LOX-1 Receptor Blockade Abrogates oxLDL-induced Oxidative DNA Damage and Prevents Activation of the Transcriptional Repressor Oct-1 in Human Coronary Arterial Endothelium*§

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Thomas Thum¹ and Jürgen Borlak³

From the ¹Medizinische Klinik I, Kardiologie, Julius-Maximilians Universität, Josef-Schneider-Strasse 2, 97080 Würzburg, Germany and the ²Center for Drug Research and Medical Biotechnology, Fraunhofer Institute of Toxicology and Experimental Medicine, Nikolai-Fuchs-Strasse 1, 30625 Hannover, Germany

Activation of the lectin-like oxLDL receptor (LOX-1) promotes atherosclerosis. Oxidized LDL (oxLDL) increases production of reactive oxygen species (ROS) and leads to the development of endothelial dysfunction. The molecular causes for oxLDL to induce oxidative DNA damage and metabolic dysfunction remain uncertain. Here we report treatment of cultured human coronary arterial endothelial cells (HCAEC) with oxLDL to cause oxidative DNA damage as determined by a 3-fold increase in 8-OH-deoxyguanosine adduct formation and a 4-fold induction of the growth arrest and DNA damage-inducible transcripts GADD45 and GADD153. Oxidative stress resulted in activation of Oct-1, a transcriptional repressor of various vascular cytochrome P450 (CYP) monooxygenases. Activation of Oct-1 was protein kinase C (PKC)-mediated. Binding of Oct-1 to promoter sequences of CYP monooxygenases was increased upon treatment of HCAEC with oxLDL. This resulted in repressed production of endothium-derived hyperpolarization factor 11,12-epoxyeicosatrienoic acid. Small interfering RNA-mediated functional knockdown of Oct-1 attenuated oxLDL-mediated endothelial DNA damage, Oct-1/DNA binding, and reversed impaired production of EDHF. Taken collectively, oxLDL induced oxidative DNA damage and activation of Oct-1 to result in metabolic dysfunction of coronary arterial endothelium.

Oxidized low density lipoproteins (oxLDL)² are atherogenic and considered to be a fundamental risk factor in the initiation of progression of atherosclerosis (1). Specifically, uptake of oxLDL into the endothelium is mediated through interaction with the lectin-like oxLDL receptor (LOX-1). This receptor was reported to be overexpressed in human atherosclerotic lesions (2). Transgenic overexpression of LOX-1 in an apoE-deficient background increases atherosclerotic plaque incidence (3), and LOX-1 expression is highly correlated with plaque instability (4). In strong contrast, deletion of LOX-1 reduces atherogenesis in LDLR knockout mice fed a high cholesterol diet (5). Furthermore, human genetic studies link LOX-1 polymorphisms to cardiovascular disease susceptibility (6).

Mechanistically, oxLDL fosters intracellular production of reactive oxygen species (ROS) (7–9) and promotes apoptosis (10). There is also evidence for ROS to cause oxidative DNA damage in endothelium (11, 12), which contributes to the pathogenesis of atherosclerosis and to instability of plaques (13–15). In patients with cardiovascular disease, the levels of oxLDL and circulating 8-hydroxy-2'-deoxyguanosine (8-OH-dG) DNA-adducts correlate well (16). Indeed, 8-OH-dG is an established marker for oxidative DNA damage (17), and 8-OH-dG is elevated in human atherosclerotic plaques of carotid endarterectomy specimens (18).

Specifically, the cellular responses to DNA damage may include regulation of transcription factors, notably, c-Jun (19), p53 (20), and NFκB (21). More recently, the homeodomain transcription factor Oct-1 was added to the growing list of transcription factors regulated upon DNA damage (22). There is evidence for Oct-1 to play an essential role in vascular biology and was found to be up-regulated in dedifferentiated endothelium (23). Furthermore, Oct-1 was shown to regulate, at least in part, LOX-1 receptor activity (24–26).

As of today, the role of Oct-1 in oxLDL-induced vascular injury is uncertain. This transcription factor recognizes an octamer sequence (ATGTAAT) in promoters of target genes (27), which encode proteins for the regulation of development, immune response, cellular repair, and general metabolism (25–31). Previous studies are suggestive for Oct-1 to function as a repressor for certain endothelial-expressed enzymes, such as cytochrome P450 epoxygenases (23, 32). These epoxygenases
catalyze production of endogenous vasoactive molecules, including epoxy fatty acids, of which 11,12-epoxyeicosatrienoic acid (11,12-EET) was reported to be an endothelial-derived hyperpolarization factor (EDHF) (9, 33). Oct-1 may therefore provide a missing link between oxidative DNA damage and impaired metabolic function of endothelium upon exposure to oxidized lipoproteins.

Here, we studied the effects of oxLDL on oxidative DNA damage and Oct-1 expression in cultures of human coronary arterial endothelial cells (HCAEC). We evaluated whether Oct-1 up-regulation after oxidative stress may be mechanistically linked to repressed CYP monoxygenases activity and EDHF production. We also studied whether LOX-1 blockade would prevent DNA damage and reverse metabolic impairment of HCAECs when exposed to oxLDL.

EXPERIMENTAL PROCEDURES

Materials—HCAECs and EGM-2MV medium were obtained from Clonetics (San Diego, CA). Human atherosclerotic aortae were obtained from cardiac explants (34). Healthy control aortic protein extracts were obtained from Biocat (Heidelberg, Germany). In addition, atherosclerotic aortae were explanted from apoE knockout mice fed with a high lipid diet for >4 months, as well as from healthy control mice (C57/BL6, Harlan-Winkelmann, Borchen, Germany). The total RNA Isolation system and the NucleoSpin Tissue kit were from Macherey-Nagel (Düren, Germany). Aprotinin, deoxyguanosine (dG), hydrolyzed samples, 2′-deoxyguanosine (dG), and 8-OH-dG standards were from Sigma. Rotiblock, skim milk powder, and Tween-20 was from Roth. Oct-1 antibody was obtained from Santa Cruz Biotechnology (Heidelberg, Germany). The anti-rabbit antibody from Chemicon (Hofheim, Germany) was from Roche Applied Science (Mannheim, Germany). Pefabloc was from Amersham Biosciences (München, Germany). T4 polynucleotide kinase was from New England Biolabs (Frankfurt am Main, Germany). DNA was from Roche Applied Science (Ingelheim am Rhein, Germany). Bovine serum albumin was from PAA (Linz, Austria).

Preparation of LDL, oxLDL, and moxLDL—LDL was isolated from human plasma by sequential gradient ultracentrifugation as described previously (9). Oxidized LDL was prepared by incubation of LDL with 5 μmol of CuSO4 for 24 h at 37 °C. moxLDL was prepared by incubation with 10 μmol of Fe2+(SO4) for 24 h at 37 °C as described (34). Oxidation was monitored using the thiobarbituric acid-reactive substances (TBARS) assay with tetrathoxypyropane as an internal standard (9). Oxidation was stopped by addition of butylated hydroxytoluene (100 μM). Note, the level of oxidation strongly differed between fully (Cu2+)-oxidized LDL (35 nm malondialdehyde/mg protein) versus minimally (Fe2+)-oxidized LDL (3 nm malondialdehyde/mg protein). For comparison the TBARS assay revealed no detectable concentration of malondialdehyde in native LDL preparations.

Cell Culture—Primary HCAECs were cultured in 75-cm² plastic flasks in EGM-2MV medium. Confluent cultures were detached by trypsin/EDTA and plated on 6 wells until 90% confluence was reached. Cultured endothelial cells (fourth passage) were checked by reverse phase contrast microscopy before and after treatment with oxLDL (magnification 20-fold). We additionally examined expression of the endothelial-specific surface protein PECAM-1 to control for cellular differentiation using fluorescence-activated flow cytometry, as described previously (23).

Endothelial cells were treated with increasing doses of oxLDL (10–100 μg/ml) for 24 h. Dose selection was based on published and clinically confirmed oxLDL plasma levels (36). As a positive control for the induction of DNA damage, we additionally subjected HCAECs in PBS to UV-C irradiation (wavelength, 254 nm; dose, 10–50 J/m²), as described (37). Then medium was changed from PBS to EGM-2MV, and 8 h later, Oct-1 expression levels were determined as described below.

RNA and cDNA—RNA was isolated from endothelial cells using a total RNA Isolation system according to the manufacturer’s recommendation. Quality and quantity of isolated RNA was checked using capillary electrophoresis (Bioanalyzer 2100, Agilent Technologies, Waldbronn, Germany) following the manufacturer’s instructions. 2 μg of total RNA from each sample were used for reverse transcription, as described previously (34). The resulting cDNA was frozen at −20 °C until further experimentation.

Real-time and Semi-quantitative PCR—Real-time RT-PCR was done with the Lightcycler (Roche Applied Science, Mannheim, Germany), as described (23). After an initial denaturation step at 95 °C for 30 s, the PCR reaction was initiated with an annealing temperature of 55 °C for 8 s followed by an extension phase for 8 s (GADD45 and GADD153) or 14 s (Oct-1) at 72 °C and a denaturation cycle at 95 °C for 1 s. The PCR reaction was stopped after a total of 40 cycles, and at the end of each extension phase, fluorescence was observed and used for quantitative measurements within the linear range of amplification. Oligonucleotide sequences were 5′-tcagcgcagcagctacagtgc (forward primer) and 5′-cagcaggcaacaccagctggtctttgctcttggt (reverse primer) for detection of the GADD45 gene, 5′-tgaccccttctttcttggt (forward primer) and 5′-ctggaggtttgctgcctggtat (reverse primer) for the GADD153 gene and 5′-gaacccct tgtctcttggt (forward primer) and 5′-gagagggccgtctgccgact (reverse primer) for the Oct-1 gene. Exact quantification was achieved by a serial dilution with cDNA produced from endothelial total RNA extracts using 1:5 dilution steps. Gene expression levels were then given as the ratio of the gene of interest (nominator) versus a stable expressed housekeeping gene (cyclophilin A, denominator) (9, 38).

Semi-quantitative gene expression of CYP1A1, CYP2B6, CYP2C8, CYP2C9, and CYP2J2 was performed and analyzed as previously described (Ref. 9 and supplemental Table S2).

Quantitative Measurement of 7,8-Dihydro-8-oxo-2′-deoxyguanosine—Genomic DNA was extracted from cultured HCAECs treated with oxLDL (10–100 μg/ml) or oxLDL (100 μg/ml) + κ-carrageenan (250 μg), as well as from appropriate controls using the NucleoSpin Tissue kit. DNA (90 μl) was digested with nuclease P1 (5 μg), zinc chloride (3 μl, 20 mmol), and HCl (3 μl, 200 mmol) for 30 min at 45 °C. Then, Tris buffer (3 μl, 500 mmol) and 2 μl of alkaline phosphatase were added and further incubated for 2 h at 37 °C to yield free deoxynucleosides. To determine the amount of dG, hydrolyzed samples and
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2′-deoxyguanosine (dG) and 8-OH-dG standards were injected onto a LiChrochart 250-4 (100RP18, 5 μm) HPLC column (Merck). Deoxyguanosine was detected at 255 nm, and calibration of the system was done with dG and 8-OH-dG as appropriate standards.

Production of 11,12-Epoxycisatrienoic Acid (EET)—We used an HPLC electrospray MS² method to detect 11,12-EET production in cultures of HCAECs as described (9). HCAECs were treated with 100 μg/ml oxLDL for 24 h, and then production of 11,12-EET was analyzed.

Intracellular ROS Measurements—After treatment with oxLDL (10, 20, and 100 μg/ml; 24 h) or native LDL (100 μg/ml; 24 h), cells were incubated with 10 μmol of 2′-7′-dichlorofluorescin diacetate (DCFH-DA) for 30 min at 37 °C. The 100 μg/ml oxLDL group was also concomitantly treated with the competitive oxLDL-receptor antagonist κ-carrageenan (250 μmol, 24 h). Endothelial cells were harvested, centrifuged for 5 min, and 1200 rpm at 4 °C, washed in PBS, and the resulting cell pellet was resuspended with 800 μl of PBS buffer. Fluorescence emission was detected at 530 nm ± 30 nm (fluorescin) after excitation of cells at 488 nm using flow cytometry (FACScan, Beckton Dickinson, Heidelberg, Germany).

Preparation of Nuclear Extracts—HCAEC nuclear extracts were prepared by the modified Dignam C method (39). 24 h after treatment with oxLDL (100 μg/ml), oxLDL (100 μg/ml) + κ-carrageenan (250 μmol), oxLDL (100 μg/ml) + chelerythrine (1 μmol), or nLDL (100 μg/ml), cells were washed twice with ice-cold PBS, scraped into microcentrifuge tubes, and centrifuged for 5 min at 1780 × g, and 4 °C. Cell pellets were resuspended in hypotonic buffer (10 mmol of Tris, pH 7.4, 2 mmol of MgCl₂, 140 mmol of NaCl, 1 mmol of dithiothreitol, 4 mmol of Pefabloc, 40 mmol of β-glycerophosphate, 1 mmol of Na₃VO₄, 10 μl of aprotinin/ml buffer, and 0.5% Triton X-100) for 10 min at 4 °C, transferred onto one volume of 50% sucrose in hypotonic buffer (see above), and centrifuged at 14,000 × g and 4 °C for 10 min. Nuclei were resuspended in Dignam C buffer (20 mmol of Hepes, pH 7.9, 25% glycerol, 420 mmol of NaCl, 1.5 mmol of MgCl₂, 0.2 mmol of EDTA, 1 mmol of dithiothreitol, 4 mmol of Pefabloc, 40 mmol of β-glycerophosphate, 1 mmol of Na₃VO₄, and 10 μl of aprotinin/ml buffer) and gently rocked for 30 min at 30 °C. Nuclear debris was removed by centrifugation at 14,000 × g and 4 °C for 10 min, and extracts were aliquoted and stored at −80 °C. Protein concentrations were determined as described (40).

Western Blotting Experiments—Western immunoblotting was done as follows: Nuclear protein (100 μg) extracts from cultured endothelial cells were denatured at 95 °C for 5 min, followed by SDS-PAGE on 12% polyacrylamide gels and blotted onto a polyvinylidene difluoride membrane (NEN, Dreieich, Germany) at 350 mA for 2 h in a buffer containing 400 mmol of glycine, 50 mmol of Tris, pH 8.3. Nonspecific binding sites were blocked with Rotiblock and 5% skim milk powder (Roth, Germany) in TBS buffer. After electrobolting of proteins, membranes were incubated with a polyclonal antibody for Oct-1 (dilution 1:50; sc-8024, Santa Cruz Biotechnology) for 1 h. For detection of human or murine LOX-1 expression we used a polyclonal LOX-1 antibody (ab60178; dilution 1:1000; Abcam, Cambridge, UK). After washing with TBS buffer containing 0.1% Tween-20. Subsequently, the membranes were incubated with a 1:5000 diluted anti-rabbit antibody (both for Oct-1 and LOX-1) for 1 h at room temperature followed by three successive washes with TBS buffer containing 0.1% Tween-20. Immunoreactive proteins were visualized with a chemiluminescence reagent kit (PerkinElmer Life Sciences, Dreieich, Germany) according to the manufacturer’s instructions, and bands were scanned with the Kodak Image Station CF 440 and analyzed using the Kodak 1D 3.5 imaging software (Eastman Kodak Company).

Electromobility Gel Shift Assay (EMSA)—The procedure for EMSA was adapted from a previously described method (39). Briefly, 5 μg of endothelial nuclear extract was incubated with binding buffer consisting of 25 mmol HEPES (pH 7.6), 5 mmol of MgCl₂, 34 mmol of KCl, 2 mmol of dithiothreitol, 2 mmol of Pefabloc, 0.5 μl of aprotinin (2.2 mg/ml), 50 ng of poly(dl-dC), and 80 ng of bovine serum albumin. The binding reaction was carried out for 20 min on ice and free DNA and DNA-protein complexes were resolved on a 6% polyacrylamide gel. Competition studies were done by adding a specific amount (50-fold) of unlabeled oligonucleotides or a specific Oct-1 antibody to the reaction mix 10 min before addition of the labeled oligonucleotides. In addition, we also incubated nuclear extracts with excess addition of mutated Oct-1 consensus binding sites. Gels were blotted to Whatman 3 MM paper, dried under vacuum, exposed to imaging screens (Imaging Screen-K, Bio-Rad) for autoradiography for 24 h at room temperature and analyzed using a phosphorimaging system (Molecular Imager FX pro plus; Bio-Rad) and the Quantity One Version 4.2.2 software (Bio-Rad).

Immunohistochemistry—HCAECs were fixed and permeabilized as described (41). Oct-1 expression was determined after addition of an anti-Oct-1 antibody (Abcam; ab51363; dilution 1:10) for 2 h at 37 °C. Thereafter, cells were washed and an Alexa Fluor 488 goat anti-mouse IgG was added for 1 h at 37 °C (dilution 1:200). In addition, nuclei were stained with 4′,6-diamidino-2-phenylindol (DAPI). Then cells were observed under appropriate fluorescence microscopy conditions as described (41).

Transcription Factor Binding Sites in Cytochrome P450 Promoters—We searched for Oct-1 binding sites in the promoters of CYP1A1, CYP2A6/7, CYP2B6, CYP2C8, CYP2C9, CYP2E1, CYP2J2, and eNOS using the transcription factor data base TRANSFAC Professional 6.2 and the program Matrix Search for Transcription Factor Binding Sites.
Small Interference RNA (siRNA)-mediated Knockdown of Oct-1 in HCAECs—HCAECs were cultured to 70–80% of confluence and were transfected with Oct-1-siRNA using the BLOCK-iTTM Transfection kit (Invitrogen). We incubated HCAECs with the siRNA oligonucleotide Oct-1 (50 nM; sc-36119, Santa Cruz Biotechnology) for 48 h to down-regulate Oct-1 expression. FITC-labeled scrambled siRNA (Control-FITC block-it fluorescent Oligo 2013, Invitrogen) was used as a negative and transfection control. Oct-1 expression was monitored by Western blotting (see above).

RESULTS

OxLDL Induced Oxidative DNA Damage in HCAECs—LOX-1 was highly expressed in atherosclerotic aortic tissue of ApoE⁻/⁻/⁻ mice fed a high lipid diet or humans aortae of patients diagnosed with ischemic heart disease when compared with appropriate controls (Fig. 1 A). This suggests pathophysiological relevance of LOX-1 up-regulation in atherosclerosis. We next focused on effects of LOX-1-mediated oxLDL uptake in HCAECs in vitro. Based on microscopic evaluation, there were no signs of altered morphology or cellular toxicity after treatment of cell cultures with increasing doses of oxLDL (10–300 μg/ml; 24 h). LDH leakage was used as a marker for membrane integrity and was assayed after treatment of cell cultures with incremental doses of oxLDL (24 h). Notably, oxLDL induced LDH leakage into culture medium with 40 units/liter at the highest dose (data not shown). Based on LDH activity, we used 100 μg/ml oxLDL as the best tolerable non-cytotoxic dose. We chose the 24-h time point for subsequent studies, because we and others (9, 42) demonstrated enhanced ROS production in HCAECs to be significantly elevated after 24 h of treatment with oxLDL. The expression of PECAM-1 (CD31) served as an endothelial differentiation marker and was expressed by >95% of cultured cells, as determined by flow cytometry (data not shown). Treatment of HCAECs with oxLDL (100 μg/ml; 24 h)
increased intracellular ROS production by 4.5-fold, whereas native (non-oxidized) LDL had no effect (Fig. 1B). ROS production was attenuated when k-carrageenan, a competitive LOX-1 inhibitor, was added (Fig. 1B). Treatment of HCAECs with oxLDL resulted in a dose-dependent 4-fold increase of the stress-inducible transcripts GADD45 and GADD153 (Fig. 2A). Both GADDs were additionally up-regulated in atherosclerotic human aortae (Fig. 2B). Likewise, moxLDL increased expression of GADDs (Fig. 3A). Production of 8-OH-dG was up to 3-fold increased after treatment of HCAECs with oxLDL (Fig. 2C). LOX-1 inhibition with k-carrageenan abrogated induction of GADD transcripts and formation of the 8-OH-dG DNA adduct (Fig. 2). Addition of native LDL (100 μg/ml) had no effect.

Induction of Oct-1 Binding Activity to Regulatory Sequences of the vWF Gene and CYP Monooxygenases—As denoted above, oxLDL induced oxidative DNA damage. We therefore probed for regulation of the transcriptional repressor Oct-1 in HCAECs after treatment with oxLDL. We observed a dose-dependent increase in Oct-1 gene and protein expression in oxLDL-treated HCAECs (Figs. 3B and 4, A and B). In addition, moxLDL increased Oct-1 protein expression to a level comparable to that of oxLDL (Fig. 3B). To further establish a link between oxidative DNA damage and Oct-1 activity, cultures of HCAECs were exposed to UV-C radiation known to induce DNA damage (positive control). A dose-dependent increase in Oct-1 protein expression was observed (Fig. 4C). We further determined increased Oct-1/DNA binding to recognition sequences within the promoter of the vWF gene (26) after treatment of HCAECs with oxLDL (see Fig. 5A). Strikingly, Oct-1/DNA binding was reduced when HCAECs were treated concomitantly with oxLDL (100 μg/ml, 24 h) and the PKC inhibitor chelerythrine (1 μM, 24 h). Notably, no regulation of Oct-1 was observed in control or unoxidized LDL (100 μg/ml, 24 h)-treated HCAEC cultures (Fig. 5, C and D).

We previously reported repression of cytochrome P450 monooxygenase expression in cultures of HCAEC after treatment with oxLDL (9). This prompted our interest to study the DNA binding of Oct-1 to endothelial expressed cytochrome P450 monooxygenases. By applying various genetic algorithms (see “Experimental Procedures”), we were able to determine putative Oct-1 binding sites in promoter sequences of CYP monooxygenases. It is of considerable importance that predicted Oct-1 binding sites could be confirmed in 6 of the 7 promoters of cytochrome P450 monooxygenases investigated, most notably for CYP1A1, CYP2A6/7, CYP2B6, CYP2C8, CYP2C9, CYP2J2; (see supplemental Table S1). We then constructed specific oligonucleotide probes (20–22 mer), which harbored the identified Oct-1 consensus binding site and used these probes for EMSAs. Upon treatment of HCAECs with oxLDL (Fig. 5, A, C, and D), we found Oct-1 DNA binding to be increased for all promoter sites investigated, albeit with different activity. Strikingly, enhanced Oct-1 DNA binding was abolished, when HCAEC cell cultures were treated simultaneously with the LOX-1 inhibitor k-carrageenan (Fig. 5, A, B, D). This compound efficiently and selectively reduces 125I-ox-LDL binding to the LOX-1 receptor (43). Indeed, we previously demonstrated that k-carrageenan is as effective as use of a specific inhibitory LOX-1 antibody to abrogate LOX-1 signaling (9).

Furthermore, treatment of cell cultures with unoxidized native LDL had no effect on Oct-1 binding (see Fig. 5D). Competition studies with unlabeled Oct-1 probe (>50-fold) visible diminished Oct-1 DNA binding and addition of an Oct-1 antibody supershifted the protein-DNA complex. In competition assays with mutated Oct-1 probes the DNA-protein complex could not be removed (see Fig. 5B and supplemental Table S1). We therefore demonstrate specificity of the assay. To study the consequences of increased DNA binding of Oct-1 to promoters of CYP monooxygenases, we transfected HCAECs with siRNA to functionally knockdown Oct-1 (Fig. 6, A and B). siRNA against Oct-1 in control cells was without significant effects on CYP gene expression (data not shown). In contrast, oxLDL-mediated silencing of the aforementioned CYPs was strongly attenuated after functional knockdown of Oct-1 (Fig. 6C). Specifically, Oct-1-siRNA prevented down-regulation of CYP epoxigenases CYP2C8, CYP2C9, and CYP2J2 after treatment.
of HCAEC with moxLDL, but no significant recovery in mRNA expression was observed in the case of CYP1A1 and CYP2B6 (Fig. 3C). In cultures of HCAEC treated with oxLDL, the production of 11,12-EET catalyzed by CYP epoxygenases was impaired (Fig. 6D). Inhibition of LOX-1 activity by \( \kappa \)-carrageenan abrogated DNA binding of Oct-1 to CYP monoxygenases, and the gene expression of endothelial-expressed CYP monoxygenases returned to normal (see Figs. 5 and 6C).

**DISCUSSION**

We studied the effects of oxLDL treatment in cultures of human primary coronary arterial endothelial cells and observed a dose-dependent increase in ROS production, an increased expression of the growth arrest and DNA damage-inducible transcripts GADD45

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**FIGURE 4.** A, semiquantitative gene expression of Oct-1 in control, nLDL (100 \( \mu \)g/ml), and oxLDL (10–100 \( \mu \)g/ml)-treated endothelial cells. Cells were also treated with oxLDL (100 \( \mu \)g/ml) + \( \kappa \)-carrageenan. Protein expression of Oct-1 in control and oxLDL (10–100 \( \mu \)g/ml)-treated endothelial cells (B) or after UV-C irradiation (C). Data represent mean ± S.D. for at least \( n = 6 \) individual cell culture experiments. *, \( p < 0.05 \).

**FIGURE 5.** EMSAs. A, Oct-1/DNA binding of control and oxLDL (100 \( \mu \)g/ml)-treated HCAECs to DNA binding sites within promoters of various CYP enzymes and the VWF (positive control). B, competition studies with excess addition of wild type (wt) or mutated (mut.; see supplemental Table S1) consensus Oct-1 binding sites. C, Oct-1/DNA binding of control, oxLDL (100 \( \mu \)g/ml), and oxLDL + \( \kappa \)-carrageenan-treated HCAECs to a sequence within the CYP2A6 and CYP2C9 promoter. D, Oct-1/DNA binding of control, oxLDL (100 \( \mu \)g/ml), oxLDL + \( \kappa \)-carrageenan, nLDL, and oxLDL + chelerythrine (1 \( \mu \)mol)-treated HCAECs to a sequence within the CYP2B6 promoter. Data are representative for at least \( n = 6 \) individual cell culture experiments with \( ~2 \) million cells per culture dish. Black arrows indicate specific Oct-1-DNA binding complexes. Note that addition of the Oct-1 antibody does not result in supershifting the complexes, but prevented complex formation. A, free probe; B, control; C, control + anti-Oct-1 antibody; D, oxLDL 100 \( \mu \)g/ml; E, oxLDL 100 \( \mu \)g/ml + anti-Oct-1 antibody; F, oxLDL 100 \( \mu \)g/ml + \( \kappa \)-carrageenan; G, oxLDL + unlabeled probe.
and GADD153, increased formation of the 8-OH-dG DNA adduct and up-regulation of the transcriptional repressor Oct-1. Additionally, activation of LOX-1 repressed production of the endothelial-derived hyperpolarization factor 11,12-EET. There is evidence for oxidative DNA damage to be an underlying cause for atherosclerotic plaque formation (18, 44–46) with high oxLDL levels in the circulation being associated with cellular stress, oxidative DNA damage to foster atherosclerosis (16). Furthermore, it was shown that oxLDL repressed base excision repair activity to result in an accumulation of damaged DNA in mouse monocytes (47). Notably, lipid-lowering diets reduced oxidative stress in rabbit atheromas (48). Although the cellular source for oxLDL-induced ROS is not entirely clear, experimental evidence points to NADPH oxidases (9) and mitochondria (49) as sites for ROS production. Next to oxLDL, other molecules such as homocysteine may further increase expression of GADD153, thereby leading to oxidative stress (50). In accordance we detected transcriptional activation of GADDs in human atherosclerotic aortae. Endothelial GADDs are also induced by irradiation (51) and shear stress, to result in repressed proliferation of endothelial cells (52). Inhibitory effects of high doses of oxLDL on endothelial cell proliferation (53) may therefore be mediated by activation of GADD factors.

Dosing of cultures of HCAEC with oxLDL was based on a previous study (35). We mainly used CuSO4 or CuCl2 for its proven ability to induce the CYP epoxygenases. Strikingly, we found expression of certain CYP monooxygenases by Oct-1. It was shown previously that minimally oxidized LDL preparations (e.g. by Fe2+(SO4) or CuSO4) are very similar in activity to oxLDL derived from human plasma of patients (54). In that study, both extensively and minimally oxidized LDL induced similar ERK1/2 activation. We show minimally and maximally oxLDL to activate Oct-1 which results in subsequent silencing of CYP epoxygenases involved in EDHF formation. Exceptions might be CYP1A1 and CYP2B6, as here oxLDL did not significantly alter expression. However, this needs to be tested in detail in future experiments. It also remains to be determined whether LDL oxidized ex vivo exerts the same biological effects on HCAECs

There is evidence for Oct-1 to foster LOX-1 expression (24). We now demonstrate induction of Oct-1 gene and protein expression in cultures of HCAECs upon treatment with oxLDL. This resulted in increased DNA binding activity of Oct-1 to recognition sites within the promoters of endothelial-expressed CYP monooxygenases. As a consequence we observed impaired metabolic competence of HCAECs as determined for the production of the EDHF 11,12-EET. We previously reported a mechanistic role of CYP monooxygenases in oxLDL-induced vascular injury and found ROS to repress NF-1 DNA binding to result in diminished transcript levels of CYP genes targeted by this factor (9). Indeed, NF-1 plays an essential role in the transcriptional regulation of CYP genes and was shown to be redox-sensitive (9). Here we report induction of Oct-1 protein expression and observed increased DNA binding activity to regulatory sequences of CYP monooxygenases to result in transcriptional repression. This could be prevented by siRNA-mediated knockdown of Oct-1 demonstrating its key role in the transcriptional control of a broad range of CYP monooxygenases in HCAECs. Consequently, oxLDL regulates two factors NF-1 and Oct-1 to impact transcription of endothelial cytochrome P450s. Strikingly, we found expression of certain CYP monooxygenases to be below the level of detection in coronary atherosclerotic blood vessel derived from explanted human heart material (9). Repression of endothelial P450 epoxygenases leads to metabolic dysfunction and impaired production of 11,12-EET. This EDHF functions as a secondary back-up system to rescue vasodilatory function in patients with low eNOS activity (33, 55). We now report increased binding of Oct-1 to an array of CYP monooxygenases, which have been shown in the past to be repressed upon oxLDL treatment (9). We propose
Oct-1 to function as a general repressor of endothelial CYP monoxygenases. Notably, repression of CYP1A1 by Oct-1 has already been shown (32). Here we report a much broader role for Oct-1 in the transcriptional repression of many CYP monoxygenases.

As oxLDL activates PKC (56), we tested the effects of chelerythrine, a specific inhibitor of PKC, on oxLDL-mediated activation of Oct-1. We investigated Oct-1 DNA binding to a recognition sequence within CYP2B6 where strong binding was observed (e.g. CYP2B6, see Fig. 5). Addition of chelerythrine abolished oxLDL-mediated activation and DNA binding of Oct-1. We conclude oxLDL-mediated activation of Oct-1 to be, at least in part, PKC-dependent. Specifically, Oct-1 participates in the cellular response to genotoxic stress and DNA damage in a variety of human cell lines (22). Furthermore, oxLDL impaired synthesis of the von Willebrand factor (vWF), e.g. a key adhesive protein with crucial roles in platelet function (57). Here we demonstrate increased binding of Oct-1 to a recognition sequence within the promoter of the vWF gene. This provided a molecular rationale for silencing of the vWF gene in oxLDL-damaged cells (57).

Loss of metabolic competence of human coronary arterial endothelium can now be traced back to repression of NF-1 and activation of Oct-1. A molecular rationale for the observed reduction in EDHF production of oxLDL-treated HCAECs can be put forward. Nonetheless, it is more than likely that additional pathways important for vascular biology are regulated by Oct-1. Microarray studies revealed an inverse relationship between Oct-1 activity and expression of TIMP3, Gpx3, Gas6, and prdx2, all of which have been linked to oxidative stress (58). This suggests up-regulation of Oct-1 by oxLDL would also lead to a repression of additional oxidative defense genes to result in exaggerated oxidative stress and atherosclerosis development. A reduction of the tissue inhibitor of metalloproteinase 3 (TIMP3) is mechanistically involved in progression of vascular inflammation (59). Glutathione peroxide 3 (Gpx3) catalyzes the reduction of hydrogen and lipid peroxides, which limits the availability of these reactive oxygen species to inactivate NO (60). As a consequence a reduction of Gpx3 results in impaired NO bioavailability and endothelial dysfunction. Reduction of the growth arrest-specific gene-6 (Gas6) increases apoptosis of vascular smooth muscle cells and destabilizes formation of stable platelet macroaggregates (61, 62). Finally, peroxiredoxin 2 (Prdx2) is an important scavenger of hydrogen peroxide (63), and its repression would lead to impaired protection against oxidative injury (64). Taken collectively, it is evident that Oct-1 may not be only important in the regulation of vascular CYP monoxygenases but may also control a broad network of genes involved in the response to oxidative stress.

In conclusion, we found oxLDL to induce oxidative DNA damage and activation of Oct-1 to result in repression of endothelial CYP monoxygenases and diminished metabolic competence, e.g. EDHF formation. This provided a molecular rationale for impaired regulation of vascular homeostasis in oxLDL-diseased coronary artery vessels. Targeting LOX-1 will likely be a remedy to protect endothelium from oxidative DNA damage and impaired metabolic function (65).

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