Online Data Supplement

Materials

Medium M199, Heparin, Fetal Bovine Serum, NADPH, Lucigenin, Native LDL, SIN-1, κ-carrageenan, apocynin were purchased from Sigma (Oakville, ON, Canada). Q Media M199 (without phenol red), Hanks Balanced Salt Solution (HBSS), were purchased from GIBCO and Dihydroethidium (DHE), DiI, Hoescht 33342, fluorescent secondary antibodies were purchased from Molecular Probes (Missisauga, ON, Canada). SIN-1 and peroxynitrite were purchased from Cayman Chemicals (Ann Arbor, MI, USA). siPORT™ NeoFX and siRNA were purchased from Ambion Inc (Streetsville, ON, Canada).

HUVEC Culture

For our study, we selected human umbilical vein endothelial cells (HUVECs) because they are of human origin, easily available, and although of fetal origin, they are in a pregnant milieu. Importantly, our pilot study showed LOX-1 receptor expression in this cell type which makes these cells an appropriate tool for our study. We examined the effects of 2% plasma from the three groups of women on these endothelial cells as this concentration of plasma minimized the cytotoxic effects while maximizing differences between groups.[1-3] Furthermore, we used plasma, but not serum to avoid the confounding effects of cellular products released into serum during blood coagulation.

Umbilical cords were obtained from normotensive pregnancies and endothelial cells were isolated as previously described.[1] Cells were grown on 0.1% gelatin-coated dishes in M199 with 1% endothelial cell growth supplement, heparin, and 20% fetal bovine serum (FBS) and used at passages 2–4. Before stimulation human umbilical vein endothelial cells were plated in six-well plates and incubated in M199 medium (without phenol red) containing 1% FBS and no endothelial cell growth supplement. At the end of each experiment, cellular protein extracts were prepared or total cellular RNA was extracted.

Experimental Protocol

To compare the effect of plasma from three groups of women on LOX-1 receptor protein expression, HUVECs grown in 6 well plates were treated with 2% plasma from non-pregnant, pregnant and preeclamptic women for 24h and LOX-1 receptor protein expression was determined by Western blot. Individual plasma samples from each of these women were assessed and not the pooled plasma from each group was used. To determine the contribution of peroxynitrite generated in response to plasma on LOX-1 receptor expression, FeTPPS (5 μmol/L)[4] a specific peroxynitrite scavenger[5, 6] was added 30 min prior to plasma treatment. To further confirm the effects of peroxynitrite, we

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conducted experiments using exogenous peroxynitrite. To determine the optimal dose of peroxynitrite, preliminary studies were conducted using 25, 50, 100 μmol/L peroxynitrite (Cayman Chemicals, Ann Arbor, MI) for 6, 12 and 24h and cells were stimulated with a dose of 25 μmol/L for 6h.[7] Incubation with higher doses or for longer periods of time resulted in cell death. Exogenous peroxynitrite, used in these experiments has a very short half life at physiological pH. We therefore, have also used SIN-1 (Cayman Chemicals, Ann Arbor, MI), a peroxynitrite donor. SIN-1 at physiological pH releases both nitric oxide and superoxide within the cell, which combine together to form peroxynitrite. Similarly, after conducting preliminary studies with 0.25, 0.50 and 1 mmol/L SIN-1 for 6, 12 and 24 hours, a dose of 0.25 mmol/L for 6h was chosen. To determine if the LOX-1 receptor expression can be regulated at the transcriptional level, LOX-1 receptor mRNA expression was also examined in response to treatment with peroxynitrite and SIN-1 for 6h using RT-PCR.

Since the LOX-1 receptor is responsible for the binding and uptake of oxLDL, we next compared the uptake of oxLDL in cells treated with plasma. Cells grown in 48 well plates were quiesced using phenol-red free media (Q-media) with 5% v/v lipoprotein deficient serum. The cells were then treated with plasma for 24h. During the last three hours of treatment, DiI-oxLDL (10 μg/ml) was added in the presence or absence of 50 ug/ml unlabelled oxLDL (for competition) or mAbLOX-1 (10 μg/ml) as appropriate. The mAbLOX-1 was a kind gift from Dr. Sawamura, Osaka, Japan.

Since the binding of oxLDL can activate the NADPH oxidase enzyme system, we compared the NADPH oxidase activity in HUVECs treated with plasma from three groups of women for 24h in the presence or absence of monoclonal antibody to the LOX-1 receptor (mAbLOX-1, 10 μg/ml) or siRNA to the LOX-1 receptor. In some experiments, a non-immune IgG was used to confirm the specificity of the mAbLOX-1.

The activation of NADPH oxidase leads to the generation of superoxide. We therefore, measured superoxide generation in HUVECs grown in 48 well plates in response to plasma treatment from three groups of women for 24h. Cells exposed to plasma from preeclamptic women were also pre-treated with k-carrageenan (250 μmol/L) a non-specific LOX-1 blocker, mAbLOX-1 (10 μg/ml), apocynin (30 μmol/L)[8] or diphenylene iodonium chloride (DPI, 10 μmol/L)[9].

Excess superoxide generated can combine with nitric oxide to generate peroxynitrite. Peroxynitrite further nitrates tyrosine residues in several proteins resulting in the formation of nitrotyrosine. We therefore, treated cells with plasma from three groups of women for 24h in the presence or absence of mAbLOX-1(10 μg/ml) or siRNA to the LOX-1 receptor and performed immunocytochemistry to detect nitrotyrosine.

Western Blot analysis for LOX-1

After HUVECs were treated with plasma, peroxynitrite or SIN-1, cells were washed in ice-cold phosphate buffered saline, and lysed using lysis buffer (25 mmol/L Tris·HCl pH 7.5 with 0.5% Triton X-100) and sonicated for ~5 s. Protein content was determined using bicinchoninic acid reagent. 10 μg of the protein was loaded on to a 10% gel for LOX-1. The gel was run at 120 V and the protein was transferred onto a nitrocellulose membrane, blocked with 5% fat free milk and the membranes were probed with primary antibodies for LOX-1 (rabbit anti-human) (1:200 Santa Cruz

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Biotechnology), overnight at 4°C. The primary antibody was then detected with a peroxidase-conjugated anti-rabbit secondary antibody (1:5,000). Membranes were scanned with Fluor S Max Multimager (Bio-Rad) and the densitometric analysis was conducted using the software.

Uptake of DiI-labeled oxLDL

Oxidation of LDL

LDL was purchased from Sigma Inc., as lyophilized protein 5mg in 150 mM NaCl and 0.01% EDTA. It was reconstituted using 5ml of sterile distilled water (to get a conc of 1mg/ml). After reconstitution, the LDL was dialyzed using 1 L autoclaved PBS for 24 hours at 4 °C changing the dialysis buffer (PBS) every 6 hours. The LDL was oxidized under aerobic conditions with 5μmol/L $CuSO_4$ for 24h at 37 °C.[10]

The reaction was stopped with 1mmol/L EDTA and the oxidized LDL was kept at 4 °C in dark. The oxidized LDL thus prepared was dialysed to remove $CuSO_4$ and EDTA.[11] The dialysis solution consisted of (in mmol/L) NaCl – 140, NaH_2PO_4 – 1.9, Na_2HPO_4 – 8.1, EDTA – 0.1.

Labeling of oxLDL with DiI

Labeling of oxLDL with DiI was carried out according to the method of Reynolds and St.Clair.[12] Both oxLDL and lipoprotein deficient serum were dialyzed against 1L of autoclaved 0.9%NaCl for 24h at 4 °C in dark, changing the dialysis solution every 6h, before labeling with DiI.[13] Ox LDL (1 mg/ml) lipoprotein deficient serum (2.5 mg/ml) were mixed in a ratio of 1:2 (vol:vol) to give a protein concentration ratio of 1:5 (wt:wt). 150μg DiI/mg oxLDL protein was gently mixed and incubated at 37 °C for 18h in dark under anaerobic conditions. The density of the mixture was adjusted to 1.080 g/ml with solid KBr and overlayed with 4ml of 1.063 g/ml KBr solution. The labeled oxLDL was isolated by centrifugation at 41500 rpm ($160831*g_{av}$) for 24 h at 4 °C using a SW 50 rotor in Beckman L8-M ultracentrifuge. The top 3ml containing DiI-labeled oxLDL was pipetted out and dialysed extensively against autoclaved 0.9% NaCl + 0.01% EDTA for 24h at 4 °C in dark and stored in dark at 4 °C until use. Before use in cell culture experiments the DiI-oxLDL was dialyze in autoclaved normal saline at 4 °C for 24h in dark.

NADPH Oxidase Activity

The NADPH oxidase activity assay was performed by modification of a previously published method.[14] After treatment with plasma, cells were washed in ice-cold Dulbecco's phosphate-buffered saline, scraped off in lysis buffer containing 20 mmol/L KH_2PO_4, 1 mmol/L EGTA, and protease inhibitors, pH 7.4. The cells were sonicated briefly, and the lucigenin-derived chemiluminescence assay was used to determine NAD(P)H oxidase activity in cell homogenates. The reaction was started by the addition of NAD(P)H (0.1 mmol/L) to the suspension (250 μL final volume) containing sample (50 μL), lucigenin (5 μmol/L), and phosphate buffer containing (in mmol/L) KH_2PO_4 -50, EGTA -1, sucrose -150, pH 7.4. Luminescence was measured

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every 4 seconds for 3 minutes in a luminometer. Buffer blank was subtracted from each reading. Activity was expressed as arbitrary units/mg protein.

Superoxide Imaging

Superoxide generated in response to plasma treatment was detected using dihydroethidium (DHE). DHE is a cell permeable dye which exhibits blue fluorescence in the cytosol. However, once this probe is oxidized to ethidium, it intercalates within the cell's DNA, staining its nucleus and emits a bright red fluorescence. During the last 40 minutes of plasma treatment, DHE (20 μmol/L final concentration) was added and incubated in the dark at room temperature. This time point and concentration of DHE was chosen based on preliminary studies. The cells were washed off thrice with Hank's Balanced Salt Solution containing calcium and magnesium. Imaging for superoxide was performed in live cells using a Olympus IX81 fluorescence microscope using a CY3 filter. Image analysis was conducted using Adobe Photoshop. The total intensity due to DHE in a field was divided by the number of cells in that field to obtain the mean fluorescence intensity and reported as arbitrary units.

Immunocytochemistry

After treatment with plasma for 24h cells were washed with ice-cold PBS and fixed with 10% formalin phosphate. After washing, the cells were blocked with 5% BSA in PBS for 3 h and incubated with primary antibody (rabbit polyclonal anti-nitrotyrosine antibody 1:200) overnight at 4°C. After washing, a FITC conjugated anti rabbit secondary antibody (Alexa Fluor 488 1: 200) was added and washed off. Nuclear counter staining using Hoechst 33342 (0.5 μg/ml) (Molecular Probes) was performed. Images were taken using a fluorescence microscope (Olympus IX 81) using the FITC and DAPI filters.

Total RNA Isolation

After treatment with peroxynitrite or SIN-1, cells were washed twice with ice-cold PBS. RNA was isolated using Trizol reagent. The RNA concentration and the quality were determined by measuring the absorbency at 260nm and 260/280nm ratio using a NanoSpek.

Analysis of mRNA expression

cDNA was synthesized by incubating total cellular RNA with random primers and dNTP at 65°C for 5 min and then by incubating the mixture with reverse transcription buffer. The cDNA obtained was amplified using LOX-1 primers (10 uM) (5'-TTACTCTCCATGGTGCC-3') (5'-AGCTTCTTCTDCTTGTTGCC-3') and human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (5'-CCCTTCATTGACCTCAACTACATGG-3') (5'-AGTCTTCTGGGTGGCAGTGATGG-3'), was used as internal standard in the PCR reaction mixture.[15] 193-base pair human LOX-1 cDNA fragment and 456-base pair human GAPDH cDNA fragments were amplified for 40 and 30 cycles, respectively. The PCR product was then subjected to

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electrophoresis on a 1% agarose gel containing ethidium bromide. The bands were imaged and analyzed using Fluor Multimager (Bio-Rad).

siRNA for LOX-1

We used siPORT NeoFX® (Ambion) Transfection agent to transfect siRNA against LOX-1 into HUVECs. HUVECs were transfected with siRNA to LOX-1 by a process called Reverse Transfection that involves simultaneously transfecting and plating cells.
Cells grown in T-75 flask were trypsinized and diluted in normal growth medium. siPORT NeoFX transfection agent was diluted in OPTI-MEM I medium (1:20) and incubated at room temperature for 10 min. siRNA was diluted in OPTI-MEM I medium. Preliminary studies were conducted using varying concentration of siRNA and a concentration of 30nM was chosen. The diluted siPORT NeoFX transfection agent and the diluted siRNA were mixed and incubated at room temperature for 10 min. Then, the mixture was dispensed into the cell culture plates and the cell suspensions were overlayed onto the transfection complexes and grown as usual.

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Table S1.

Characterisitcs of subjects from whom fat biopsies were obtained

Characterisitics	Non-Pregnant	Pregnant	Preeclamptic
Maternal age (y)	33.3 ± 2.5	29.6 ± 1.5	28.4 ± 2.0
Prepregnant weight (kg)	71.6 ± 3.2	67.69 ± 5.77	76.34 ± 8.0
Body Mass Index (kg/m^2)	26.69 ± 1.7	27.27 ± 2.7	29.88 ± 1.72
Term BP			
Systolic BP	107.8 ± 3.5	112.1 ± 1.0	155.7 ± 6.7†,*
Diastolic BP	70.67 ± 3.3	72.1 ± 1.5	98.7 ± 3.6†,*
Parity	1.5 ± 0.7	1.85 ± 0.34	1.42 ± 0.2
Proteinuria	NA	NA	2.9 ± 0.3
Gestational age at delivery (wk)	NA	38.9 ± 0.24	33.2 ± 2.0*
Infant birth weight (g)	NA	3356 ± 148.3	1986 ± 270.8*

The characteristics of subjects, 6 from each group of women are shown in the table. Data are shown as mean ± SEM. † denotes $P<0.01$ vs. non-pregnant women; * denotes $P<0.01$ vs. pregnant women.

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Table S2.

Characteristics of subjects from whom plasma samples were obtained

Characterisitics	Non-Pregnant	Pregnant	Preeclamptic
Maternal age (y)	28.4 ± 2.0	27.6 ± 1.5	30.6 ± 4.3
Prepregnant weight (kg)	59.4 ± 2.8	60.6 ± 4.6	62.6 ± 4.3
Body Mass Index (kg/m^2)	27.42 ± 2.37	28.02 ± 1.03	28.03 ± 2.18
Term BP			
Systolic BP	115.6 ± 3.6	106.2 ± 5.4	$148 \pm 3.5^{\dagger,*}$
Diastolic BP	78.8 ± 0.8	64.2 ± 1.8	$101.0 \pm 4.0^{\dagger,*}$
Parity	1.0 ± 0.0	1.8 ± 0.2	1.4 ± 0.4
Proteinuria	NA	NA	3+
Gestational age at delivery (wk)	NA	38.4 ± 0.2	32.0 ± 2.2*
Infant birth weight (g)	NA	3196 ± 119.7	2017 ± 210.6*

The characteristics of subjects, 6 from each group of women are shown in the table. Data are shown as mean ± SEM. † denotes $P<0.01$ vs. non-pregnant women; * denotes $P<0.01$ vs. pregnant women.

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Impaired Vascular Function in Small Resistance Arteries of LOX-1 Overexpressing Mice on High-Fat Diet

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Abstract

Aims: LOX-1 is a major vascular receptor for oxidized low-density lipoprotein (oxLDL). In this study, we analyzed the impact of LOX-1 overexpression and high dietary fat intake on vascular function in small resistance arteries.

Methods and Results: Relaxation of mesenteric arteries was measured using a wire myograph. Compared with the control group, mice overexpressing LOX-1 on a high-fat diet (FD) had preserved vascular smooth muscle relaxation, but impaired endothelium-dependent relaxation via NO. Vascular NO availability was decreased by exaggerated formation of reactive oxygen species and decreased endothelial NO synthase expression. Endothelium-derived hyperpolarizing factor (EDHF)-mediated relaxation via cytochrome P450 metabolites was increased in LOX-1+FD animals, but did not completely compensate for the loss of NO. Currents of calcium-activated potassium channels with large conductance (BK_{Ca} channels) were measured by the voltage-clamp method. The BK_{Ca} current amplitudes were not altered in endothelial cells, but highly increased in vascular smooth muscle cells from resistance arteries of LOX-1-overexpressing mice on FD. BK_{Ca} currents were activated by low-dose H_2O_2 and cytochrome P450 metabolites 11,12-EET and 14,15-EET as EDHF in control mice.

Conclusion: LOX-1 overexpression and FD caused functional changes in endothelial and vascular smooth muscle cells of small resistance arteries.

Key Word: Endothelial function; K^+-channel; Lipoproteins; Smooth muscle; lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1)

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1. Introduction

Atherosclerosis, with its clinical manifestation in cardiovascular diseases, is the major cause of death in industrialized countries. Functional changes in endothelial cells (EC) and vascular smooth muscle cells (VSMCs) contribute to the initiation and early progression of cardiovascular diseases like atherosclerosis.[1-3] Resistance arteries do not show morphological alterations in response to high-fat diet or oxLDL, but rather develop functional impairment.[4] Several changes in the early phase of endothelial dysfunction are associated with high plasma levels of lipoproteins. Circulating low-density lipoproteins (LDL) can be modified to oxidized LDL (oxLDL). The major receptor of oxLDL is the lectin-like oxLDL receptor-1 (LOX-1) in the vessel wall.[5-7] LOX-1 mediates endocytosis of oxLDL in endothelial cells (ECs)[8], VSMCs and monocytes.[9] Basal LOX-1 expression is low, but several pathophysiological conditions like hypertension, diabetes mellitus and hyperlipidemia and the development of atherosclerotic lesions have been linked with an increased vascular LOX-1 expression.[10] The G501C mutation in the lectin-like oxidized low-density lipoprotein receptor gene (LOX-1/OLR1) has been associated with the risk of myocardial infarction,[11] but not with the risk for stroke.[12] LOX-1 expression is increased in human atherosclerotic lesions (in early lesions mainly in endothelial cells, in advanced lesions also in vascular smooth muscle cells and macrophages).[13] Moreover plaque formation is enhanced in coronary arteries of mice overexpressing LOX-1 against a genetic background of apolipoprotein E deficiency.[14] Cell culture studies have shown that the endothelial generation of reactive oxygen species (ROS) by NAD(P)H oxidase complexes in response to oxLDL is mediated by LOX-1.[15] Furthermore, in vitro studies indicate that activation of LOX-1 also initiates a reduction in NO release.[15] However, little is known about the contribution of LOX-1 to vascular homeostasis and endothelial dysfunction in small vessels. The intact endothelium plays an important role in vascular function by synthesizing and releasing vasodilating factors.[16]

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Major vasodilating factors in arteries are the endothelium-derived hyperpolarizing factor (EDHF), nitric oxide (NO) and prostacyclin. The reduced NO availability in atherosclerosis can be mediated via decreased expression of the endothelial NO synthase (eNOS), or via inactivation of NO by ROS.[2] In addition to eNOS expression, there is also evidence of reduced eNOS activity by lack of cofactors (e.g. BH_4) and increased formation of endogenous inhibitors.[17] The impaired NO-mediated relaxation in different vessels of hypercholesterolemic and atherosclerotic animal models or patients[18,19] can be compensated by EDHF. Several components for EDHF signalling have been proposed including electrical coupling through gap junctions, certain ROS as for instance H_2O_2, cytochrome P450 metabolites, and vascular Ca^{2+}-activated K^+ channels (K_{Ca})[20] in particular those with large conductance (BK_{Ca} channels).[21-23] Activation of BK_{Ca} channels facilitates relaxation by cell membrane hyperpolarization. Nevertheless their role in endothelial and smooth muscle dysfunction is not completely understood. Binding of oxLDL to LOX-1 was shown to modulate BK_{Ca}-channels in endothelial cells in vitro.[15]

In this study, we have used mice overexpressing bovine LOX-1 that were fed a high-fat diet to examine a potential functional impairment of small resistance arteries (mesenteric arteries). Based on our experimental findings we provide evidence, that LOX-1 receptors can cause vascular dysfunction in resistance vessels.

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2. Materials and Methods

2.1. Animals

Male wild-type mice (C57BL/6, WT) and mice overexpressing bovine LOX-1 in a C57BL/6 background under control of the murine preproendothelin-1 promoter (LOX-1 mice[14]), aged 8 weeks, were fed standard chow EF R/M CD88137 or high-fat diet (FD) EF R/M TD88137 (Ssniff Spezialitäten GmbH, Soest, Germany) for 10 weeks. LOX-1 mice were kindly provided by Dr. T. Sawamura, Department of Vascular Physiology, National Cardiovascular Center Research Institute Fujishirodai, Suita, Osaka, Japan. The generation and characterization of the mice has been recently described by Inoue *et al.*[14] The LOX-1 mice carry 24 copies of the transgene, resulting in a ~8-fold higher mRNA-expression and a marked upregulation of endothelial LOX-1 protein expression. A similar upregulation of the LOX-1 protein has been described in endothelial cells of human carotid arteries, covering early atherosclerotic lesions.[13] Overexpression of the bovine LOX-1 was verified by PCR in mesenteric arteries (Figure 1 online data supplement). All performed experiments are in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). The animal research ethics committee of the Dresden University of Technology and the Regierungspräsidium Dresden approved the animal facilities and the experiments according to institutional guidelines and German animal welfare regulations (AZ: 24-9168.24-1-2003-13, 24D-9168.24-1/2006-16).

2.2. Serum lipid measurements

Serum lipids were measured at the Institute of Clinical Chemistry and Laboratory Medicine (University of Technology Dresden) using kits for triglycerides, cholesterol, high-density

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lipoproteins (HDL) and low-density lipoproteins (LDL; Roche Diagnostics GmbH, Mannheim, Germany).

2.3. Preparation of mesenteric arteries for in vitro studies

Arteries (3rd order branch) were dissected and maintained in Ca^{2+}-free physiological salt solution (PSS; mmol/L: NaCl 119.0; KCl 4.7; $MgSO_4$ 1.17; $NaHCO_3$ 25.0; KH_2PO_4 1.18; glucose 5.5 and ethylenediaminetetraacetic acid (EDTA) 0.027; pH 7.4).

2.4. Superoxide anions

Dissected vessels were incubated in Krebs-Henseleit buffer (mmol/L: NaCl 115.0; $NaHCO_3$ 25.0; KCl 4.0; KH_2PO_4 0.9; $MgSO_4x7H_2O$ 1.1; $CaCl_2$ 2.6; glucose 5.5; pH: 7.4) for 30 min at 37°C. Lucigenin (5 µmol/L – a concentration below the threshold of redox cycling[24]) and NADPH (100 µmol/L) were dissolved in Krebs-Henseleit buffer for determination of ROS. Lucigenin solution containing additional 200 U/mL superoxide dismutase (SOD) and 380 U/mL catalase was used to examine superoxide anions and the resulting H_2O_2 formation.[25] Solution without tissue served as control. Photoemission was detected every second for 30 min in a Fluorescence Microplate Reader Fluorimeter FLUOstar OPTIMA (BMG LABTECH, Jena, Germany). The length of the blood vessels was measured with an eye piece scale (ZEISS, Jena, Germany) and used for data normalization. The increase in ROS production in animals fed with a high-fat diet was normalized as 100% of the corresponding control.

2.5. Measurement of contractile function

Small sections of mesenteric arteries (length 2 mm) were mounted in microvascular myographs for isometric tension recordings as described previously[26] and maintained in oxygenated PSS (5% CO_2 in 95% O_2; 1.6 mmol/L $CaCl_2$) at 37°C. During equilibration of the vessels, tension (T) corresponding to a pressure (P) of 70 mm Hg according to the equation $P=T2\pi U^{1}$ (U = inner circumference) was adjusted. In all experiments cyclooxygenase-

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mediated relaxation was blocked with cyclooxygenase inhibitor diclofenac (0.1 mmol/L; Sigma, Taufkirchen, Germany). The vessel rings were contracted with cumulatively increasing concentrations of phenylephrine (PE). Relaxation was measured by increasing concentrations of acetylcholine (ACh) or sodium nitroprusside (SNP) in PE-precontracted (10 μmol/L) vessels. Relaxing effects of ACh were studied in the absence and presence of NO synthase inhibitor nitro-L-arginine-methylester (L-NAME; 30 μmol/L), BK_{Ca} inhibitor paxilline (1 μmol/L), cytochrome P450 inhibitor N,N-diethyl-aminoethyl-2,2-diphenylvalerate (proadifen; 50 μmol/L) and epoxygenase inhibitor 6-(2-propargyloxyphenyl)hexanoic acid (PPOH; 30 μmol/L). The effects of ACh or SNP are expressed in percent of the response to PE (=100%).

2.6. Isolation of vascular cells

Mesenteric arteries were stored in low Ca^{2+}-containing PSS (0.16 mmol/L Ca^{2+}) at 4°C. Enzymatic dissociation was carried out in two steps. The first solution (1 mL PPS) contained 0.7 mg papain; 1.5 mg dithioerythritol (Roth, Karlsruhe, Germany), bubbled with O_2, 20 min, 37°C. The second solution (1 mL PPS) contained 1.2 mg collagenase type F, 1.5 mg trypsin inhibitor, 0.5 mg elastase (Serva, Heidelberg, Germany) and 1.0 mg bovine albumin fraction V (Serva, Heidelberg, Germany) gassed with O_2, 12 min, 37°C. Single VSMCs and ECs (Figure 3) were obtained by trituration in PSS.

2.7. Electrophysiological experiments

Potassium outward current through the BK_{Ca} channels ($I_{BK,Ca}$) was measured using a HEKA-EPC8 amplifier (HEKA Elektronik, Lambrecht, Germany) in voltage-clamp mode. Bath superfusion buffer (mmol/L): NaCl 127.0; KCl 5.9; $CaCl_2$ 2.4; $MgCl_2$ 1.2; glucose 11.0; HEPES 10.0, pH 7.4. Pipette solution (mmol/L): KCl 134.0; NaCl 6.0; $MgCl_2$ 1.2; $CaCl_2$ 4.2, EGTA 5.0; glucose 11.0; Mg-ATP 3.0; HEPES 10.0; pH 7.4. Pipette tip resistance was 3.0-4.0 MΩ. Experiments were carried out at 21°C. Effects of the following compounds were

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studied: 1,3-Dihydro-1-[2-hydroxy-5-(trifluoromethyl)phenyl]-5-(trifluoromethyl)-2H-benzimidazol-2-one (NS1619; 30 µmol/L), paxilline (1 µmol/L), H_2O_2 (1 µmol/L), 11,12-Epoxy-(5Z,8Z,14Z)-eicosatrienoic acid (11,12-EET; 300 nmol/L) and 14(R),15(S)-Epoxy-(5Z,8Z,11Z)-eicosatrienoic acid (14,15-EET; 300 nmol/L). The cell capacity was used for data normalization. Unless stated otherwise, all substances were purchased from Sigma, Taufkirchen, Germany.

2.8. RNA isolation and real-time PCR

Total RNA of mesenteric arteries was isolated using the EZNA Total RNA-Kit (Peqlab, Erlangen, Germany). For quantification of mRNA expression real-time PCR was performed using the QuantiTect SYBR Green RT-PCR kit (Qiagen, Hilden, Germany) in a thermal cycler (Corbett Research, Mortlake, Australia). Primers: bovine LOX-1 (sense: 5`-CCAGGAGAACTGCTTGTCTT-3`, antisense: 5`-GTGCTCTCAATAGATTCGCC-3`), eNOS (sense: 5´-TTCCGGCTGCCACCTGATCCTAA-3´, antisense: 5´-AACATATGTCCTTGCTCAAGGCA-3´), KCNMA (sense: 5´-CGATAAGCTGTGGTTCTGGC-3´, antisense: 5´-AAGAAGACCATGAAGAGGCGTC-3´) and KCNMB1 (sense: 5´-AGAAGCGCGGAGAGACACGA-3´, antisense: 5´-CAGCTCTTCCTGGTCCTTGATA-3´). Quantification by one step RT real-time PCR included 50°C for 30 min, 95°C for 15 min, subsequent cycles of 94°C for 30 s, 60°C for 30 s and finally 72°C for 50 s. Internal RNA standards were produced as previously described.[27]

2.9. Data analysis and statistics

Potency of agonists was determined as $-\log EC_{50}$ [mmol/L]. All results are expressed as mean±standard error of the mean (SEM). Unless stated otherwise, number of experiments is given as arteries or cells from *n* mice. Student´s *t*-test (unpaired) was used for statistical analysis, differences with $P<0.05$ were considered significant. Multiple comparisons were done by one-way ANOVA followed by Bonferroni post-hoc test.

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3. Results

3.1. Body weight and serum lipids

Table 1 summarizes body weight and serum lipid concentrations in the four investigated animal groups. LOX-1 mice on normal diet were heavier than WT mice, but did not differ in serum lipid concentrations. FD significantly increased body weight in WT and in transgenic mice as compared with control diet. While serum concentrations of triglycerides were not different between the groups, free cholesterol, LDL and HDL concentrations were significantly higher in WT and LOX-1 animals on high-fat compared with standard diet. The ratio of LDL:HDL increased during FD from 1:7 to 1:4 in WT mice and from 1:8 to 1:4 in LOX-1 mice. Although high-fat diet caused significantly larger weight gain in LOX-1 mice compared with WT animals, no statistically significant differences were found in serum lipid parameters.

3.2. Vascular function

Basal tone of mesenteric arteries was unaffected by FD (compare pre-PE control values in Figure 2A online data supplement).

Endothelium-dependent and -independent relaxation in mesenteric arteries was studied in PE-preconstricted vessels. In order to determine the optimum concentration for precontraction, we measured contractile responses to cumulatively increasing PE concentrations. All vessels contracted in the same concentration range (average -logEC_{50} [mol/L] values between 5.5 and 5.7). However, the maximum contractile response to PE was significantly lower in the LOX-1+FD mice than in the other groups (LOX-1+FD: 1.4±0.1 mN/mm versus WT: 1.7±0.3 mN/mm; WT+FD: 1.8±0.1 mN/mm; LOX-1: 2.0±0.2 mN/mm; $P<0.05$; Figure 2A,B online data supplement). Subsequently, arteries in all further experiments were precontracted with 10 μmol/L PE. Values of maximum contraction produced by 80 mmol/L KCl were similar in the four groups (Figure 2C online data supplement). Absolute values of the constriction

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induced by 100 μM PE and 80 mmol/L KCl in WT and LOX-1 mice are consistent with previously published results.[28,29]

Endothelium-dependent relaxation was studied by exposing the arteries to increasing concentrations of ACh. The resulting concentration-response curves (CRC; Figure 1A, B) revealed incomplete relaxation by ACh with significantly reduced relaxation in LOX-1+FD animals (maximum relaxation, Eff_{max}: 67.8±3.4%; $P<0.01$) compared with WT, WT+FD and LOX-1 (Eff_{max}: 89.3±5.0%; 88.1±4.7%; and 88.1±3.6%; Figure 1C). Interestingly, potencies were similar in all groups (average -logEC_{50} [mol/L] values between 6.8 and 7.0). The relaxing response to ACh was reduced in the presence of the NO synthase (NOS) blocker L-NAME (30 μmol/L). Relaxation was reduced in WT (42.2±5.6%), WT+FD (39.1±7.5%), and LOX-1 mice (40.1±6.1%), but not in LOX-1+FD arteries (5.5±5.2%). These results suggest that NO-mediated relaxation was significantly impaired in LOX-1+FD animals compared with the other groups (Figure 1D). In addition the basal tone during L-NAME incubation increased only in arteries from WT, WT+FD and LOX-1 animals, but not in LOX-1+FD mice. In presence of L-NAME, the differences in PE-induced contractions between LOX-1+FD mice and the other three animal groups persisted (Figure 2D,E online data supplement).

ACh-induced relaxation in the presence of NOS and cyclooxygenase inhibitors is mediated by EDHF. The EDHF-mediated fraction of relaxation was largest in LOX-1+FD mice (62.6±3.5%) compared with the other groups (WT: 47.0±3.2%; WT+FD: 49.0±4.0%; LOX-1: 48.0±4.1%; $P<0.01$; Figure 1E). Cytochrome P450 enzymes are a substantial source of EDHF, they are responsible for the transformation of arachidonic acid into epoxyeicosatrienoic acids (EETs).[30,31] The role of these enzymes in ACh-induced relaxation was examined with proadifen, a non-specific blocker of cytochrome P450 isoenzymes and PPOH, a specific epoxygenase blocker. Proadifen and PPOH did not significantly block EDHF-mediated relaxation in WT, WT+FD and LOX-1 animals, but significantly reduced

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EDHF-mediated relaxation in LOX-1+FD (proadifen 41.3±6.3%; $P<0.01$ and PPOH 28.7±6.3%; $P<0.05$; Figure 2A-D). Both compounds changed the efficacy of ACh to a larger extent in LOX-1+FD mice compared with the other groups, leading to similar levels of relaxation in all four groups.

The contribution of BK_{Ca} channels as potential EDHF targets in LOX-1+FD, was tested by blocking the channels with the specific BK_{Ca} blocker paxilline (Figure 2E-H). Paxilline reduced ACh-mediated relaxation only in LOX-1+FD (18.42±3.6%; $P=0.0026$), but not in WT (0.1±3.3%); WT+FD (1.6±2.5%) and LOX-1 animals (1.1±3.9%), indicating that BK_{Ca} channels were activated only in LOX-1+FD mice. Even in the presence of L-NAME, paxilline was able to inhibit relaxation, indicating a BK_{Ca} channel involvement in the EDHF-mediated relaxation. During paxilline incubation the basal tone significantly elevated in arteries from LOX-1+FD mice compared with WT, WT+FD and LOX-1 animals (Figure 2F online data supplement). PE-induced contraction in presence of paxilline was similar in all four groups (Figure 2G online data supplement).

Endothelium-independent relaxation was measured using the NO-donor SNP. Potency and efficacy of SNP were calculated from cumulative CRCs (Figure 3 online data supplement) and were found to be similar in all groups ($-logEC_{50}$ [mol/L]: WT: 7.4±0.2; WT+FD: 7.4±0.1; LOX-1: 7.2±0.2; LOX-1+FD: 7.2±0.1; Eff_{max}: WT: 0.6±2.5%; WT+FD: 1.2±2.6%; LOX-1: 2.8±2.9%; LOX-1+FD 1.7±0.5%).

3.3. Reactive oxygen species (ROS)

Since vascular dysfunction is associated with increased ROS production, we have examined the formation of ROS by chemiluminescence in mesenteric arteries. ROS formation was increased in WT+FD and LOX-1+FD, and the increase was even higher in LOX-1+FD than in WT+FD animals (Figure 4 online data supplement). We have also determined superoxide anions, because this particular fraction of ROS has been previously shown to result in H_2O_2

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formation and act directly as an EDHF component.[32] We found that H_2O_2 levels were significantly increased in LOX-1+FD mice (56.2±5.8%) compared with the other groups (WT: 33.2±3.4%; WT+FD: 40.6±8.3%; LOX-1: 39.8±5.7%; Figure 4 online data supplement).

3.4. BK_{Ca} currents

Our results provide evidence that EDHF formation is involved in the contractile responses and that EDHF has the largest impact in the LOX-1+FD group. Since EDHF activates BK_{Ca} channels, we measured BK_{Ca} currents in smooth muscle and endothelial cells in order to elucidate their contribution to the observed EDHF-mediated response in LOX-1+FD mice.

All vascular smooth muscle cells exhibited robust BK_{Ca} currents. The current amplitude was completely suppressed by the selective BK_{Ca} channel blocker paxilline (1 µmol/L).[33] Current densities of $I_{BK,Ca}$ in VSMCs at a test potential of +60 mV were significantly higher in LOX-1+FD mice (623±93 pA/pF) compared with WT (356±54 pA/pF), WT+FD (346±47 pA/pF) and LOX-1 (324±54 pA/pF; Figure 3A,B). In LOX-1+FD $I_{BK,Ca}$ voltage dependence was shifted to lower voltages suggesting that the current is already activated at more negative potentials. Moreover, opening of BK_{Ca}-channels with the selective channel activator NS1619 (30 µmol/L)[34] did not further increase current density in LOX-1+FD mice, but increased the current in the other groups to almost the same levels as previously observed in LOX-1+FD (Figure 3C). Interestingly, the $I_{BK,Ca}$ current densities in ECs were similar in all four mice groups (WT: 64±31 pA/pF; WT+FD: 61±12 pA/pF; LOX-1: 63±27 pA/pF; LOX-1+FD: 68±32 pA/pF), but much smaller than in VSMCs (Figure 3D,E). Next, we examined the effects of three supposed EDHFs, i.e. H_2O_2, 11,12-EET and 14,15-EET, on BK_{Ca} channel activity in WT mice. At +60 mV H_2O_2 (1 µmol/L) significantly increased current density, but not to the same value as observed with NS1619 or in LOX-1+FD mice. 11,12-EET and

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