Preservation of Endothelium-Dependent Relaxation in Atherosclerotic Mice with Endothelin-Restricted Endothelin-1 Overexpression

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Received May 21, 2013; accepted July 30, 2013

ABSTRACT

In human atherosclerosis, which is associated with elevated plasma and coronary endothelin (ET)-1 levels, ETA receptor antagonists improve coronary endothelial function. Mice overexpressing ET-1 specifically in the endothelium (eET-1) crossed with atherosclerosis-prone apolipoprotein E knockout mice (ApoE<sup>-/-</sup>) exhibit exaggerated high-fat diet (HFD)-induced atherosclerosis. Since endothelial dysfunction often precedes atherosclerosis development, we hypothesized that mice overexpressing endothelial ET-1 on a genetic background deficient in apolipoprotein E (eET-1/ApoE<sup>-/-</sup>) would have severe endothelial dysfunction. To test this hypothesis, we investigated endothelium-dependent relaxation (EDR) in eET-1/ApoE<sup>-/-</sup> mice. EDR in mesenteric resistance arteries from 8- and 16-week-old mice fed a normal diet or HFD was improved in eET-1/ApoE<sup>-/-</sup> compared with ApoE<sup>-/-</sup> mice. Nitric oxide synthase (NOS) inhibition abolished EDR in ApoE<sup>-/-</sup>. EDR in eET-1/ApoE<sup>-/-</sup> mice was resistant to NOS inhibition irrespective of age or diet. Inhibition of cyclooxygenase, the cytochrome P450 pathway, and endothelium-dependent hyperpolarization (EDH) resulted in little or no inhibition of EDR in eET-1/ApoE<sup>-/-</sup> compared with wild-type (WT) mice. In eET-1/ApoE<sup>-/-</sup> mice, blocking of EDH or soluble guanylate cyclase (sGC), in addition to NOS inhibition, decreased EDR by 36 and 30%, respectively. The activation of 4-aminopyridine-sensitive voltage-dependent potassium channels (K<sub>Ca</sub>) during EDR was increased in eET-1/ApoE<sup>-/-</sup> compared with WT mice. We conclude that increasing ET-1 in mice that develop atherosclerosis results in decreased mutual dependence of endothelial signaling pathways responsible for EDR, and that NOS-independent activation of sGC and increased activation of K<sub>Ca</sub> are responsible for enhanced EDR in this model of atherosclerosis associated with elevated endothelial and circulating ET-1.

Introduction

Endothelin (ET)-1 is an endothelium-derived contracting peptide with potent vasoconstrictor effects. Endothelin type A (ET<sub>A</sub>) and type B (ET<sub>B</sub>) receptors are responsible for its physiologic effects on the vasculature. In vascular smooth muscle cells (VSMCs), ET<sub>A</sub> activation and, to a lesser extent, ET<sub>B</sub> activation can induce contraction and proliferation of VSMC, although the main role of ET<sub>B</sub> is in endothelium to stimulate release of vasorelaxant agents [nitric oxide (NO) and prostacyclin (PGI<sub>2</sub>)]. Rautureau and Schiffrin (2012). ET-1 is involved in the pathophysiology of cardiovascular disease. In hypertension, ET-1 induces hypertrophic remodeling of small arteries (Li et al., 1994; Schiffrin et al., 1996; Schiffrin, 2005). In the coronary circulation, there is a correlation

ABBREVIATIONS: ACh, acetylcholine; 4-AP, 4-aminopyridine; ApoE<sup>-/-</sup>, apolipoprotein E knockout mouse; AUC, area under the curve; COX, cyclooxygenase; EDH, endothelin-dependent relaxing factor; ET, endothelin; ETA, type A ET receptor; ETB, type B ET receptor; HFD, high-fat diet; HNO, nitroxy; Ibtx, iberiotoxin; K<sub>Ca</sub>, calcium-activated potassium channel; K<sub>ir</sub>, inward-rectifier potassium channel; L-NAME, N<sup>-</sup>-nitro-L-arginine methyl ester; ND, normal diet; NE, norepinephrine; NOS, NO synthase; ODQ, 1H-[1,2,4]oxadiazolo[4,3-b]quinoxalin-1-one; PGL<sub>2</sub>, prostacyclin; sGC, soluble guanylate cyclase; SK<sub>Ca</sub>, small conductance calcium-activated potassium channel; SNP, sodium nitroprusside; TRAM-34, 1-[2-chlorophenyl][2-(4-methylphenyl)]-1H-pyrazole; VSMC, vascular smooth muscle cell; WT, wild-type.
between the severity of atherosclerosis and plasma ET-1 concentration (Lerman et al., 1995). ET-1 antagonists have been shown to decrease the size of atheroma in humans (Yoon et al., 2013). In a mouse model of endothelial ET-1 overexpression (eET-1), we showed that ET-1 is associated with vascular injury characterized by enhanced vascular remodeling, an increase in the formation of reactive oxygen species, and inflammation (Amiri et al., 2004, 2008). In eET-1 mice, we demonstrated an increase in the expression of genes associated with lipid synthesis in the vasculature (Simeone et al., 2011). This change in gene expression was associated with an exacerbation of high-fat diet (HFD)–induced aortic atherosclerotic lesions when eET-1 mice were crossed with apolipoprotein E knockout mice (Apoe^-/-), a model to study the progression of atherosclerosis (Plump et al., 1992). We proposed that eET-1–induced lipids synthesis could be involved in this proatherosclerotic effect (Simeone et al., 2011).

Endothelial dysfunction is associated with the development of atherosclerosis (Freiman et al., 1986; Ross, 1993). One of the main consequences of endothelial dysfunction is impairment of endothelium-dependent relaxation (EDR) (Freiman et al., 1986). EDR is dependent on the activation of several signaling pathways, resulting in the production of endothelium-derived relaxing factors (EDRFs) by endothelial cells and leading to relaxation of VSMC. These signaling pathways include the production of NO by endothelial NO synthase (eNOS), PGJ2 by cyclooxygenase (COX), and epoxygenasie acid (EDTs) by the cytochrome P450 epoxygenase (Vanhoutte et al., 2009). Endothelium-dependent hyperpolarization (EDH) (Feletou et al. and Vanhoutte, 2013) is an additional vasorelaxation mechanism involving the activation of endothelial small (SKCa) and intermediate conductance (IKCa) of calcium-activated potassium channels (KCa), which are known to be inhibited by apamin and TRAM-34 [1-[(2-chlorophenyl)diphenylmethyl]-1H-pyrazole], respectively (Feletou et al., 2003). Opening of SKCa and IKCa increases extracellular potassium, which activates inward rectifier K+ channels (KCa) and the Na+/K+ exchanger to hyperpolarize and relax VSMC (Edwards et al., 2010). In general, endothelium signaling pathways lead to the activation of several classes of K+ channels that are important effectors of EDR as they are responsible for the repolarization of membrane potential and the decrease in calcium concentrations in VSMC (Cox, 2005).

In conduit vessels of Apoe^-/- mice, there is a decrease of NO-mediated EDR (D’Uscio et al., 2001) that is restored by blockade of ETa receptor (Barton et al., 1998; D’Uscio et al., 2002). In resistance arteries, decreased NO-mediated EDR may be substituted by an EDH mechanism (Beleznaï et al., 2011). Although it is known that ET-1 impairs EDR, a specific role of ET-1 in resistance arteries, decreased NO-mediated EDR may be mediated by the loss of relaxation induced by ET-1 (D’Uscio et al., 2001). Therefore, we investigated the mechanisms for the restoration of EDR in eET-1/Apoe^-/- mice, which exhibit transgenic overexpression of ET-1, resulting in high levels of ET-1 in endothelial cells and in the circulation.

**Material and Methods**

**Generation of EET-1/Apoe^-/- Mice and Procedures.** Protocols were approved by the Animal Care Committees of the Lady Davis Institute for Medical Research and McGill University, followed the guidelines of the Canadian Council for Animal Care, and were in agreement with the recommendations for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health. Mice were housed in the Lady Davis Institute for Medical Research Animal Facility. Transgenic mice generated in our laboratory and overexpressing human preproET-1 (eET-1) in endothelial cells were described previously (Amiri et al., 2004). Human ET-1 is processed similarly to the endogenous protein as the consensus sequence necessary for the maturation of the prepropeptide are identical in mice and humans (Chan et al., 1995). Apolipoprotein E knockout (Apoe^-/-) mice were obtained from The Jackson Laboratory (B6.129P2-Apoe^-/-, Bar Harbor, ME). Apoe^-/- mice were crossed with eET-1 mice to obtain eET-1/Apoe^-/- mice. An additional cross of eET-1/Apoe^-/- with Apoe^-/- mice produced the mice used in the present experiments. Eight-week-old male eET-1, Apoe^-/-, eET-1/Apoe^-/-, and littermate wild-type (WT) mice were used for experiments or were fed either a high-fat, cholesterol-rich diet (HFD; 35% fat, 1.25% cholesterol, D12336; Research Diets, Inc., New Brunswick, NJ) or regular chow (normal diet, ND) for 8 weeks. All mice were on C57BL/6 genetic background.

**Drugs.** Apamin and TRAM-34 were purchased from Toceis BioScience (Minneapolis, MN). Acetylcholine (ACh), 4-aminopyridine (4-AP), iberotoxin (Ibtx), BaCl2, flunacozone, 1-NAME (N^N-nitro-arginine methyl ester) hydrochloride, sodium salt of meclofenamic acid (meclo), norepinephrine (NE), ODQ (1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one), and TBA were purchased from Sigma-Aldrich (St. Louis, MO). 14,15-Epoxyeicos-5(Z)-enoic acid (14,15-EEZ) was purchased from Cayman Chemical (Ann Arbor, MI). Stock solutions (10^-2 M) of ODQ and TRAM-34 were made in ethanol. Stock solution (6.7 x 10^-3 M) of flunacozone was made in dimethyl sulfoxide. All the other products were dissolved in water.

**Collection of Tissues and Study of Vascular Reactivity on a Pressurized Myograph.** Second-order mesenteric vessels were dissected and mounted on a pressurized myograph system to study vascular reactivity, as previously described (Diep et al., 2002; Leibovitz et al., 2009). Mice were anesthetized using isoflurane, and the complete mesenteric arterial bed was collected and placed in ice-cold Krebs’ solution, pH 7.4, containing (in mM): NaCl (120), NaHCO3 (25), KCl (25), CaCl2 (1.25), MgSO4 (1.18), glucose (5.5). Second-order mesenteric vessels (150–250 μm, 2-mm-long sections) were cleaned of surrounding fat, mounted on glass micropipettes on a pressurized myograph, and perfused with Krebs’ solution at an intraluminal pressure of 45 mm Hg. Pressurized vessels were kept in a chamber with a superfusion flow of warmed (37°C) and oxygenated (95% air, 5% CO2) control solution (Krebs’ or Krebs’ containing the pharmacological agents. After mounting, vessels were equilibrated for 60 minutes in Krebs’ solution. The lumen diameter was measured using a computer-based video imaging system (Living Systems Instrumentation, Burlington, VA). All vessels were initially constricted with Krebs’ solution containing 10^-5 M NE and 120 mM KCl. Only vessels constricting at least 60% of the initial resting diameter were used for further studies. Viable vessels were used to perform concentration-response curves to NE or were preconstricted in the presence of 5 x 10^-6 M NE to study concentration-responses to ACh or sodium nitroprusside (SNP). For some concentration-response studies, the vessels were pretreated with inhibitors before NE or SNP. For some concentration-response studies, the vessels were pretreated with inhibitors before NE or SNP. For some concentration-response studies, the vessels were pretreated with inhibitors before NE or SNP.
Experimental animals. pEC\textsubscript{50} and area-under-the-curve (AUC) values of concentration-response curves were calculated using SigmaPlot 12.0 (Systat Software, Inc., Chicago, IL). The effects of strain, diet, or pharmacological inhibitors were analyzed by comparing the AUCs, pEC\textsubscript{50}, and E\textsubscript{max} of concentration-response curves using one-way analysis of variance with SigmaPlot 12.0. Unless otherwise specified, only statistical analyses of the AUCs of concentration-response curves are discussed or represented. E\textsubscript{max} differences of concentration-response curves were calculated as the difference between the percentage of maximum relaxation of each curve. The effect of apamin + TRAM-34 treatment in WT and eET-1/Apo\textsuperscript{-/-} was determined using two-way analysis of variance for repeated measures. P values less than 0.05 were considered significant.

Results

**eET-1 Restores EDR in Atherosclerotic Mice.** NE-induced contraction of mesenteric arteries from 8-week-old mice and 16-week-old mice on ND or HFD was similar in WT, eET-1, Apo\textsuperscript{-/-}, and eET-1/Apo\textsuperscript{-/-} mice (Supplemental Figs. 1 and 2A). EDR to ACh 10\textsuperscript{-4} M in mesenteric arteries of 8-week-old (E\textsubscript{max} 84.2 ± 6.5%), 16-week-old ND (82.5 ± 5.6%), and HFD wild-type (WT) mice (88.0 ± 2.6%) was comparable (Fig. 1A and B; Supplemental Fig. 2B). Eight-week- (Supplemental Fig. 2B) and 16-week-old (Fig. 1A) eET-1 mice presented similar ACh-induced vasorelaxation when fed an ND (E\textsubscript{max} 72 ± 4.3%) compared with WT mice (82.5 ± 5.6%). ACh-induced vasorelaxation was significantly inhibited in eET-1 mice fed an HFD (eET-1 51.8 ± 5.6%, WT 88.0 ± 2.6%, P < 0.05). In Apo\textsuperscript{-/-} mice, relaxation was decreased by 30.5% in 8-week-old (Supplemental Fig. 2B) and by 43.5 and 64.2% (Fig. 1, A and B) in 16-week-old mice fed an ND or HFD, respectively, compared with the corresponding WT mice (P < 0.01). In Apo\textsuperscript{-/-} mice overexpressing eET-1 (eET-1/Apo\textsuperscript{-/-}), ACh-induced EDR was restored at 8 weeks (74.0 ± 13.0%, Supplemental Fig. 2B), 16 weeks ND (88.3 ± 4.2%, Fig. 1A), and 16 weeks HFD (70.9 ± 9.8%, Fig. 1B) compared with Apo\textsuperscript{-/-} mice (P < 0.01). NO-dependent, endothelium-independent relaxation of VSMC in response to the NO donor SNP was similar in WT, eET-1, Apo\textsuperscript{-/-}, eET-1/Apo\textsuperscript{-/-} mice at 8 weeks (Supplemental Fig. 2C) and 16 weeks on ND (Fig. 1C). In 16-week-old mice fed an HFD, WT, eET-1, and eET-1/Apo\textsuperscript{-/-} presented similar SNP-induced relaxation (Fig. 1D). Only arteries from Apo\textsuperscript{-/-} mice presented a decreased sensitivity to SNP (pEC\textsubscript{50} 5.7 ± 0.1) compared with WT (6.3 ± 0.1, P < 0.01) (Fig. 1D). However, E\textsubscript{max} values were not significantly different (WT, 98.1 ± 1.2%, Apo\textsuperscript{-/-}, 87.2 ± 5.8%; Fig. 1D).

**EDR in Mesenteric Arteries from eET-1/Apo\textsuperscript{-/-} Is Resistant to eNOS Inhibition.** In 16-week-old mice fed an ND, eNOS inhibition by 1-NAME predominantly abolished the maximal ACh-induced EDR in WT (E\textsubscript{max} 2.8 ± 0.4%), eET-1 (3.5 ± 1.1%), and Apo\textsuperscript{-/-} (10.4 ± 5.5%) mice (Fig. 2A). In contrast, ACh-induced EDR in eET-1/Apo\textsuperscript{-/-} mice was resistant to 1-NAME (68.6 ± 9.9%, P < 0.01 versus Apo\textsuperscript{-/-}). Similar results were found for the four groups of mice at 8 weeks (Supplemental Fig. 2D) and in 16-week-old mice on HFD (Fig. 2B).

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**TABLE 1**

<table>
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<tr>
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<td>SK\textsubscript{Ca}</td>
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<td>30</td>
<td>K\textsubscript{r}</td>
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<tr>
<td>BaCl\textsubscript{2} + ouabain</td>
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<td>20</td>
<td>Kir</td>
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<td>30</td>
<td>EETs</td>
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<td>20</td>
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<td>Na\textsuperscript{+}/K\textsuperscript{+} pump</td>
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<td>TRAM-34</td>
<td>10\textsuperscript{-5}</td>
<td>20</td>
<td>IK\textsubscript{Ca}</td>
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**Fig. 1.** ET-1 overexpression restores endothelium-dependent relaxation (EDR) in Apo\textsuperscript{-/-} mice. EDR to acetylcholine ACh (A and B) and endothelium-independent relaxation to sodium nitroprusside (SNP) (C and D) of mesenteric arteries from 16-week-old WT, eET-1, Apo\textsuperscript{-/-}, and eET-1/Apo\textsuperscript{-/-} mice receiving either ND (A and C) or HFD (B and D) for 8 weeks. Values are means ± S.E.M. *P < 0.05; **P < 0.01 versus WT; ††P < 0.01 versus Apo\textsuperscript{-/-} within same diet groups; n = 3–6.
Remodeling of EDRF and EDH-Mediated EDR in eET-1/Apoε<sup>2/2</sup> Mice. By use of specific pharmacological inhibitors, we studied the role of signaling pathways involved in ACh-induced EDR in 16-week-old WT and eET-1/Apoε<sup>2/2</sup> mice fed an ND. In WT mice, incubation with meclofenamate, an inhibitor of COX1, decreased maximal EDR to ACh by 27.0 to 55.7 ± 3.1% (P < 0.05 versus vehicle-treated; Fig. 3A). In contrast, maximal EDR to ACh was preserved in mesenteric arteries from eET-1/Apoε<sup>2/2</sup> mice incubated with meclofenamate (E<sub>max</sub> 83.3 ± 7.8%) (Fig. 3B) compared with control conditions. Similarly, fluconazole, which inhibits the synthesis of epoxyeicosatrienoic acids (EETs) by cytochrome P450, tended to decrease ACh-induced EDR in WT mice (Fig. 3C, nonsignificant) but did not alter it in eET-1/Apoε<sup>2/2</sup> mice (E<sub>max</sub> 83.7 ± 16.3%; Fig. 3D). Incubation with 14,15-EE5ZE, an antagonist of EETs (Gauthier et al., 2002), decreased vasorelaxation to ACh (10<sup>-6</sup> M) to the same extent in WT (15.7%) and eET-1/Apoε<sup>2/2</sup> mice (18.3%), without, however, achieving significance but essentially confirming fluconazole results and the minor role of the EET signaling pathway in enhanced EDR to ACh observed in eET-1/Apoε<sup>2/2</sup> mice (Supplemental Fig. 3).

We next investigated the role of endothelial hyperpolarization mediated by IKCa and SKCa activation in EDR. Apamin and TRAM-34 incubation inhibited maximal EDR to ACh by 46% in WT (Fig. 3E). In eET-1/Apoε<sup>2/2</sup> mice, apamin and TRAM-34 did not modify maximal EDR but induced a rightward shift in the concentration-relaxation curve to ACh from a pEC<sub>50</sub> of 7.0 ± 0.2 to 6.1 ± 0.2 (For pEC<sub>50</sub>, P < 0.05) (Fig. 3F; Supplemental Table 1). In the presence of apamin + TRAM-34, ACh-induced relaxation was greater in eET-1/Apoε<sup>2/2</sup> than in WT mice (P < 0.01). BaCl<sub>2</sub> + ouabain were used to inhibit VSMC hyperpolarization activated by the potassium cloud formed by the activation of endothelial IKCa and SKCa (Edwards et al., 1998). In WT and in eET-1/Apoε<sup>2/2</sup> mice, BaCl<sub>2</sub> + ouabain decreased maximal ACh-induced EDR by 30 to 52.1 ± 2.5% and 20 to 62.4 ± 10.3%, respectively (both P < 0.01 versus vehicle) (Fig. 3, G and H).

In eET-1/Apoε<sup>2/2</sup> mice fed an HFD, we performed additional experiments using apamin and TRAM-34 in addition to l-NAME. In the presence of l-NAME, maximal EDR to ACh was 82.8 ± 8.4%. The addition of apamin + TRAM-34 + l-NAME decreased maximal EDR to ACh by 36.3 to 43.0 ± 8.3% (Fig. 4A) (P < 0.01 versus l-NAME alone). We also examined the role of soluble guanylate cyclase (sGC) on EDR independent of eNOS activation. Addition of the sGC inhibitor ODQ to l-NAME decreased EDR to ACh by 30.6 to 48.6 ± 19.2% (Fig. 4B) (P < 0.01 versus l-NAME alone; Supplemental Table 2).

The Role of 4-AP-Sensitive K<sub>v</sub> Channels Mediating ACh-Induced EDR Is Enhanced in eET-1/Apoε<sup>2/2</sup> Mice. Several classes of K<sup>+</sup> channels mediate EDR (Ko et al., 2008).
We used selective inhibitors of different classes of K⁺ channel to investigate their involvement in 16-week-old ND WT and eET-1/Apoε²/² mice. We first tested TEA, an inhibitor of voltage-dependent (Kᵥ) and calcium-dependent potassium channels (KᵥCa) (Cox, 2005). TEA inhibited maximal EDR to ACh by 28.3 to 54.3 ± 6.0% in WT mice (P < 0.05; Fig. 5A). In eET-1/Apoε²/² mice, TEA inhibition also decreased maximal ACh-induced EDR by 14.8 to 73.5% (P < 0.05; Fig. 5B).

We then studied the effect of iberiotoxin (Ibtx), a selective inhibitor of large-conductance KᵥCa (BKᵥCa), and 4-aminopyridine (4-AP), a selective inhibitor of Kᵥ. In WT mice, Ibtx inhibited maximal EDR to ACh by 50.5 to 38 ± 14% (P < 0.05) (Fig. 5C), whereas 4-AP did not significantly modify EDR (Rmax by 69.3 ± 8.2%) (Fig. 5E) compared with control. In contrast, both Ibtx and 4-AP significantly inhibited maximal ACh-induced EDR by ∼38% in eET-1/Apoε²/² mice (P < 0.05 for both; Fig. 5, D and F).

Inward rectifier potassium channels (Kir) are important regulators of the resting membrane potential and are activated by an increase in extracellular potassium concentration (Ko et al., 2008). We used BaCl₂ at 100 µM to investigate selectively the role of Kir. EDR to ACh was significantly inhibited by BaCl₂ in WT (reduction of 31.3%, P < 0.05) (Fig. 5G). In eET-1/Apoε²/² mice, Kir function appeared relatively preserved with a nonsignificant reduction of 24.1% in the amplitude of the response) (Fig. 5H).

**Discussion**

Our findings indicate that overexpression of eET-1 in Apoε²/² mice unexpectedly restores EDR in a model of eET-1-mediated exacerbation of atherosclerosis. This restoration of endothelial function is associated with remodeling of endothelial signaling pathways mediating ACh-induced EDR. We found that overexpressing eET-1 in Apoε²/² mice makes ACh-induced EDR more resistant to eNOS, COX, cytochrome P450, and EDH inhibition compared with WT. We propose that this endothelial remodeling could be explained by a decrease in mutual dependence between endothelial signaling pathways. The reduction of ACh-induced EDR in vessels treated with sGC inhibitor and l-NAME (Fig. 4B) suggests that eNOS-independent activation of sGC is involved in ACh-induced EDR in eET-1/Apoε²/² mice.

Unlike in the aorta and other conduit arteries, neither atheroma nor the early occurring fatty streaks develop in small mesenteric resistance arteries and would not be expected nor are found in the Apoε²/² and eET-1/Apoε²/² mice used in this study. The use of small mesenteric arteries is an established model to study the reactivity of arteries involved in the control of microvascular regional blood flow, which can be affected by endothelial dysfunction in atherosclerosis. In aortae of Apoε²/² mice, reduction in EDR is dependent on
increased production of superoxide that reduces NO bioavailability (Barton et al., 1998; D’Uschio et al., 2001; Laursen et al., 2001). A decrease in EDR has been reported in mesenteric arteries of Apoe−/− mice fed a Western-type diet for 30 weeks (D’Uschio et al., 2002). In the latter study and in 16-week-old Apoe−/− mice on HFD in the present work, decreased EDR was accompanied by a diminished endothelium-independent vasorelaxation in response to an NO donor, suggesting that there is a decreased NO relaxant response of VSMC in Apoe−/− mice. In other studies, however, no modification of ACh-induced EDR in mesenteric arteries was observed in 10- to 16-week-old Apoe−/− mice (Morikawa et al., 2005; Beleznaiz et al., 2011). It is important to point out that our study design differed from these other studies. For example, we used NE to precontract vessels, whereas Morikawa et al. (2005) used prostaglandin F2α, and Beleznaiz et al. (2011) and Ding et al. (2005) used phenylephrine. Dysfunction in α2-adrenoceptor-mediated EDR has been reported in Apoe−/− mice (Lee et al., 2011). The absence of α2-adrenoceptor stimulation by NE in phenylephrine and prostaglandin F2α preconstricted arteries could be related to the impaired EDR observed.

In our study, the application of L-NAME (10−4 M) abolished EDR in WT and Apoe−/− mice. Whereas some groups have reported similar findings (Andrews et al., 2009; Hosoya et al., 2010), others have shown that in mesenteric arteries from WT (Morikawa et al., 2005; Ohashi et al., 2012) and Apoe−/− mice (Beleznaiz et al., 2011), EDR to ACh is resistant to eNOS inhibition. Such discrepancies could be explained by different housing conditions, intestinal microbiota differences, or technical aspects of myography.

We did not observe impairment of endothelial function in 8-week-old eET-1 mice and only a minor impairment at 16 weeks, in contrast to other reports (Leung et al., 2011) and our own previous work (Amiri et al., 2004, 2008). Loss of endothelial dysfunction in eET-1 mice in this study could have been caused by additional back-crossing into the C57BL/6 background or by accidental selection of mice resistant to eET-1–induced endothelial dysfunction (Javeshghani et al., 2013).

Atherosclerosis development in eET-1/Apoe−/− mice was much more extensive than in Apoe−/− mice at comparable ages (Simeone et al., 2011). Since endothelial dysfunction occurs early in the development of atherosclerosis, we hypothesized that eET-1 overexpression in Apoe−/− mice would induce a worsening of endothelial dysfunction. Unexpectedly, we observed that EDR was fully restored in eET-1/Apoe−/− mice, compared with Apoe−/− mice, at all endpoints. This vasorelaxation was resistant to L-NAME inhibition. ODQ coinubcation with L-NAME decreased ACh-induced EDR, indicating that there was eNOS-independent activation of sGC. Several mechanisms can account for this finding. It has been previously shown that NO stores can mediate EDR (Danser et al., 1998; Chauhan et al., 2003). Nitrite and nitrate are endogenous sources of NO as they can be reduced to form bioactive nitrogen oxides (Lundberg et al., 2008). The heme-containing proteins, myoglobin, hemoglobin, xanthine oxidase, and cytochrome P450 enzyme system, generate NO from nitrite and nitrate. Nitrite- and nitrate-dependent generation contributes to endothelium-dependent, eNOS-independent regulation of vascular tone (Zhao et al., 2013). S-Nitrosothiols, derived in part from S-nitrosoglutathione, have also been shown to be an eNOS-independent source of NO that could account for ACh-mediated NO release (Ng et al., 2007). Carbon monoxide, an endogenous weak activator of sGC produced by heme oxygenases 2 and 3 (Foresti et al., 2004), could also be involved in eNOS-independent, sGC-mediated EDR (Foresti et al., 2004). In eET-1/Apoe−/− mice, superoxide production by aorta is significant (unpublished observations). Uncoupled eNOS produces superoxide that can be reduced to hydrogen peroxide by superoxide dismutase (Takaki et al., 2008). NO-derived hydrogen peroxide has been shown to be involved in EDR (Takaki et al., 2008). Hydrogen peroxide can mediate dimerization and activation of G-kinase (Prysyazhna et al., 2012) by means of disulfide bridge formation (Cohen et al., 1997; McCulloch et al., 1997; Leo et al., 2008). Heterodimerization of α and β subunits of sGC is augmented in presence of enhanced oxidative stress (Zheng et al., 2011). Hydrogen peroxide–mediated dimerization and activation of sGC could therefore be involved in L-NAME–resistant EDR. It has also been proposed that residual NOS activation in response to ACh could occur in the presence of L-NAME (Cohen et al., 1997; McCulloch et al., 1997; Leo et al., 2008). Since we observed complete inhibition of EDR in WT, eET-1, and Apoe−/− mice, decreased affinity of eNOS for L-NAME or an increased concentration of L-arginine, which can compete with L-NAME, could play a role in L-NAME–resistant eNOS activation in eET-1/Apoe−/− mice.

Because the eNOS pathway involved in EDR was altered in eET-1/Apoe−/− mice compared with WT mice, we investigated other endothelial pathways mediating relaxation. By use of specific pharmacological inhibitors, we found that COX and, to a lesser extent, cytochrome P450 pathways/ETs were involved in EDR in WT mice. In contrast, no involvement of COX and cytochrome P450 pathways was found in eET-1/Apoe−/− mice. As inhibition of EDRP pathways was unable to modify EDR in eET-1/Apoe−/− mice, we investigated EDH mechanisms mediated by endothelial hyperpolarization/K+ cloud (Edwards et al., 1998). In WT mice, both ouabain + BaCl2 and apamin + TRAM-34 efficiently inhibited EDR. In eET-1/Apoe−/− mice, ouabain + BaCl2 inhibition was less effective than in WT mice, and there was a decreased sensitivity to ACh in presence of apamin + TRAM-34. Incubation with L-NAME + apamin + TRAM-34 potentiated the inhibition of EDR (Fig. 4A). This suggests that eNOS- and EDH-signaling mechanisms are less mutually dependent in eET-1/Apoe−/− than in WT mice.

Potassium channels are major regulators of vascular tone in EDR. We hypothesized that changes in various potassium channels involved in EDR accompanied remodeling of endothelial function in eET-1/Apoe−/− compared with WT mice. In the presence of TEA, a nonselective inhibitor of Kv and BKCa (Cox, 2005), EDR was inhibited to the same extent in WT and eET-1/Apoe−/− mice. We then looked at the specific roles of BKCa and Kv, using the selective inhibitors, Ibtx and 4-AP, respectively. Ibtx induced similar inhibition in both strains of mice, but the inhibitory effect of 4-AP was potentiated in eET-1/Apoe−/− mice compared with WT. Consistent with our results using BaCl2 + ouabain, blockade of Kv with BaCl2 induced a more pronounced inhibition in WT than in eET-1/Apoe−/− mice (Fig. 5, G and H). Therefore, we conclude that an increase in Kv and a decrease in Kir contribute to remodeling of endothelial signaling pathways in eET-1/Apoe−/− mice. Inhibition of EDR by 4-AP is a hallmark of nitroryl (HNO) action. HNO activates sGC to induce vasorelaxation (Bullen et al., 2011). In a model of vessel
injury (angiotensin II–treated mice), HNO-mediated relaxation has been shown to be inhibited by 4-AP, whereas in sham mice, HNO donor-induced relaxation is insensitive to 4-AP (Wynn et al., 2012). As 1-NAM-resistant EDR is partially inhibited by ODQ in eET-1/Apo-1/− mice, it will be necessary in the future to investigate the role of HNO in 4-AP-sensitive EDR in these mice.

Overall, our studies demonstrate that in a model of atherosclerosis, exacerbation of atherosclerosis induced by overexpression of eET-1 is accompanied by preservation and remodeling of endothelial signaling pathways and K+ channels mediating EDR. ET-1 overexpression induces the development of compensatory mechanisms in the arteries of eET-1/Apo-1/− mice, which allows eNOS, EDH mechanisms, or the COX pathway to mediate independently EDR.

Acknowledgments

The authors thank Marie-Ève Deschènes and Heather Mynarski for animal care.

Authorship Contributions

Participated in research design: Mian, Idris-Khodja, Li, Paradis, Rautureau, Schiffrin.

Conducted experiments: Mian, Idris-Khodja, Li, Leibowitz, Rautureau.

Performed data analysis: Mian, Idris-Khodja, Li, Leibowitz, Paradis, Rautureau, Schiffrin.

Wrote or contributed to the writing of the manuscript: Mian, Idris-Khodja, Paradis, Rautureau, Schiffrin.

References


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ONLINE SUPPLEMENT

PRESERVATION OF ENDOTHELIUM-DEPENDENT RELAXATION IN A MODEL OF ENDOTHELIN-1-MEDIATED EXACERBATION OF ATHEROSCLEROSIS

Mian et al., ET-1 and endothelial function in atherosclerosis.

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Supplemental Figure 1:
Contractile responses to norepinephrine (NE) of mesenteric arteries from 16-week-old wild type (WT), eET-1, Apoe\textsuperscript{-/-} and eET-1/Apoe\textsuperscript{-/-} mice, receiving normal diet (ND) (A) or high fat diet (HFD) (B) during 8 weeks, n = 3-6.
Supplemental Figure 2:
Contractile responses to NE (A), endothelium-dependent relaxation responses to acetylcholine (ACh) (B), endothelium-independent relaxation responses to sodium nitroprusside (SNP) (C) and endothelium-dependent responses to ACh in presence of L-NAME (D) of mesenteric arteries from 8-week-old WT, eET-1, Apoe<sup>-/-</sup> and eET-1/Apo<sub>e<sup>-/-</sup></sub> mice receiving normal diet. Values are means ± SEM. **P<0.01 vs. WT, ††P<0.01 vs. Apoe<sup>-/-</sup>, n = 4-8.
Supplemental Figure 3:
Endothelium-dependent relaxation to $10^{-6}$ M ACh in the absence and presence of 14,15-EE5ZE ($10^{-5}$ M, antagonist of epoxyeicosatrienoic acids) of mesenteric arteries from 16-week-old WT and eET-1/Apoe$^{-/-}$ mice receiving normal diet. Values are means ± SEM, n = 4.
Supplement table 1:
pEC50, E_max and AUC of acetylcholine concentration-response curves in mesenteric arteries from 16-week-old WT and eET-1/Apoe⁻/⁻ mice receiving normal diet, in the presence of indicated inhibitors. Values are means ± SEM. *P<0.05 and **P<0.01 vs. vehicle, †P<0.05 and ††P<0.01 vs. corresponding WT mice, n = 3-7.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>pEC50</th>
<th>E_max</th>
<th>AUC</th>
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<tr>
<td></td>
<td>WT</td>
<td>eET-1/Apoe⁻/⁻</td>
<td>WT</td>
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<tr>
<td>Vehicle</td>
<td>6.8 ± 0.2</td>
<td>7.0 ± 0.2</td>
<td>82.6 ± 5.6</td>
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<td>L-NAME</td>
<td>6.5 ± 1.5</td>
<td>7.3 ± 0.5</td>
<td>2.8 ± 0.5 **</td>
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<tr>
<td>Meclofenamic acid</td>
<td>6.8 ± 1.1</td>
<td>6.3 ± 0.3</td>
<td>55.7 ± 3.1 **</td>
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<td>Fluconazole</td>
<td>6.5 ± 0.4</td>
<td>7.4 ± 0.4</td>
<td>64.1 ± 16.7</td>
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<td>Apamin + TRAM-34</td>
<td>5.7 ± 0.3 **</td>
<td>6.1 ± 0.2</td>
<td>35.8 ± 8.5 **</td>
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<tr>
<td>BaCl₂ + Ouabain</td>
<td>5.4 ± 0.3 **</td>
<td>6.3 ± 0.2 †</td>
<td>52.1 ± 2.5 **</td>
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<tr>
<td>TEA</td>
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<td>5.7 ± 0.2 **</td>
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<td>BaCl₂</td>
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<td>6.7 ± 0.3</td>
<td>51.3 ± 10.5</td>
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<td>Ibtx</td>
<td>5.3 ± 0.4 **</td>
<td>6.4 ± 0.3 †</td>
<td>38.2 ± 13.6 *</td>
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<td>4-AP</td>
<td>6.8 ± 0.4</td>
<td>6.4 ± 0.2</td>
<td>69.3 ± 08.2</td>
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Supplement table 2:
pEC50, Emax and AUC of acetylcholine concentration-response curves in mesenteric arteries from 16-week-old eET-1/Apoel−/− mice receiving high-fat diet, in the presence of indicated inhibitors. Values are means ± SEM. **P<0.01 vs. L-NAME, n = 4-9.

<table>
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<th>Treatment</th>
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<th>Emax</th>
<th>AUC</th>
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<tr>
<td>L-NAME</td>
<td>7.7 ± 0.4</td>
<td>79.3 ± 9.9</td>
<td>282.0 ± 34.0</td>
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<td>L-NAME + Apamin + TRAM-34</td>
<td>6.6 ± 0.3</td>
<td>43.0 ± 8.3**</td>
<td>113.6 ± 19.8**</td>
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<td>L-NAME + ODQ</td>
<td>6.8 ± 0.4</td>
<td>48.6 ± 19.2</td>
<td>132.2 ± 52.5**</td>
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