemistry analyzer (Roche Diagnostic, Mannheim, Germany). Results are expressed in units per liter.

Statistical Analysis

In vivo microscopy data, ICAM-1 staining score, fenestrae numbers, and sinusoidal endothelial thickness were analyzed by Mann Whitney test and one-way ANOVA. The post hoc tests were Dunnett (in vivo microscopy, immunohistochemistry, endothelial thickness data) and linear trend (fenestrae numbers). Anatomical distribution data of intravenously injected radiolabeled lipoproteins were evaluated by two-way ANOVA and Bonferroni post hoc tests. P values <0.05 were considered statistically significant. The statistical analyses were performed with GraphPad Prism version 4.00 for Macintosh (GraphPad Software, San Diego, CA).

RESULTS

Blood Clearance and Organ Distribution of oxLDLs

Blood clearance. The blood radioactivity decay was monitored for 60 min following intravenous injection of radiolabeled LDLs or oxLDLs (3.6 μg/g body wt) (Fig. 1). Nonoxidized LDLs (nLDL and cLDL) were removed very slowly from the circulation with ~70% of the injected ligand radioactivity still circulating 60 min after injection (Fig. 1, A and B). There was no difference in the rate of blood clearance of nLDL or cLDL, both of which showed a monophasic exponential decay pattern.

The blood clearance of 125I-oxLDL3 and 125I-oxLDL24 exhibited a two-phase exponential decay (Fig. 1, C and D). However, the elimination of oxLDL24 was very fast compared with that of oxLDL3; after 10 min 90% of the oxLDL24 radioactivity was cleared from the circulation compared...
with \( \sim 20\% \) for oxLDL\(_3\) (Fig. 1C). Notably, the removal of oxLDL\(_3\) was significantly faster than that of nonoxidized LDLs \( (P < 0.01) \), which showed no decrease in blood radioactivity after 10 min.

**Organ distribution.** The organ distribution of \( ^{125}\text{I}-\text{nLDL}, ^{125}\text{I}-\text{cLDL}, ^{125}\text{I}-\text{oxLDL}_3, \) and \( ^{125}\text{I}-\text{oxLDL}_{24} \) was measured 60 min after injection (Fig. 2). Whole organ analysis revealed the following distribution of radioactivity for mildly and heavily oxidized LDLs: oxLDL\(_3\), liver 14.9\%, carcass 15.4\%, blood 61.6\%; and oxLDL\(_{24}\), liver 31.4\%, carcass 46.1\%, blood 5.1\%. The uptake in other organs (lung, heart, kidneys, gastrointestinal tract, and spleen) was very low. The nonoxidized LDLs were found mainly in blood; the liver uptake was 6.3\% (nLDL) and 9\% (cLDL), respectively, which was significantly different from that of oxLDL\(_3\) and oxLDL\(_{24}\) \( (P < 0.05) \).

**Effects of oxLDL on the Mouse Hepatic Microcirculation**

To assess the effects of mildly modified and heavily modified oxLDLs on the hepatic microcirculation, 3.6 µg/g body wt of nLDL \( (n = 6), \) cLDL \( (n = 5), \) oxLDL\(_3\) \( (n = 6), \) or oxLDL\(_{24}\) \( (n = 6) \) were injected in the tail vein of mice, and the number of perfused sinusoids, adhering leukocytes, and swollen endothelial cells (SwEC) was evaluated by in vivo microscopy in periportal and centrilobular areas of the left liver lobe (Fig. 3). cLDL and nLDL showed corresponding results, and the observations measured for these two ligands were therefore pooled in a nonmodified LDL group.

**Perfused sinusoids.** A slight but significant \( (P < 0.05) \) decrease in the number of perfused sinusoids was found in the centrilobular areas after treatment with oxLDL\(_3\) compared with the LDL group (Fig. 3B), whereas no significant differences were noted in the periportal areas.

**Leukocyte adhesion.** A significant increase \( (P < 0.05) \) in the number of leukocytes adhering to the sinusoidal endothelium was observed in both the oxLDL\(_{24}\) and oxLDL\(_3\)-treated groups compared with the LDL controls. OxLDL\(_{24}\) treatment yielded leukocyte adhesion in both periportal and centrilobular areas, whereas increased leukocyte adherence was significant only in the centrilobular areas following oxLDL\(_3\) treatment (Fig. 3, C and D). Rolling of leukocytes along the endothelium was not observed. Examples of adherent leukocytes are shown in Fig. 4. Transmission EM showed that most of the leukocytes recruited to the hepatic sinusoids were lymphocytes, but neutrophils (Fig. 4C), eosinophils, monocytes/macrophages, and a few platelets were also observed.

**Endothelial swelling.** A significant increase in the number of SwEC per microscope field was observed in the centrilobular areas after injection of oxLDL\(_3\) or oxLDL\(_{24}\) compared with LDL controls (Fig. 3F). An example of a SwEC is shown in Fig. 4B.

**Serum ALT Levels**

The ALT activity in serum samples from 10 mice used in the in vivo microscopy study was measured to evaluate hepatocellular injury, and the following results were obtained: LDL =

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**Fig. 4.** Leukocyte adherence to the liver sinusoidal endothelium after oxLDLs treatment. A and B: in vivo micrographs of hepatic sinusoids from mouse treated with oxLDL\(_3\) (A, centrilobular area) or oxLDL\(_{24}\) (B, periportal area). Arrows in A and B show leukocytes sticking to the sinusoidal wall. One swollen liver sinusoidal endothelial cell is indicated by arrowhead in B (magnified and outlined in the inset). S, sinusoids. Original magnification: \( \times 800.\) C: transmission electron micrograph of liver section from an oxLDL\(_3\)-injected mouse showing a lymphocyte (L) and a neutrophil granulocyte (G) trapped in a sinusoid. Scale bar: 5 µm.
ICAM-1 Expression

ICAM-1, a leukocyte adhesion molecule in endothelial cells, is constitutively expressed in LSECs (15) and can be upregulated in inflammation and aging (19, 58). Immune labeling of paraffin-embedded liver sections for ICAM-1 showed positive staining of the endothelium of larger liver vessels both in oxLDL- and LDL-injected animals, although the staining intensity along the sinusoids varied among animals and treatments (Fig. 5). The sinusoidal staining was evaluated on a scale from zero (negative) to five (strong). In general, ICAM-1 expression was highest in centrilobular areas; here it was most intense in the oxLDL3-injected group (*P < 0.01 vs. LDL) (Fig. 5A).

Small foci of leukocyte aggregates in the sinusoids were seen after oxLDL3 injection and to a lesser extent after oxLDL24 injection. These foci were associated with strong ICAM-1 staining (Fig. 5, C–E).

Effect of oxLDLs on Sinusoidal Endothelium Thickness

Measurements of endothelial thickness were performed on transmission EM sections (Fig. 6). A significant increase in liver sinusoidal endothelial thickness was observed in the oxLDL3-treated mice (256.13 ± 5.22 nm, n = 6) (mean ± SE) compared with LDL controls (194.44 ± 1.44 nm, n = 8) (Fig. 6A). OxLDL24 injection also led to increased endothelial thickness (228.94 ± 5.68 nm, n = 4) (not significant vs. LDL).

Effect of oxLDLs on LSEC Fenestration

To examine the effect of mildly and heavily oxLDLs on LSEC fenestration, the number of fenestrae per 10 μm² was counted on scanning electron micrographs of mouse livers fixed 60 min after intravenous injections of nLDL, cLDL, oxLDL3, or oxLDL24 (Fig. 7). No differences were found between the cLDL- and nLDL-injected mice; therefore, the LDL groups were pooled. Generally, the centrilobular areas showed a higher fenestration compared with periportal areas (*P < 0.05). With increased oxidation of LDL, a trend toward reduced fenestration was observed in the centrilobular areas (*P for trend <0.05), whereas no such trends were observed in the periportal areas (Fig. 7A).

DISCUSSION

OxLDL, which is generally held as a major atherogenic substance, is present both in atherosclerotic lesions and in...
circulation (1, 63). The liver is the major clearance organ for these substances (present study and Refs. 35, 56, and 57), and we report here for the first time the in vivo effect of oxLDLs on the hepatic microcirculation. OxLDLs are heterogeneous particles, and different forms may be present in plasma (6). We therefore tested the effects of both mildly and heavily oxLDLs. A cross-sectional study among patients with acute coronary syndromes indicated that patients with acute myocardial infarction present plasma levels of oxLDL approximately fourfold higher than control subjects (14). The injected doses in our study gave initial plasma concentrations that resembled the oxLDL concentrations reported in these patients (14). In addition, the mildly oxLDL (i.e., oxLDL3) used by us carried a net negative charge (REM: 1.46 ± 0.17 compared with nLDL) corresponding to that of the oxLDL24 fraction isolated from mice treated with LDL (B) or oxLDL3 (C). h, Hepatocytes, sd, space of Disse, s, sinusoid. Arrows point to sinusoidal endothelium and arrowheads to fenestrae. Scale bars: 2 μm.

Fig. 6. Effects of oxLDLs on liver sinusoidal endothelial thickness. The mice used for in vivo microscopy were killed after 60 min, and the median liver lobe was prepared for transmission electron microscopy. A: the thickness of the sinusoidal endothelium was measured as described in MATERIALS AND METHODS, and results were presented as means ± SE. Treatments are as follows: open bars, LDL (n = 8); gray bars, oxLDL3 (n = 6); and black bars, oxLDL24 (n = 1). *P < 0.05 compared with LDL. B and C: electron micrographs of liver sinusoids from mice treated with LDL (B) or oxLDL3 (C). h, Hepatocytes, sd, space of Disse, s, sinusoid. Arrows point to sinusoidal endothelium and arrowheads to fenestrae. Scale bars: 2 μm.

indicating endothelial activation. The effects were most pronounced in the central areas of the liver lobules. However, serum ALT values were low and did not differ between the LDL and oxLDL groups, suggesting normal hepatocyte function.

The injection of mildly oxLDL but not heavily oxLDL further led to a decrease in the number of perfused sinusoids in centrilobular areas of the liver lobuli, and the mildly oxLDL-treated mice also showed the most intense sinusoidal ICAM-1 staining. This difference between the oxLDL-treated groups may be explained by the very different elimination rates of the two ligands from the circulation. The in vivo microcirculatory responses were evaluated from 30 to 60 min after the intravenous ligand injections. At this time, heavily oxLDL was almost completely removed from the circulation, whereas 46% of intravenously injected mildly oxLDL was still circulating after 60 min. These clearance rates correspond to those reported elsewhere (57). We recently found that mildly oxLDL was not recognized by KCs in vitro but was taken up by LSECs via the scavenger receptors stabilin-1 and stabilin-2 (32). However, the LSEC capacity for endocytosis of mildly oxLDL is rather low compared with heavily oxLDL and other LSEC ligands (32). It is also reported that the net negative charge of mildly oxLDL is lower than that of heavily oxLDL. This finding, along with the fact that the affinity for scavenger receptors increases with increasing net negative charge of the ligand (7, 32), may explain why the presence of oxLDL in human plasma is dominated by mildly oxidized forms, as well as the slow removal of this ligand from the mouse circulation. This also lends support to the hypothesis that the liver clearance system is insufficient to reduce the high plasma oxLDL levels reported in cardiovascular disease patients. The long circulatory life of mildly oxLDL prolongs its exposure time to blood cells and vascular endothelial cells and hence enhances the chances of negative effects in different vascular beds.

Leukocyte adherence to the hepatic sinusoidal lining in centrilobular areas was induced by both heavily and mildly oxLDL, whereas only heavily oxLDL significantly increased the number of adherent leukocytes in periportal areas. Increased adhesion of leukocytes to the endothelium a short time after oxLDL exposure has also been observed in other microcirculatory beds (29–31, 33, 34), e.g., in the mesenteric microcirculation of rats after local infusion of heavily oxLDL in the superior mesentery artery (33, 34). An increase in leukocyte rolling and subsequent firm adhesion of leukocytes following intravenous injections of oxLDL was also observed in postcapillary venules and arterioles of skin striated muscle of mice and hamsters (29–31), and in the aorta macrovascular endothelium of hamsters (29), suggesting a systemic effect of oxLDL in the promotion of leukocyte adhesion. The proposed mechanism was that oxLDL induced leukocyte adhesion to the microvascular endothelium by formation of leukocyte aggregates with platelets and the involvement of P-selectin in the mediation of cell-cell (platelet to leukocyte, aggregates to endothelium) interactions (31). However, in the present study, we did not observe platelet aggregation or rolling of leukocytes along the hepatic sinusoids. The latter can be explained by the finding that LSECs lack the expression of selectins (8). Moreover, leukocyte rolling was not observed in the liver sinusoids (61).
In our study, as well as in the intravital microscopy studies in Refs. 29–31, 33, 34, human LDL and oxLDL were used, and one may raise the question of potential deleterious effects of using human LDL in rodent models. However, native human LDL did not have negative effects in the circulatory systems examined (29–31, 33, 34), and a study of oxLDL effects on the hamster cheek pouch microcirculation showed that also homologous oxLDL was a potent inducer of inflammatory changes in microcirculatory vessels (54).

Our transmission EM analysis suggested that lymphocytes were the most prominent leukocyte recruited to the hepatic sinusoids, but neutrophils and other leukocytes were also observed. We found increased expression of ICAM-1 in centrilobular areas of oxLDL-treated mice (significant for mildly oxLDL) and in areas with leukocyte accumulation in the two oxLDL groups. ICAM-1 is expressed constitutively in LSECs (15), and its expression is further upregulated in inflammation (58). It is a counter receptor for leukocyte integrins, such as CD11b/CD18 (Mac-1) on neutrophils and monocytes, and CD11a/CD18 (LFA-1) on lymphocytes (5, 60). Intravital microscopy studies in rat mesentery have suggested the involvement of ICAM-1 in promotion of leukocyte adhesion induced by oxLDL to the microvascular endothelium (34). The role of ICAM-1 in leukocyte adhesion to hepatic sinusoids has also been demonstrated in inflamed livers (43, 61). However, antibody inhibition studies in a mouse endotoxin model suggested that ICAM-1 was not involved in the adhesion of neutrophils to the hepatic sinusoidal endothelium (20). Instead, neutrophil attachment in liver was suggested to be caused by physical trapping (21). This hypothesis has been challenged by two recent reports indicating that neutrophil attachment in liver is mediated by Mac-1 and ICAM-1 or CD44 and hyaluronan, depending on whether the inflammatory stimulus is local or systemic (38, 40).

In addition to ICAM-1, LSEC is also reported to express VCAM-1 and VAP-1 (25, 58), which may be involved in lymphocyte recruitment to inflamed livers (2). Recently, Shetty et al. (48) presented in vitro evidence for stabilin-1 (also known as CLEVER-1 and FEEL-1) involvement together with ICAM-1 and VAP-1 in the transmigration of CD4 FoxP3+ regulatory T cells through cultured human LSECs. Previously, the stabilin-1 homologous protein stabilin-2 was reported to support lymphocyte adhesion to human LSECs in vitro via interactions with α4β2-integrin (22). Stabilin-1 and stabilin-2 are constitutively expressed in LSECs at high levels (44), and their in vivo role in hepatic recruitment of lymphocytes is at present unknown. We have shown that uptake of oxLDL in rat LSEC is mediated by these two receptors (32). The stabilins may therefore have a dual role in the hepatic microcirculation, both as scavengers of oxLDL and as promoters of lymphocyte recruitment to the hepatic sinusoids remains to be elucidated.

The observed increase in SwEC numbers may lead to sinusoid obstruction with the subsequent retention of leukocytes as suggested by Ito et al. (19). Some physical trapping of leukocytes can therefore not be excluded. Both mildly and heavily oxLDLs induced an increase in the number of SwECs in centrilobular areas, which may indicate an early toxic effect of oxLDLs on LSECs.

Image analysis of transmission EM micrographs revealed an ~30% increase in the sinusoidal endothelial thickness, after
the injection of mildly oxLDL. The more pronounced effects of mildly oxLDL compared with heavily oxLDL may be because of the slower blood clearance, prolonging the exposure time to the former molecule. In addition, a slight reduction ($P$ for trend <0.05) of the number of fenestrae was observed in the centrilobular areas of livers exposed to mildly and heavily oxLDL. Defenestration and thickening of the sinusoidal endothelium is also associated with liver ageing in human and laboratory animals (19, 28, 50), and LSEC defenestration has been shown to impair the blood clearance of chylomicron remnants, leading to increased risk of cardiovascular complications (9, 11, 27). In the present study, we have examined the acute in vivo effects on the hepatic microcirculation of relatively high doses of oxLDL. Increased levels of circulating oxLDL are also reported with ageing in healthy people (4), and one may speculate if long-term exposure of low levels of oxLDL can lead to defenestration and other vascular changes observed in the old liver (28).

We found that oxLDL, at clinically relevant plasma concentrations, induced acute changes in the hepatic microcirculation of mice, such as decreased numbers of perfused sinusoids, increased adhesion of leukocytes to the sinusoidal endothelium, and increased number of SwEC, endothelial thickening, and reduced fenestration, indicating hepatic microvascular dysfunction. Thus, circulating oxLDL has significant negative effects on its most important clearance system, which may increase the risk of negative effects also in extrahepatic vascular beds.

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DISCLOSURES
No conflicts of interest are declared by the authors.

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