Regulation of Coronary Endothelial Function by Interactions between TNF- α , LOX-1 and Adiponectin in Apolipoprotein E Knockout Mice

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Key Words

Adiponectin · Endothelial dysfunction · LOX-1 · TNF- α

Abstract

Background/Aims: Although individual contributions of TNF- α , LOX-1 and adiponectin to the regulation of endothelial function were previously studied, their interactions in the regulation of coronary endothelial function remain unclear. The aim of this study is to investigate the interactions between TNF- α , LOX-1 and adiponectin in endothelial dysfunction in atherosclerosis. **Methods:** Vasodilator function was assessed in coronary arterioles isolated from wild-type, apolipoprotein (ApoE) knockout (KO) mice, ApoE KO null for TNF- α (ApoE KO^{TNF-/-}) and ApoE KO mice treated with neutralizing antibodies to either TNF- α and LOX-1, or recombinant adiponectin. Western blot analysis and immunofluorescence staining were used for mechanistic studies. **Results:** Acetylcholine (ACh) dilation was impaired in ApoE KO mice. KO of TNF- α , anti-TNF- α anti-LOX-1 or adiponectin restored impaired ACh vasodilation without affecting endothelium-derived hyperpolarizing factor-mediated vasodilation. Immunofluorescence staining demonstrated colocal-

ization of TNF- α with vascular smooth muscle cells, and adiponectin with endothelial cells. ApoE KO mice showed increased protein expression of LOX-1, NF- κ B, NADPH oxidase subunit NOX4 and nitrotyrosine (N-Tyr) levels in coronary arterioles. Treatment with anti-TNF- α , anti-LOX-1 and adiponectin suppressed protein expression of LOX-1, NOX4, NF- κ B and N-Tyr levels. **Conclusion:** Adiponectin, anti-TNF- α and anti-LOX-1 exert vasoprotective effects in atherosclerotic ApoE KO mice.

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Introduction

Atherosclerosis is a complex, multifactorial disease with both genetic and environmental determinants. Endothelial dysfunction is one of the earliest manifestations of atherosclerosis, and the assessment of endothelial function potentially serves as a useful prognostic tool for

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coronary artery disease [1]. Oxidized low-density lipoprotein (ox-LDL) is recognized as a major contributor to endothelial dysfunction in atherogenesis [2]. Ox-LDL has been implicated in impaired endothelium-dependent vascular tone through decreasing production/bioavailability of endothelium-derived nitric oxide (NO) and mediates several of its biological effects via LOX-1 (lectin-like ox-LDL receptor-1) [3, 4]. When ox-LDL is bound to LOX-1, it induces the generation of superoxide anions (O_2^-) [5] that reduce the bioavailability of NO and activate nuclear factor (NF)- κ B [6] and CD40/CD40L [7], and subsequently stimulate the expression of vasoconstrictor molecules such as endothelin-1, adhesion molecules, including P-selectin, vascular cell adhesion molecule-1, intracellular adhesion molecule-1 and chemokines (monocyte chemoattractant protein-1) [8, 9] in endothelial cells. We previously reported that oxidative stress was significantly higher in isolated coronary arterioles of apolipoprotein E (ApoE) knockout (KO) mice, which lay the foundation for further mechanistic study of microvascular pathology in atherosclerosis [10, 11]. Despite the crucial role of LOX-1 in endothelial dysfunction, particularly within an atherosclerotic environment, the mechanism by which the expression of LOX-1 is regulated in the vasculature is poorly understood. It is plausible that multiple inflammatory cytokines and novel regulators of vascular function may converge on LOX-1 signaling, forming a complex circuit to regulate LOX-1-mediated coronary microcirculatory dysfunction in atherosclerosis. Indeed, *in vitro* experiments have suggested that the proinflammatory cytokine TNF- α increases LOX-1 expression in a concentration-dependent manner in bovine aortic endothelial cells [12]. Despite the profound proinflammatory effects of TNF- α , an adipose-derived hormone, adiponectin, has been shown to exhibit inhibitory effects on TNF- α expression in aortas of db/db mice [13]. The above evidence suggests that a potential interaction among adiponectin/TNF- α /LOX-1 is responsible for the regulation of endothelial function, but the nature of this interaction in microvessels in atherosclerosis is uncertain.

In previous studies, we have established that anti-LOX-1 treatment reverses coronary arteriolar endothelial dysfunction in ApoE KO mice by preventing NADPH oxidase-mediated oxidative stress thereby restoring NO availability. We also found that anti-LOX-1 treatment improved aortic endothelial function in ApoE KO mice [11, 14]. Further, adiponectin treatment reduced LOX-1 protein expression in aortas of ApoE KO mice and in TNF- α -stimulated mouse coronary artery endothelial

cells, suggesting a reciprocal relationship between adiponectin and TNF- α in the regulation of LOX-1 expression. In this study, we further delineate the role of this reciprocal regulatory mechanism in the coronary microcirculation. We hypothesize an interactive circuit amongst adiponectin, TNF- α and LOX-1 regulating coronary arteriolar function in atherosclerosis. To test this hypothesis, we used genetic mouse models, including the ApoE KO mouse, ApoE KO mice null for TNF- α (ApoE KO^{TNF-/-}) and ApoE KO mice treated with a neutralizing antibody to TNF- α (anti-TNF- α) to neutralize TNF- α signaling, with exogenous recombinant adiponectin and with a neutralizing antibody to LOX-1 (anti-LOX-1) to inhibit LOX-1 signaling. Using these models, we aimed to determine whether (1) anti-LOX-1 and anti-TNF- α neutralizing antibody or adiponectin supplementation improve coronary arteriolar endothelial function in ApoE KO mice; (2) adiponectin and TNF- α are colocalized with vascular cells of coronary arterioles, and (3) there is reciprocal regulation in the expression of adiponectin, TNF- α and LOX-1.

Methods

Animal Models

The procedures followed were in accordance with approved guidelines set by the University of Missouri Animal Care and Use Committee. Wild-type (WT; C57BL/6) control mice, ApoE KO mice and ApoE KO null for TNF- α (ApoE KO^{TNF-/-}) between 6 and 8 weeks of age were obtained from the Jackson Laboratory (Bar Harbor, Maine, USA). The ApoE and TNF- α double KO mice (ApoE KO^{TNF-/-}) were established by breeding of ApoE KO mice (heterozygous) with TNF- α KO mice (homozygous) and were genotyped by PCR as we described [14]. To accelerate lesion formation in atherosclerosis-prone mice on the ApoE KO background, all animals were treated with a western-type diet (adjusted calorie diet; Harlan Teklad TD88137; 42% from milk fat, 0.15% cholesterol) for 12 weeks. Male mice were used in this study and animals had free access to water and were kept at a 12-hour light/dark cycle. Body weight, abdominal circumference and glucose levels were recorded prior to euthanasia and the data were reported in a previous study by our group [10].

TNF- α , LOX-1 Neutralization and Adiponectin Treatment

TNF- α was neutralized using a 2E2 monoclonal antibody (2E2 MAb 94021402; NCI Biological Resources Branch, Frederick, Md., USA). A neutralizing antibody (AF1564; R&D), which blocks the binding of ox-LDL to LOX-1, was used to suppress LOX-1 activity. After 12 weeks of western diet, ApoE KO mice were treated with anti-TNF- α antibody (0.625 mg/ml per kg per day *i.p.* for 3 days) [14], anti-LOX-1 antibody (16 μ g/ml, 0.1 ml/day *i.p.* for 7 days) [11] or recombinant murine globular adiponectin (15 μ g/day, *s.c.* for 8 days, gAcrp30; PeproTech) as we previously described [10, 15, 16].

Fig. 1. Anti-TNF- α , anti-LOX-1 and adiponectin improved endothelium-dependent vasodilation of coronary arterioles in ApoE KO. **a** Isolated coronary arterioles from WT ($n = 11$) and ApoE KO ($n = 9$) mice dilated in response to ACh (endothelium-dependent vasodilator) in a concentration-dependent manner. ACh-induced vasodilation was significantly attenuated in ApoE KO mice compared to WT mice. Treatment with anti-LOX-1 ($n = 10$) improved ACh-induced vasodilation in ApoE KO, but did not affect the vascular function of WT mice ($n = 3$). **b** ACh-induced dilation in

ApoE KO^{TNF-/-}/TNF- α ($n = 6$) was comparable to that in WT mice, and impaired ACh-induced vasodilation in ApoE KO mice was restored after treatment with neutralizing antibody to TNF- α ($n = 3$). Treatment with anti-TNF- α did not affect the vascular function of WT mice ($n = 3$). **c** Adiponectin treatment in ApoE KO mice ($n = 8$) partially improved ACh-induced vasodilation. **d** The endothelium-independent vasodilator (NO donor) SNP-induced vasodilation was not significantly different among the groups. Means $\pm$ SEM. * $p < 0.05$ vs. WT, # $p < 0.05$ vs. ApoE KO.

Functional Assessment of Isolated Coronary Arterioles

Coronary arterioles (40–100 μm in diameter) from murine cardiac tissue were identified, carefully microdissected and cannulated on glass pipettes as previously described [14]. The cannulated vessels were then pressurized at 60 cm H_2O in the absence of intraluminal flow for measuring vasodilatory function from the spontaneous level of pressure-induced myogenic tone. Those measurements were performed using video microscopy [14]. To determine whether TNF- α , LOX-1 or adiponectin regulate coronary vascular function in ApoE KO mice, vasodilation to the endothelium-dependent vasodilator, acetylcholine (ACh; 0.1 nmol/l to 10 $\mu\text{mol/l}$), and endothelium-independent vasodilator, sodium nitroprusside (SNP; 0.1 nmol/l to 10 $\mu\text{mol/l}$), was assessed in coronary arterioles isolated from WT, ApoE KO and ApoE KO mice treated with anti-TNF α , anti-LOX-1 or adiponectin. The contribution of NO to vasodilation was determined by incubating the vessels with the NO synthase (eNOS and nNOS) inhibitor L-NAME (10 $\mu\text{mol/l}$, 20 min) [17, 18]. To determine the response of coronary arterioles to endothelium-derived hyperpolarizing factor (EDHF)-dependent mechanisms, ACh vasodilation was performed following pretreatment (30 min) with both L-NAME (10 $\mu\text{mol/l}$) and the cyclooxygenase inhibitor indomethacin (10 $\mu\text{mol/l}$). At the end of each experiment, the vessel was relaxed with 100 $\mu\text{mol/l}$ SNP to obtain its maximal diameter at 60 cm H_2O intraluminal pressure. All diameter changes were normalized to the vasodilation in response to 100 $\mu\text{mol/l}$ SNP and expressed as a percentage of maximal dilation. For the functional experiments, all drugs were administered extraluminally in the physiological salt solution superfusate.

Immunofluorescence Staining

Transverse sections of the murine heart were stained immunohistochemically to identify and localize adiponectin and TNF- α expression with markers of endothelial cells, vascular smooth muscle cells and macrophages. Briefly, excised hearts were embedded in OCT and sectioned at 5 μm . Slides were incubated with blocking solution (10% donkey serum in PBS). Primary antibodies to adiponectin (goat polyclonal, 1:500, AF1119; R&D Systems) [19], TNF- α (goat polyclonal; R&D Systems) [14], the endothelial cell marker, von Willebrand factor (vWF; rabbit polyclonal, 1:1,000, ab6994; Abcam) [14], smooth muscle α -actin (rabbit polyclonal, 1:800, ab5964; Abcam) [14] or macrophage marker F4/80 (rat monoclonal, 1:400, MCA497R; AbD Serotec) were used for sequential double immunofluorescence staining. The specificity of the adiponectin antibody was confirmed in adiponectin^{-/-} mice as we previously described [19], and specificity of TNF- α antibody was previously validated [14]. Secondary antibodies were conjugated with the fluorophores, FITC or Texas red. For negative controls, primary antibodies were replaced with IgG isotype controls at the same concentration. Sections were finally mounted in an antifading agent (Slowfade gold with DAPI; Invitrogen). Slides were observed and analyzed using a fluorescence microscope (IX81; Olympus) with a $\times 40$ objective (0.90 numerical aperture) [20].

Western Blot Analysis of Protein Expression

Coronary arterioles (4–6 vessels per sample) were homogenized in lysis buffer (CellyticTM MT mammalian tissue lysis/extraction reagent; Sigma). Protein concentrations were assessed with a BCATM protein assay kit (Pierce) and samples were subsequently separated by SDS-PAGE and transferred to PVDF membranes.

Protein expression was detected using the appropriate primary antibody: TNF- α (1:500; R&D Systems), adiponectin (1:500; R&D Systems), LOX-1 (1:1,000; R&D Systems), NF- κB p65 (1:1,000; Abcam), NOX4 (1:500; Santa Cruz), and anti-nitro tyrosine (N-Tyr, 1:500; Abcam) and β -actin (1:2,000; R&D Systems). Horseradish peroxidase-conjugated secondary antibodies were used and signals were visualized by enhanced chemiluminescence (Santa-Cruz). Quantification was performed following scanning with a Fuji LAS3000 densitometer and using MultiGauge software (Fuji-film). Relative amounts of protein expression were normalized to those of the corresponding WT control, which was set to a value of 1.0.

Data Analysis

All diameter changes to pharmacological agonists were normalized to the passive vessel diameter at 60 cm H_2O . Statistical comparisons of vasomotor responses between groups were performed with two-way analysis of variance (ANOVA) for repeated measures, and intergroup differences were tested with the Bonferroni inequality using SPSS v11.5. All data are presented as means $\pm$ SEM if not stated otherwise. Significance was accepted at $p < 0.05$.

Results

Contribution of TNF- α , LOX-1 and Adiponectin to Coronary Endothelial Dysfunction in ApoE KO Mice

Vasodilation to the endothelium-dependent vasodilator, ACh, was significantly impaired in coronary arterioles of ApoE KO mice. Conversely, anti-LOX-1 (fig. 1a), genetic deficiency in TNF- α in ApoE KO mice (ApoE KO^{TNF- α /TNF- α}), anti-TNF- α (fig. 1b) or adiponectin (fig. 1c), each partially restored ACh-induced vasodilation in ApoE KO mice. SNP-induced, endothelium-independent vasodilation was similar among WT, ApoE KO, or ApoE KO treated with anti-TNF, anti-LOX-1 or adiponectin (fig. 1d). Improvement in vasodilator function in ApoE KO mice following treatment with anti-TNF- α (fig. 2a), anti-LOX-1 (fig. 2b) or adiponectin (fig. 2c) was abolished by L-NAME. This result indicates that (1) vasodilation to ACh was NO-mediated in the coronary arterioles of both WT and ApoE KO mice, and (2) anti-TNF- α , anti-LOX-1 or adiponectin restored ACh-induced, NO-mediated, vasodilation in atherosclerotic mice. We also investigated whether EDHF interacts with anti-TNF- α , anti-LOX-1 or adiponectin in coronary arteriolar dysfunction in ApoE KO mice. Incubation with L-NAME and indomethacin had no apparent effect on basal arteriolar tone. Unlike NO, EDHF-induced vasodilation was not significantly different in all groups suggesting EDHF-mediated responses are preserved in atherosclerotic coronary arte-

Fig. 2. Improved NO but not EDHF-dependent vasodilation accounts for the vascular protective effects by anti-TNF- α , anti-LOX-1 and adiponectin. Improvement in ACh-induced vasodilation in coronary arterioles in ApoE KO treated with anti-TNF- α antibody (**a**), anti-LOX-1 antibody (**b**) or adiponectin (**c**) was abol-

ished by incubation with the NO synthase inhibitor L-NAME. **d** EDHF-induced vasodilation (ACh-induced dilation in the presence of L-NAME and indomethacin) was not significantly different among the groups. Means $\pm$ SEM. $n = 6-7$ mice. * $p < 0.05$ vs. WT.

Table 1. Vessel characteristics

	Maximal diameter, μm	Percent tone		
		at baseline	with L-NAME	with L-NAME + INDO
WT	110.5±4.4	29.9±1.8	34.9±1.0*	35.6±1.4*
WT + anti-LOX-1	111.8±5.2	32.3±1.5	36.6±1.1	38.0±1.5
WT + anti-TNF-α	102.7±9.7	27.5±1.2	35.0±1.4*	34.9±2.7*
ApoE KO	116.7±6.0	32.7±0.7	35.6±0.3	37.1±1.1*
ApoE KO + anti-LOX-1	106.5±4.2	28.3±1.9	34.7±0.5*	36.8±1.8*
ApoE KO + anti-TNF-α	113.0±5.9	29.9±1.7	33.9±2.1	36.3±1.2*
ApoE KO + adiponectin	101.6±6.0	31.0±1.1	34.8±1.1	35.3±1.8
ApoE KO ^{TNF-/-/TNF-/-}	98.8±6.5	30.7±2.9	36.2±1.3	36.2±1.2

Means ± SEM. INDO = Indomethacin. * p < 0.05 vs. percent tone at baseline.

Fig. 3. Interactive regulation of TNF-α, LOX-1 and adiponectin in coronary arterioles. LOX-1 protein expression was higher in coronary arterioles of ApoE KO mice. Anti-TNF-α, anti-LOX-1 and adiponectin reduced LOX-1 protein expression. Treatment with adiponectin, but not anti-TNF-α or anti-LOX-1, increased adiponectin levels in the coronary arterioles of ApoE KO mice. NF-κB and NOX4 levels were higher in ApoE KO and suppressed by anti-TNF-α, anti-LOX-1 and adiponectin treatment. N-Tyr signals showed three bands at 50, 37 and 19 kDa, respectively. Both 37- and 19-kDa signals were higher in ApoE KO mice compared with WT mice. Anti-LOX-1, anti-TNF-α and adiponectin reduced levels of 37- and 19-kDa signals. In contrast, the 50-kDa signal was similar between WT and ApoE mice. Representative blots of 4 separate experiments.

rioles (fig. 2d). Vessel characteristics are shown in table 1. There were no significant differences in maximal diameter and diameter after tone among the experiment groups (table 1)

TNF-α, LOX-1 and Adiponectin Expression in Coronary Arterioles of ApoE KO Mice

Protein expression of LOX-1 and adiponectin (fig. 3) in isolated coronary arterioles was determined for WT, ApoE KO and ApoE KO mice treated with anti-TNF-α, anti-LOX-1 or adiponectin. Anti-TNF-α, anti-LOX-1 or adiponectin treatment remarkably decreased LOX-1 expression, while adiponectin expression was identical in WT and ApoE KO mice. Although anti-TNF-α or anti-LOX-1 did not affect adiponectin expression, adiponectin treatment increased adiponectin expression in coronary arterioles of ApoE KO mice.

TNF-α, LOX-1 and Adiponectin Regulate Protein Expression of NF-κB, NOX4 and N-Tyr

The protein expression of NF-κB p65, NOX4 and N-Tyr (37 and 19 kDa) was significantly increased in coronary arterioles in ApoE KO mice. Anti-TNF-α, anti-LOX-1 and adiponectin treatment dramatically attenuated NF-κB, NOX4 and N-Tyr (37 and 19 kDa) levels in the ApoE KO mice (fig. 3).

Colocalization of TNF-α and Adiponectin with Vascular Cells in Atherosclerosis

Immunostaining results showed that TNF-α (red, fig. 4a–l; colors are shown in the online version) was colocalized with vascular smooth muscle cells, and adiponectin (red, fig. 5a–l) was colocalized with endothelial

Fig. 4. TNF- α is colocalized with vascular smooth muscle cells in coronary arterioles as shown by dual fluorescence labeling for TNF- α and markers for endothelial cells (vWF) and vascular smooth muscle cells (α -actin) on frozen heart sections. **a–d** Dual labeling of TNF- α (red; see online version for colors) and α -actin (green) in control mice. Dual labeling of TNF- α (red) and α -actin (green) in ApoE KO mice (**e–h**) and ApoE KO + adiponectin mice (**i–l**). Arrows show staining of TNF- α (red; **e, i**) and vascular smooth muscle cells (green; **b, f, j**). Blue arrows indicate colocalization of TNF- α and vascular smooth muscle cells (yellow; **h, l**). **m–p** Dual labeling of TNF- α (red) and vWF (green) in ApoE KO mice. **d, h, l, p** Enlargements of panels **c, g, k, o**, respectively. The pink arrows in **n** and **p** show specific vWF staining with absence of TNF- α staining. **q, r** Negative control (Ctl): the arrows show absence of staining with primary antibodies being replaced by IgG isotype controls. **s** Nuclear staining with DAPI (blue) in control mice. $\times 40$. Data shown are representative of 4 separate experiments.

cells in WT, ApoE KO and ApoE KO^{TNF- α /TNF- α} mice. TNF- α was not colocalized with endothelial cells (fig. 4m–p), while adiponectin was not expressed in vascular smooth muscle cells (fig. 5m–p) of coronary microvessels or in macrophages (fig. 5q–t). For negative controls, primary antibodies were replaced with IgG isotype controls (fig. 4q–s, 5u–w).

Discussion

The major findings of this study include: (1) in addition to anti-LOX-1 treatment, adiponectin supplementation and anti-TNF- α treatment improved coronary en-

dothelial function of ApoE KO mice through NO, but not through an EDHF-mediated mechanism; (2) both adiponectin and anti-TNF- α reduced LOX-1 expression; (3) adiponectin was colocalized with vascular endothelial cells, but TNF- α was colocalized with vascular smooth muscle cells; (4) adiponectin, anti-LOX-1 and anti-TNF- α treatment reduced oxidative stress possibly through NOX4, a mechanism not previously reported in coronary microvessels in ApoE KO mice. The results of this study provide the first evidence of interactive regulation among adiponectin, TNF- α and LOX-1. Furthermore, the results reveal the complexity of mechanisms underlying coronary microvascular dysfunction in atherosclerosis.

Fig. 5. Adiponectin is colocalized with endothelial cells in coronary arterioles. Dual fluorescence labeling for adiponectin and markers for endothelial cells (vWF) and vascular smooth muscle cells (α-actin) on frozen heart sections. **a–d** Dual labeling of adiponectin (red; see online version for colors) and vWF (green) in control mice. **e–h** Dual labeling of adiponectin (red) and vWF (green) in ApoE KO mice. **i–l** Dual labeling of adiponectin (red) and vWF (green) in ApoE and TNF-α double KO mice (ApoE KO^{TNF-/-TNF-/-}). White arrows (**c, g, k**) indicate colocalization of adiponectin and endothelial cells (yellow). **m–p** Dual labeling of adiponectin (red) and α-actin (green) in ApoE KO mice. **o** Arrow shows specific α-actin staining with absence of adiponectin staining. **q–t** Dual labeling of adiponectin (red) and F4/80, marker of macrophages. **s** Arrow indicates specific F4/80 staining with absence of adiponectin staining. **u, v** Negative control (Ctl): the arrows show absence of staining with primary antibodies being replaced by IgG isotype controls. **w** Nuclear staining with DAPI (blue) in control mice. ×40. Data shown are representative of 4 separate experiments.

Vascular Effects of TNF-α, LOX-1 and Adiponectin

LOX-1, the main receptor for ox-LDL in endothelial cells, plays an important role in atherosclerosis [21, 22]. However, the precise mechanisms and effects of LOX-1 on vascular dysfunction remain unclear. Mehta et al. [23]

reported that basal relaxation of aortic rings from LOX-1 KO mice in response to ACh was enhanced compared to WT mice and anti-LOX-1 antibody pretreatment protected the WT mouse aortic rings from the adverse effects of ox-LDL. Our previous studies showed that LOX-1 ex-

pression was higher in coronary arterioles from ApoE KO mice [11], and serum LOX-1 levels were significantly enhanced in ApoE KO mice [10]. These results support our current finding that anti-LOX-1 treatment in ApoE KO mice is vasoprotective by partially restoring impaired ACh-induced coronary vasodilation in ApoE KO mice (fig. 1). Thus, the results implicate the role of LOX-1 in the progression of endothelial dysfunction in both macro- and microvasculature.

TNF- α , a proinflammatory cytokine, has been reported to be associated with plaque vulnerability [24, 25]. Herein, we found that TNF- α is colocalized with vascular smooth muscle cells of coronary arteries (fig. 4) in ApoE KO mice. Furthermore, coronary endothelial function was partially restored in ApoE KO null for TNF- α or ApoE KO mice treated with anti-TNF- α (fig. 1b, 2a) further supporting the interactive regulation at both expression and functional levels.

By treating the mice with recombinant globular adiponectin, we found that repetitive adiponectin administration partially rescues coronary microvascular dysfunction in ApoE KO mice (fig. 1c, 2c). This vasoprotection by adiponectin may, in part, be attributed to the direct stimulation of endothelial NO production. However, the current study does not fully support that this enhanced vasodilatory function is primarily due to an increase in adiponectin expression in the endothelium. This vasoprotective effect could be obtained from any secondary mechanism evoked by adiponectin administration.

In contrast, endothelium-independent vasodilatory effects of anti-LOX-1, anti-TNF- α or adiponectin do not appear to contribute to vascular dysfunction in ApoE KO mice since SNP-induced vasodilation was similar in control mice, ApoE KO mice and ApoE KO mice treated with adiponectin, LOX-1 or anti-TNF- α (fig. 1d). Our results (fig. 2a–c) also showed that anti-TNF- α , anti-LOX-1 or adiponectin restored ACh-induced NO-mediated vasodilation in atherosclerotic mice.

Unlike NO-mediated vasodilation, EDHF-induced vasodilation was not significantly different in the groups (fig. 2d), suggesting EDHF-induced vasodilation is preserved in coronary arterioles in our model of atherosclerosis. It suggests that the rescue of endothelium-dependent vasodilation by anti-TNF- α , anti-LOX-1 or adiponectin in ApoE KO mice was mainly through NO-dependent mechanisms but not that of EDHF. Thus, our results indicate that in our model, LOX-1, adiponectin and TNF- α exert vascular effects primarily by affecting vascular NO bioavailability but not EDHF-induced vasodilation in coronary arterioles in atherosclerosis. In con-

trast, EDHF-mediated vasodilation was previously shown to be significantly compromised in coronary microvessels of db/db mice (leptin receptor-deficient mice as models of type 2 diabetes). However, the relative proportion of EDHF-mediated vasodilation in db/db mice was substantially higher compared to normal WT mice (50 vs. 81%) suggesting that EDHF in these diabetic mice is a key factor in compensating for severely diminished NO-dependent vasodilation [26]. Thus, mechanisms of endothelial dysfunction may vary greatly between different disease processes/models.

Reciprocal Regulation among TNF- α , LOX-1 and Adiponectin

Reciprocal regulation among TNF- α , LOX-1 and adiponectin has been implicated in vitro, but has not been extensively investigated in vivo. Up-regulation of LOX-1 by TNF- α has been documented in bovine aortic endothelial cells [12], and human and murine macrophages [27]. TNF- α inhibited the expression and secretion of adiponectin from adipocytes [28, 29]. In contrast, adiponectin suppresses LPS-induced secretion of TNF- α from macrophages [30]. Furthermore, adiponectin KO mice showed higher levels of TNF- α mRNA in adipose tissue and higher plasma TNF- α levels [31]. In the current study, we first determined the cellular localization of TNF- α and adiponectin in coronary vessels of ApoE KO mice by immunostaining. The results showed that TNF- α (fig. 4) was predominantly colocalized with vascular smooth muscle cells while adiponectin (fig. 5) was colocalized with endothelial cells. Although, in our model, we were not able to obtain a strong immunofluorescent signal for LOX-1 expression to confirm its cellular localization, Li et al. [32] previously reported that LOX-1 was colocalized with macrophages and proliferating smooth muscle cells. Furthermore, Western blotting of isolated coronary arterioles suggested that anti-TNF- α , anti-LOX-1 and adiponectin treatment reduced LOX-1 expression in coronary arterioles of ApoE KO. Adiponectin expression was not markedly different in coronary arterioles of ApoE KO versus WT. Thus, these data indicate that LOX-1, adiponectin and TNF- α interact to participate in the complex regulation of their vascular expression in coronary arterioles.

Regulation of Arteriole Function by TNF- α , LOX-1 and Adiponectin Involves NF- κ B and NOX4

NADPH oxidase, a class of multicomponent enzymes, has been considered as one of the main sources of reactive oxygen species (ROS) in vascular cells, especially in endo-

thelial cells [33, 34]. The NADPH oxidase subunits (NOX1, NOX2, NOX4 and NOX5) are expressed in virtually all cardiovascular cells and regulate diverse functions, including differentiation, proliferation, apoptosis, senescence, inflammatory responses and oxygen sensing [35]. In regard to isoform specificity, particular interest has been focused on NOX4 due to its high level of expression in cardiovascular tissues and unique enzymatic properties in ROS formation [36]. Both TNF- α and LOX-1 lie downstream of NADPH oxidase-derived ROS [5, 37] while adiponectin has been shown to suppress hyperglycemia-induced ROS production in endothelial cells [38]. LOX-1 is known to mediate the activation of NF- κ B through an increased production of intracellular ROS [5, 39]. Furthermore, the binding of ox-LDL to LOX-1 reduces the intracellular concentration of NO through increased superoxide production in endothelial cells [5, 39]. We previously [11] demonstrated that LOX-1 leads to superoxide production and suggested NADPH oxidase to be the major source of superoxide. Here, we found that NOX4 protein expression was increased in coronary arterioles in ApoE KO mice, which was suppressed by anti-TNF- α , anti-LOX-1 and adiponectin administration (fig. 3). This suggests that NOX4 might contribute to the ROS formation in ApoE mice. We also previously demonstrated that TNF- α contributed to endothelial dysfunction in type 2 diabetes by inducing activation of NADPH oxidase and production of ROS in aortas and the coronary microcirculation [40]. These observations were confirmed by the findings in our present study. Most importantly, NF- κ B and N-Tyr protein expression (fig. 3) were increased in coronary arterioles of ApoE KO mice, which were inhibited by anti-TNF- α , anti-LOX-1 and adiponectin (fig. 3). These results suggest that the impaired endothelium-dependent vasodilation in ApoE KO might be

due to increased vascular oxidative stress induced by NOX4/NF- κ B signaling. This is partially supported by previous studies showing that impaired coronary vasodilation to ACh in ApoE KO mice was partially restored by NADPH oxidase inhibitors, apocynin or diphenyleneiodonium [11]. In order to support the direct involvement of oxidative stress in endothelial dysfunction of ApoE KO mice and the antioxidant effects by anti-TNF- α , anti-LOX-1 and adiponectin in vasoprotection, further studies are needed to examine the effects of superoxide scavengers or other specific NADPH oxidase inhibitors on coronary endothelial function of ApoE KO mice and ApoE KO mice treated with anti-TNF- α , anti-LOX-1 and adiponectin.

In conclusion, adiponectin, TNF- α , and LOX-1 exert complex regulatory effects on the coronary microvascular endothelial function in atherosclerotic ApoE KO mice. Administration of exogenous adiponectin, adiponectin agonist or inhibition of TNF- α and LOX-1 may serve as effective therapeutic strategies to ameliorate endothelial dysfunction in atherosclerosis.

Acknowledgments

This study was supported by grants from the Pfizer Atorvastatin Research Award (2004-37, to C.Z.), the American Heart Association (AHA) SDG (110350047A, to C.Z.) and NIH grants (RO1-HL077566 and RO1-HL085119, to C.Z. and M.A.H.). H.Z. was supported by an AHA predoctoral fellowship (10PRE4300043) and is currently supported by an AHA postdoctoral fellowship (15POST25620017).

Disclosure Statement

The authors declare no conflicts of interest.

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