14,15-EET (300 nmol/L each) activated $I_{BK,Ca}$ to the same maximum as NS1619 (Figure 4A-C) in WT mice and as the observed amplitude in LOX-1+FD animals.

3.5. mRNA expression of BK_{Ca} channel subunits and eNOS

Differences in current densities could be due to different expression levels of the relevant channel subunits. Therefore, we have measured mRNA expression of the BK_{Ca}-channels using real-time PCR (Figure 5A,B). Both the pore-forming α-subunits and the accessory β1-subunits were expressed in similar amounts in all groups. In contrast mRNA expression of eNOS was lower in LOX-1+FD mice compared with WT, WT+FD and LOX-1 animals (Figure 5C). This finding is in agreement with the reduced NO-dependent vascular relaxation in this group of animals. In heart and kidney, we did not detect statistically significant differences in the mRNA and protein expression of the BK_{Ca} channel α- and β1-subunits and eNOS between all groups.

4. Discussion

The main findings of our study show that mice overexpressing the LOX-1 in combination with FD have markedly increased body weight, display impaired NO-mediated and enhanced EDHF-dependent relaxation in mesenteric arteries, and have increased vascular ROS production in LOX-1+FD compared with WT+FD animals. Furthermore, BK_{Ca} channel activity in VSMCs was higher in LOX-1+FD, which could be increased by the EDHFs H_2O_2 and EETs. In contrast, endothelial BK_{Ca} channel activity was unchanged.

Body weight and plasma lipids

As expected, WT and LOX-1 overexpressing mice gained weight after FD. However, LOX-1 mice on standard diet were also heavier than their corresponding WT animals suggesting an impact of LOX-1 on weight control or metabolic status. Higher body weight in LOX-1 mice was not associated with a significant shift in plasma lipid parameters to a more harmful lipid profile as observed with FD in both WT and LOX-1 mice. In the latter 2 groups, elevated total plasma cholesterol, LDL and HDL are in agreement with previous studies.[35,36] Furthermore the LDL/HDL quotient shifted to higher values suggesting an enhanced risk for endothelial dysfunction and atherosclerosis.[37]

Vascular constriction and relaxation

None of the animals had grossly visible atherosclerotic lesions. Therefore, we tested for vascular dysfunction as an early sign of cardiovascular disease, by measuring contraction and endothelium-dependent as well as endothelium–independent relaxation of PE-preconstricted small resistance arterioles. The impact of oxLDL on vascular tone is not fully resolved. OxLDL enhances the basal tone in rabbit cerebral arteries.[38] In contrast, oxLDL has no effect on basal tone in rabbit aorta.[39] We observed no differences in basal vascular tone in the animal groups fed standard or high-fat diet. However, the maximum contractile response to

PE was lower in LOX-1+FD than in the other animals, but potencies were similar. This difference in the vessel contraction between LOX-1+FD mice and the other three animal groups was not observed in the presence of high, depolarizing potassium concentration or by the combination of phenylephrine and paxilline suggesting a mechanism that involves potassium channel activity. BK_{Ca} channels also participate in ACh-/ endothelium-mediated relaxation.[21-23]

ACh-stimulated, NO-mediated dilatation was lower in LOX-1+FD mice than in WT, WT+FD and LOX-1 animals, suggesting reduced NO availability in LOX-1+FD. In accordance basal tone during L-NAME incubation was less increased in LOX-1+FD mice compared to WT, WT+FD and LOX-1 animals. Effectivity of L-NAME to eliminate NO production has been questioned because in some species, especially in pig and rabbit, endothelium-dependent/NO-mediated relaxation was resistant to eNOS-inhibitors.[40] In our experiments with mouse mesenteric arteries, however, relaxation persisting after inhibition of eNOS with L-NAME was fully suppressed with apamin and charybdotoxin, the combination of which is commonly used for EDHF block (data not shown). Possible explanations for decreased NO-mediated relaxation in mesenteric arteries of LOX-1+FD mice are (i) that ROS production is elevated , (ii) that expression of eNOS mRNA is reduced and (iii) that EDHF can compensate for the loss of NO.[41] In contrast to the reduced NO-mediated relaxation, the EDHF-dependent response was elevated in LOX-1+FD mice compared with WT, WT+FD and LOX-1 animals, but could be abolished by a general cytochrome P450 blocker or by an epoxygenase inhibitor. This finding suggests a potential role of cytochrome P450 and its epoxygenases as a compensatory mechanism that makes up for loss of NO and subsequent endothelial dysfunction in LOX-1+FD mice. This same amount of EDHF, which was abolished by the cytochrome P450 inhibitors, was also blocked by paxilline in presence and absence of L-NAME suggesting an essential role of the BK_{Ca} channel in the EDHF-mediated relaxation. Block of cytochrome P450 or BK_{Ca} channels in LOX-1+FD led to similar levels of persisting

EDHF-mediated relaxation in all four groups. The nature of the persisting EDHF-mediated relaxation was not investigated in this study. As reported before, release of EDHF from endothelial cells is associated with the endothelial activity of calcium-activated potassium channels of small and intermediate conductance,[21] but the activation of BK_{Ca} channels is involved in the EDHF-mediated relaxation of vascular smooth muscle cells. No relevant differences in endothelium-independent relaxation were detected, i.e. potency and efficacy of SNP were similar in all groups, suggesting comparable properties of VSMC-relaxation in mice on FD and standard diet. Interestingly, an EETs-mediated relaxation in response to 11,12-EET and 14,15-EET was significantly increased in mesenteric arteries with and without endothelium of LOX-1+FD mice compared to WT, WT+FD and LOX-1 animals (Figure 5 online data supplement). This could be mediated by the compensatory enhanced activity of the BK_{Ca} channels in vascular smooth muscle cells of LOX-1+FD mice. However, activation is not sufficient to compensate for the loss of NO in the LOX-1+FD mice.

Vascular ROS productions

Our results and several previous studies have shown that hypercholesterolemia is associated with impaired endothelium-dependent relaxation in experimental and clinical studies.[1,42-44] Rapid degradation of endothelium-derived NO by high levels of ROS is thought to be a major mechanism underlying impaired endothelium-dependent vasodilatation under hypercholesterolemic conditions. It should also be responsible for decreased endothelium-dependent relaxation.[45,46] In our experiments, ROS was indeed increased in LOX-1+FD and WT+FD mice, and to a larger extent in the former than the latter. ROS leads to oxidation of LDL resulting in increased oxLDL levels. Uptake of oxLDL via LOX-1 into endothelial cells activates NAD(P)H oxidases and enhances production of ROS,[47,48] starting a vicious cycle finally leading to endothelial dysfunction. However, exaggerated ROS production has not only deleterious effects. Growing evidence supports an import role of redox-sensitive

signaling in vascular function.[49] Furthermore Shimokawa and Matoba[32] have suggested that the ROS product H_2O_2 could act as EDHF. H_2O_2 amount was higher in LOX-1+FD mice than in WT, WT+FD and LOX-1 animals, suggesting a potential role in the elevated EDHF-mediated relaxation.

Role of EDHF and BK_{Ca} channel activity

In contrast to reduced NO-mediated responses, EDHF-mediated relaxation was most pronounced in LOX-1+FD mice suggesting that this mechanism might partly compensate the impaired NO-mediated relaxation. Since different EDHFs, like EETs and H_2O_2 are known to activate BK_{Ca} channels in VSMCs,[21] we studied BK_{Ca} currents directly. At any activating potential, the amplitude of $I_{BK,Ca}$ was indeed larger in VSMCs from LOX-1+FD than in the other groups. Moreover, $I_{BK,Ca}$ amplitude in LOX-1+FD mice could not be further increased by the BK_{Ca} channel opener NS1619, suggesting that increased current amplitude resulted from enhanced channel open probability. This interpretation is supported by the fact that mRNA expression of the BK_{Ca} channel α- and β1-subunits was similar in all mice. Because of the limited availability of vascular tissue from the mesenteric arteries, no additional data on protein level could be obtained. However, similar mRNA and protein expression of BK_{Ca} channels have been demonstrated in human arteries and veins.[50] The $I_{BK,Ca}$ amplitudes in ECs were smaller than in VSMCs and did not reveal any differences between the four groups. Therefore BK_{Ca} channels in ECs do not contribute to vascular dysfunction in LOX-1+FD mice. The activation of endothelial BK_{Ca}-channels contributes to the EDHF-mediated relaxation. Endothelial hyperpolarization can be transmitted directly via gap junctions to the vascular smooth muscle cells.[51,52] In addition, the K^+ outward current through BK_{Ca} channels increases the extracellular potassium concentration.[53] The subsequent activation of the inward-rectifier potassium channel (K_{IR}) and the Na^+/K^+ pump overcomes the minor depolarising effects linked to the K^+ increase[54,55] and relaxes smooth muscle cells.

The possible EDHF H_2O_2 significantly increased $I_{BK,Ca}$ in VSMCs, although the level did not reach the maximum current amplitude of NS1619 or as in LOX-1+FD mice. Therefore, H_2O_2 plays only a minor compensatory role in EDHF-mediated relaxation of this model. Recently, an inhibiting effect of H_2O_2 on cytochrome P450 has been suggested, indicating a negative feedback mechanism of EET production.[56] This could not be confirmed in our experimental model.

Reduced NO levels have been reported to disinhibit cytochrome P450 and hence elevate two other possible EDHF components, 11,12-EET and 14,15-EET.[57-59] These two EETs were able to enhance $I_{BK,Ca}$ to similar maximum current amplitude as observed in the presence of NS1619 and in LOX-1+FD. In agreement with previous findings in isolated renal arteries of hypercholesterolemic rabbits,[18,19] we suggest that enhanced formation of EDHF represents a compensatory mechanism of the decreased NO-mediated vessel relaxation. In contrast, Urakami-Harasawa *et al.*[60] found a significant inhibition of endothelium-dependent hyperpolarization in isolated gastroepiploic arteries from atherosclerotic patients. These contradictory results could be explained by the longer duration of hypercholesterolemic conditions and the progressive development of vascular diseases.

In the last few years, vascular BK_{Ca} channels were considered as potential therapeutic targets in the treatment of hypertension, endothelial dysfunction, and other cardiovascular diseases,[61] because aldosterone overexpression induces a nitric oxide-independent coronary dysfunction with decreased VSMC BK_{Ca} expression and coronary BK_{Ca}-dependent relaxation.[62] However activation of LOX-1 in clinical manifestation of atherosclerosis could activate VSMC BK_{Ca} channels. In contrast, enhanced LOX-1 expression does not influence endothelial BK_{Ca} channels.

In conclusion, we have consistently detected significant changes in contractile and electrophysiological properties of small resistance vessels only in the combination of LOX-1 overexpression and high-fat diet. The endothelium-mediated relaxation via NO release was

impaired, but partly compensated by the higher release of EDHF. The consequence of this compensatory mechanism was a higher $I_{BK,Ca}$ due to increased open probability of BK_{Ca} channels in VSMCs, but not in ECs. Our results clearly demonstrate that LOX-1 overexpression and FD cause functional changes in ECs and VSMCs of small resistance arteries leading to vascular dysfunction as an early sign of cardiovascular diseases.

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Conflict of Interest

Conflict of interest: none declared.

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Figure 1: Effects of acetylcholine in mesenteric arteries of WT and LOX-1 mice.

A: Concentration-response curves (CRCs) for acetylcholine in arteries of WT mice on standard and high-fat diet without and with L-NAME.

B: CRCs for acetylcholine in arteries of LOX-1 mice on standard and high-fat diet without and with L-NAME.

Maximum effects of acetylcholine-induced (C), NO-mediated relaxation (D) and EDHF-mediated relaxations (E) of arteries from WT and LOX-1 mice on standard and high-fat diet.

*** $P<0.001$ control versus L-NAME of mice on standard diet.

$P<0.001$ control versus L-NAME of mice on high-fat diet.

Figure 2: Concentration-response curve for acetylcholine in presence of BK_{Ca} channel blocker and cytochrome P450 blocker in mesenteric arteries of WT and LOX-1 mice.

Concentration-response curves (CRCs) for acetylcholine in the presence of cytochrome P450 blocker proadifen and epoxygenase blocker PPOH in arteries of A: WT; B: WT+FD; C: LOX-1; D: LOX-1+FD in combination with L-NAME.

CRCs for acetylcholine in the presence of BK_{Ca}-channel blocker paxilline on arteries of E: WT; F: WT+FD; G: LOX-1 and H: LOX-1+FD and in presence and absence of L-NAME.

*$P<0.05$ proadifen+L-NAME versus L-NAME;**$P<0.01$ PPOH+L-NAME versus L-NAME.

Figure 3: Characterization of electrophysiological properties in vascular smooth muscle cells (VSMCs) and endothelial cells (ECs) of mesenteric arteries from WT and LOX-1 mice on standard and high-fat diet.

A: Current density in pA/pF of BK_{Ca} currents in VSMCs from WT, WT+FD, LOX-1 and LOX-1+FD.

B: Current voltage relationships of BK_{Ca} currents of VSMC from WT, WT+FD, LOX-1 and LOX-1+FD. $^{**}P<0.01$ standard diet versus high-fat diet.

C: Current density in pA/pF of BK_{Ca} channels in VSMCs in response to clamp steps to +60 mV under control condition, with NS1619 (30 µmol/L) and paxilline (1 µmol/L) in WT, WT+FD, LOX-1 and LOX-1+FD. Please note the different scaling factors for the ordinate. $^{**}P<0.01$ control versus NS1619.

D: Current density in pA/pF of BK_{Ca} channels in ECs from WT, WT+FD, LOX-1 and LOX-1+FD.

E: Comparison of the BK_{Ca} currents in VSMCs and ECs from WT.

F: Above: Pulse protocol. Below: Typical BK_{Ca} current tracings of a VSMC from WT.

Figure 4: EDHF-mediated activation of BK_{Ca} currents in vascular smooth muscle cells (VMSCs) of mesenteric arteries from WT.

A: Hydrogen peroxide (1 µmol/L) activated the BK_{Ca} currents in VSMCs from WT, and even further increased with additional NS1619 (30 µmol/L).

B: 11,12-EET (300 nmol/L) significantly activated BK_{Ca} currents in VSMCs from WT to the same maximum like NS1619 (30 µmol/L).

C: Activation of BK_{Ca} currents by 14,15-EET (300 nmol/L) in VSMCs from WT to the similar values like NS1619 (30 µmol/L).

Figure 5: Expression of BK_{Ca} channel subunits and eNOS in mesenteric arteries

A: BK_{Ca} channel α-subunit (KCNMA) mRNA expression was analyzed by real-time PCR using an internal standard.

B: Amount of BK_{Ca} channel β_1-subunit (KCNMB1) mRNA was quantified by real-time PCR using an internal standard.

C: Expression of eNOS mRNA determined by real-time PCR using an internal standard.

WT- wildtype; LOX-1 – mice overexpressing LOX-1; FD – high-fat diet

Table 1: Characterization of body weight and serum parameters

	WT		LOX-1	
	Control	**+FD**	**Control**	**+FD**
Body weight [g]	27.3±0.8 (n=8)	33.1±1.1** (n=22)	30.7±1.2$^{\#}$ (n=7)	36.3±1.5*$^{\#}$ (n=18)
Triglycerides [mmol/L]	1.1±0.1 (n=7)	1.3±0.1 (n=24)	1.1±0.03 (n=7)	1.3±0.1 (n=18)
Cholesterol [mmol/L]	3.4±0.2 (n=7)	5.6±0.3*** (n=24)	3.3±0.1 (n=7)	5.9±0.3*** (n=18)
HDL [mmol/L]	2.7±0.2 (n=7)	4.5±0.2*** (n=24)	2.4±0.1 (n=7)	4.8±0.3*** (n=18)
LDL [mmol/L]	0.3±0.1 (n=7)	1.1±0.1*** (n=24)	0.3±0.04 (n=7)	1.1±0.1*** (n=18)

*P<0.05,**P<0.01,***P<0.001 standard diet versus high-fat diet;

$^{\#}P$<0.05 WT versus LOX-1

Figure 1

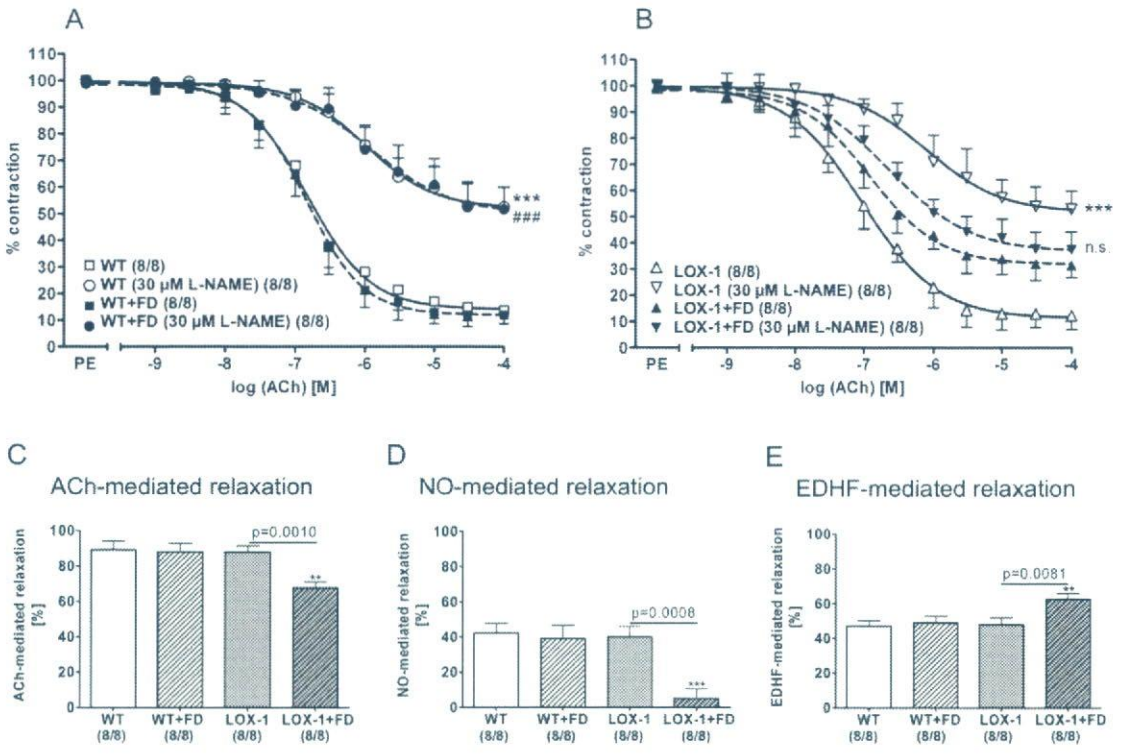

Figure 2