Endothelin-1 Overexpression Exacerbates Atherosclerosis and Induces Aortic Aneurysms in Apolipoprotein E Knockout Mice

Melissa W. Li,* Muhammad Oneeb Rehman Mian,* Tlili Barhoumi, Asia Rehman, Koren Mann, Pierre Paradis, Ernesto L. Schiffrin

Objective—Endothelin (ET)-1 plays a role in vascular reactive oxygen species production and inflammation. ET-1 has been implicated in human atherosclerosis and abdominal aortic aneurysm (AAA) development. ET-1 overexpression exacerbates high-fat diet–induced atherosclerosis in apolipoprotein E−/− (Apoe−/−) mice. ET-1–induced reactive oxygen species and inflammation may contribute to atherosclerosis progression and AAA development.

Approach and Results—Eight-week-old male wild-type mice, transgenic mice overexpressing ET-1 selectively in endothelium, and eET-1/Apoe−/− mice were fed high-fat diet for 8 weeks. eET-1/Apoe−/− had a 45% reduction in plasma high-density lipoprotein (P<0.05) and presented ≥2-fold more aortic atherosclerotic lesions compared with Apoe−/− (P<0.01). AAs were detected only in eET-1/Apoe−/− (8/21; P<0.05). Reactive oxygen species production was increased ≥2-fold in perivascular fat, media, or atherosclerotic lesions in the ascending aorta and AAs of eET-1/Apoe−/− compared with Apoe−/− (P<0.05). Monocyte/macrophage infiltration was enhanced ≥2.5-fold in perivascular fat of ascending aorta and AAs in eET-1/Apoe−/− compared with Apoe−/− (P<0.05). CD4+ T cells were detected almost exclusively in perivascular fat (3/6) and atherosclerotic lesions (5/6) in ascending aorta of eET-1/Apoe−/− (P<0.05). The percentage of spleen proinflammatory Ly-6Chi monocytes was enhanced 26% by ET-1 overexpression in Apoe−/− (P<0.05), and matrix metalloproteinase-2 was increased 2-fold in plaques of eET-1/Apoe−/− (P<0.05) compared with Apoe−/−.

Conclusions—ET-1 plays a role in progression of atherosclerosis and AAA formation by increasing high-density lipoprotein, and increasing oxidative stress, inflammatory cell infiltration, and matrix metalloproteinase-2 in perivascular fat, vascular wall, and atherosclerotic lesions.

Key Words: cholesterol, HDL ■ elastin ■ fibronectin ■ inflammation ■ monocytes ■ oxidative stress ■ T lymphocytes

Endothelin (ET)-1 is a 21-aa peptide discovered in endothelium but also produced by many other tissues. It is one of the most potent endogenous vasoconstrictors.1 ET-1 plays an important role in the pathophysiology of cardiovascular disease by causing vascular damage.2,3 We have previously generated transgenic mice overexpressing ET-1 selectively in endothelium (eET-1). Apoe−/− mice, and eET-1/Apoe−/− mice were fed high-fat diet for 8 weeks. eET-1/Apoe−/− had a 45% reduction in plasma high-density lipoprotein (P<0.05) and presented ≥2-fold more aortic atherosclerotic lesions compared with Apoe−/− (P<0.01). AAs were detected only in eET-1/Apoe−/− (8/21; P<0.05). Reactive oxygen species production was increased ≥2-fold in perivascular fat, media, or atherosclerotic lesions in the ascending aorta and AAs of eET-1/Apoe−/− compared with Apoe−/− (P<0.05). Monocyte/macrophage infiltration was enhanced ≥2.5-fold in perivascular fat of ascending aorta and AAs in eET-1/Apoe−/− compared with Apoe−/− (P<0.05). CD4+ T cells were detected almost exclusively in perivascular fat (3/6) and atherosclerotic lesions (5/6) in ascending aorta of eET-1/Apoe−/− (P<0.05). The percentage of spleen proinflammatory Ly-6Chi monocytes was enhanced 26% by ET-1 overexpression in Apoe−/− (P<0.05), and matrix metalloproteinase-2 was increased 2-fold in plaques of eET-1/Apoe−/− (P<0.05) compared with Apoe−/−.

Endothelin-1 levels are increased in Western diet–induced atherosclerosis in apolipoprotein E knockout (Apoe−/−) mice14 and low-density lipoprotein receptor knockout (Ldlr−/−) mice.15 Consistent with these observations, the development of atherosclerosis has been shown to be reduced by mixed ETA/B and selective ETA receptor antagonists in Western-type diet–fed Apoe−/− mice,14,16 Ldlr−/− mice,15 and hamsters.17 Recently, we reported using DNA microarrays that eET-1 mice present changes in the expression of genes involved in lipid biosynthesis in the vascular wall.18 In pilot experiments, to demonstrate the biological significance of this alteration in gene expression, we found that increased ET-1 expression in the endothelium of Apoe−/− mice exacerbates high-fat diet (HFD)–induced lipid–containing lesions in the descending thoracic aorta. However, it remains unclear how increased ET-1 expression worsens atherosclerosis progression in HFD-fed Apoe−/− mice.

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*These authors contributed equally.

The online-only Data Supplement is available with this article at http://atvb.ahajournals.org/lookup/suppl/doi:10.1161/ATVBHA.113.302028/-/DC1. Correspondence to Ernesto L. Schiffrin, CM, MD, PhD, Sir Mortimer B. Davis-Jewish General Hospital, #B-127, 3755 Côtes-St-Catherine Rd, Montreal, PQ, Canada H3T 1E2. E-mail ernesto.schiffrin@mcgill.ca

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2306
Inflammation in perivascular fat (PVAT) may play a role in atherosclerosis,\textsuperscript{9,20} Angiotensin (Ang) II and aldosterone induce oxidative stress and T lymphocyte and monocyte/macrophage infiltration in PVAT.\textsuperscript{21–23} It is unknown, however, whether ET-1 has similar effects.

ET-1 plasma levels are increased in patients with abdominal aortic aneurysms (AAAs).\textsuperscript{24–26} Patients with larger AAAs displayed higher levels of ET-1.\textsuperscript{21,25} Accordingly, a role for ET-1 has been suggested in the development of AAAs in humans. Whether ET-1 overexpression indeed causes AAAs has not been demonstrated.

We hypothesized that ET-1–induced PVAT and vascular oxidative stress and inflammation contribute to the progression of atherosclerosis and increased incidence of AAAs. To test this hypothesis, we crossed eET-1 with Apoe\textsuperscript{−/−} mice, to determine whether ET-1 overexpression exaggerates HFD-induced atherosclerosis in the latter, and establish whether this is associated with enhanced PVAT and vascular oxidative stress and inflammation, and whether increased incidence of AAAs will occur.

**Materials and Methods**

Materials and Methods are available in detail in the online-only Supplement.

**Results**

**Physiological Parameters**

HFD decreased body weight gain by 8%, 9%, 21%, and 24% in wild-type (WT), eET-1, Apoe\textsuperscript{−/−}, and eET-1/Apoe\textsuperscript{−/−} mice, respectively (Table I in the online-only Data Supplement). This was not attributable to decreased growth because tibia length (TL) was similar in all groups. The eET-1/Apoe\textsuperscript{−/−} mice presented a 20% increase in heart weight and heart weight/TL compared with WT. HFD decreased heart weight and heart weight/TL by \textasciitilde10% in WT and eET-1 mice and 25% in Apoe\textsuperscript{−/−} and eET-1/Apoe\textsuperscript{−/−} mice. HFD decreased kidney weight by 32% and 56% and kidney weight/TL by 31% and 37% in Apoe\textsuperscript{−/−} and eET-1/Apoe\textsuperscript{−/−} mice, respectively. HFD resulted in increased spleen weight and spleen weight/TL by 38% in eET-1 mice. Spleen weight/TL was increased by 9% in Apoe\textsuperscript{−/−} mice, and spleen weight and spleen weight/TL were augmented by 14% in eET-1/Apoe\textsuperscript{−/−} mice fed a HFD compared with regular chow.

As previously reported,\textsuperscript{33} circulating ET-1 levels were \textasciitilde10-fold higher in eET-1 mice (Figure 1A) compared with WT mice fed a regular chow. The increase in plasma ET-1 was not affected by crossing eET-1 with Apoe\textsuperscript{−/−} mice or by the diet. Systolic blood pressure (BP) and heart rate were not affected by genotype or diet (Figure 1B; Figure II in the online-only Data Supplement). However, when regular chow and HFD groups were combined, systolic BP was 10 to 15 mm Hg higher in eET-1, Apoe\textsuperscript{−/−}, and eET-1/Apoe\textsuperscript{−/−} compared with WT mice (Figure 1B). Plasma cholesterol was unaltered in eET-1 mice but was increased 2.6- and 3-fold in Apoe\textsuperscript{−/−} and eET-1/Apoe\textsuperscript{−/−}, respectively, compared with WT mice fed a regular chow (Figure 1C). HFD increased plasma cholesterol 1.5- and 1.8-fold in WT and eET-1 mice, respectively. As expected, HFD increased plasma cholesterol 7-fold in Apoe\textsuperscript{−/−} mice, which was not affected by ET-1 overexpression.

Triglyceride levels were unaffected by ET-1 overexpression, Apoe knockout, or both in regular chow–fed animals (Figure III in the online-only Data Supplement). However, triglyceride levels were decreased \textasciitilde50% in WT and eET-1/Apoe\textsuperscript{−/−} mice and tended to be lower in eET-1 and Apoe\textsuperscript{−/−} mice in HFD-fed animals compared with regular chow. High-density lipoprotein (HDL) levels were unchanged by ET-1 overexpression, Apoe knockout, or both in regular chow–fed animals (Figure 1D). HDL levels were unaffected by the HFD in WT, eET-1, or Apoe\textsuperscript{−/−} mice but were decreased 45% in eET-1/Apoe\textsuperscript{−/−} mice.

**ET-1 Overexpression Exacerbated HFD-Induced Atherosclerosis and Triggered AAA Formation in Apoe\textsuperscript{−/−} Mice**

HFD-induced atherosclerotic lesions were characterized in cryosections of aortic sinus and 4 cryosections at 80-µm intervals of ascending aorta. No atherosclerotic lesions were detected in the aortic sinus and ascending aorta sections of WT or eET-1 mice (Figure 2A and 2B). Minor lipid deposition was observed in the aortic sinus of 2 of 9 WT and 3 of 9 eET-1 mice (Figure IV in the online-only Data Supplement). Atherosclerotic lesions were detected in cryosections of aortic sinus and ascending aorta of HFD-fed Apoe\textsuperscript{−/−} mice. HFD-induced an additional 2- and 6-fold increase in lesion size in the aortic sinus and ascending aorta, respectively, of eET-1/Apoe\textsuperscript{−/−} mice compared with Apoe\textsuperscript{−/−} mice. Atherosclerotic plaques in aortic arch sections of HFD-fed Apoe\textsuperscript{−/−} and eET-1/Apoe\textsuperscript{−/−} mice were further characterized by determining collagen distribution. Atherosclerotic plaques were observed in all 8 eET-1/Apoe\textsuperscript{−/−} mice examined compared with 3 of 7 Apoe\textsuperscript{−/−} mice (P<0.05). Plaques in eET-1/Apoe\textsuperscript{−/−} mice contained necrotic cores surrounded by a fibrous cap (Figure VI in the online-only Data Supplement).
AAAs were found at the supraprenal level in 8 of 21 eET-1/Apoε−/− mice fed a HFD (Figure 3A; Table 1). No AAAs were detected in the other groups fed a regular chow or HFD. All AAAs were associated with atherosclerosis plaques (Figure 3A). Aortic circumference at the supraprenal aortic level was 2-fold greater in eET-1/Apoε−/− mice with AAAs than in other groups fed a HFD (Figure 3B). Stretching and fragmentation of elastin was detected only in AAAs of eET-1/Apoε−/− mice (Figure 4A). The media collagen fraction was decreased 57% in HFD-fed Apoε−/− mice, which was not further changed by ET-1 overexpression (Figure V in the online-only Data Supplement). Fibronectin expression in the media tended to decrease in HFD-fed Apoε−/− mice, and was decreased by 42% in HFD-fed eET-1/Apoε−/− mice (Figure 4B).

**ET-1 Overexpression in HFD-Fed Apoε−/− Mice Exacerbated Vascular Inflammation in Ascending and Abdominal Supraprenal Aorta**

In HFD animals, reactive oxygen species (ROS) generation measured as dihydroethidium fluorescence tended to be higher in the media and PVAT of ascending and abdominal supraprenal aorta in eET-1 compared with WT mice (Figure 5A and 5B). ROS production was increased 4.6- and 3.3-fold in the media and PVAT of ascending aorta, respectively, in Apoε−/− compared with WT mice (Figure 5A). ROS production was also detected in atherosclerotic plaques of Apoε−/− mice. The increase in ROS production was exaggerated 2.7-fold in the media and 1.9-fold in the PVAT and atherosclerotic lesions in eET-1/Apoε−/− compared with Apoε−/− mice. Similar changes in ROS generation were observed in abdominal supraprenal aorta (Figure 5B). ROS production was increased 26-fold in the media and PVAT in Apoε−/− compared with WT mice. This was further elevated 3.4- and 2.8-fold in the media and PVAT, respectively, in eET-1/Apoε−/− compared with Apoε−/− mice. Comparable levels of ROS were detected in atherosclerotic plaques at the level of abdominal supraprenal aorta and ascending aorta in eET-1/Apoε−/− mice (Figure 5A and 5B).

**ET-1 Overexpression in HFD-Fed Apoε−/− Mice Exacerbated Superoxide Production in Ascending and AAAs**

Monocyte/macrophage infiltration, measured in HFD-fed animals, was minimal in the ascending aorta of WT and eET-1 mice (Figure 6A). Monocyte/macrophage infiltration was detected in 2%, 8%, and 52% of the surface of media, PVAT, and atherosclerotic plaques, respectively, in Apoε−/− mice. In the ascending aorta of eET-1/Apoε−/− mice, monocyte/macrophage infiltration was further increased 5.2- and 8.3-fold in the media and PVAT, respectively, compared with Apoε−/− mice. The level of monocyte/macrophage infiltration in atherosclerotic lesions was similar in eET-1/Apoε−/− and Apoε−/− mice. In abdominal supraprenal aorta of Apoε−/− mice, monocyte/macrophage infiltration was detected in ≥12% of the surface of the media and PVAT (Figure 6B). This was further exaggerated 2.6-fold in the PVAT but not in the media of abdominal supraprenal aorta in eET-1/Apoε−/− mice compared with Apoε−/− mice. Similar levels of monocyte/macrophages were found in atherosclerotic lesions at the level of the abdominal supraprenal aorta and ascending aorta in eET-1/Apoε−/− mice (Figure 6A and 6B). CD4+ cells, measured in HFD-fed animals, were undetectable in ascending or abdominal supraprenal aorta of WT and eET-1 mice (Table 2). Very few CD4+ cells were found in ascending aortic plaques of Apoε−/− mice. CD4+ infiltration was present more frequently in ascending and abdominal supraprenal aorta sections of eET-1/Apoε−/− mice. CD4+ cells were detected in ascending aorta sections of media in 3 of 6 and PVAT in 5 of 6 eET-1/Apoε−/− mice (Figure 6C; Table 2). CD4+ cell infiltration was also found in abdominal supraprenal aortic plaques of 4 of 6 eET-1/Apoε−/− mice (Figure 6D; Table 2). However, few CD4+ cells were detected in PVAT.

**ET-1 Overexpression in HFD-Fed Apoε−/− Mice Exacerbated Spleen Proinflammatory Ly6C+ Monocytes but Did Not Affect Plasma Cytokine Levels or T Cell Subsets**

Plasma interleukin (IL)-6 was decreased by 61%, whereas tumor necrosis factor-α and monocyte chemotactic protein-1...
Endothelin-1, Atherosclerosis, and Aneurysms

Li et al

Table 1. ET-1 Overexpression Increased the Prevalence of Abdominal Aortic Aneurysms in Apoe−/− Mice

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<tr>
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<th>Ctrl</th>
<th>HFD*</th>
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<tr>
<td>WT</td>
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<td>eET-1</td>
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<td>0/17</td>
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<td>0/20</td>
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<tr>
<td>eET-1/Apoe−/−</td>
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The prevalence of abdominal aortic aneurysms was determined in 16-week-old wild-type (WT), transgenic mice overexpressing endothelin (ET)-1 selectively in endothelium (eET-1), Apoe−/−, and eET1/Apoe−/− mice fed regular chow (Ctrl) or a high-fat diet (HFD) for 8 weeks. Values are ratios of number of animals with aneurysms over total number of mice studied.

*Higher rate of abdominal aortic aneurysm in eET-1/Apoe−/− with \( P < 0.01 \) by \( \chi^2 \).

Figure 3. Endothelin (ET)-1 overexpression increased incidence of abdominal aortic aneurysms (AAAs) in Apoe−/− mice fed a high-fat diet. A. Example of AAAs in transgenic mice overexpressing ET-1 selectively in endothelium (eET-1)/Apoe−/− mouse is presented. The top left picture shows whole aorta containing AAAs. A magnification of AAAs is presented in bottom left picture. The bottom right picture shows an oil red O-stained section of AAAs. Arrowheads indicate AAAs. Incidence of AAAs is shown in Table 1. B. The abdominal suprarenal aorta circumference was determined in the same groups as in Figure 1. Representative images of Sirius red-stained aorta sections and abdominal suprarenal aorta circumference for each individual (closed circle) and mean circumference for wild type (WT), eET-1, Apoe−/−, and eET1/Apoe−/− with normal circumference (open circle), and eET1/Apoe−/− with circumference >1.5-fold of WT mean circumference indicated by the dashed gray line (gray-filled circle) are shown. Values are mean±SEM, n=8 to 13. *\( P < 0.001 \) vs WT, †\( P < 0.001 \) vs eET-1, and ‡\( P < 0.001 \) vs Apoe−/−.

FOXP3+ T regulatory cells was increased 2-fold in Apoe−/− and eET-1/Apoe−/− compared with WT mice. The percentage of spleen-activated CD4+CD69+ T cells was increased 3.3-fold in Apoe−/− and eET-1/Apoe−/− compared with WT mice. The percentage of spleen-activated CD8+CD69+ T cells was 2.4-fold higher in Apoe−/− compared with WT mice, and tended to be further increased in eET-1/Apoe−/− mice. The percentage of spleen proinflammatory Ly-6C+ monocytes was increased 1.7-fold in Apoe−/− compared with WT mice, and was further increased 26% in eET-1/Apoe−/− mice (Figure IX in the online-only Data Supplement). ET-1 overexpression alone did not alter spleen T cell and monocyte subtype profiles.

Discussion

Our study extends our previous findings by demonstrating that increased expression of ET-1 in endothelium, in addition to exaggerating HFD-induced atherosclerosis, triggers AAA formation in Apoe−/− mice, an atherosclerosis-prone model that resembles human disease. These phenomena were associated with decreased plasma HDL, elevated oxidative stress in the media, plaque, and PVAT, increased monocyte/macrophage infiltration primarily in the PVAT, CD4+ T cell infiltration in plaque and PVAT, and greater percentage of spleen proinflammatory Ly-6C+ monocytes. Our findings provide support to the hypothesis that ET-1 overexpression worsens the progression of atherosclerosis and contributes to the pathogenesis of AAAs via pro-oxidant and inflammatory mechanisms, and lowering of HDL.

Plasma ET immunoreactivity in patients with early and advanced atherosclerosis has been shown to be >2-fold higher than in healthy subjects, and has been correlated with the extent of atherosclerosis, indicating a possible role of ET-1 in atherogenesis. Animal studies have shown that ET-1 is increased in plasma and the vessel wall from Apoe−/− mice, and in pigs fed a HFD. Increased ET-1 expression in the vascular wall has also been associated with...
Atherosclerosis development in 1-year-old \textit{Apoe}^{−/−} mice fed a regular chow. In the present study, 8 weeks of HFD induced an increase in plasma ET-1 levels in WT and \textit{Apoe}^{−/−} mice, but this was not sufficient to accelerate the development of abdominal aortic aneurysms. Endothelin (ET-1) overexpression–induced abdominal aortic aneurysms are associated with elastic laminae disruption and flattening and decreased media fibronectin expression. The structure of elastin (A) and expression of fibronectin (B) were determined in abdominal suprarenal aorta of the same groups as in Figure 1. Representative images of Verhoeff’s elastic–stained sections (A) and fibronectin immunofluorescence (red; B) are shown. Blue and green fluorescence represent nuclear DAPI staining and autofluorescence of elastin, respectively. RFU indicates relative fluorescence units. Values are mean±SEM, n=4 to 5 for A and 4 to 6 for B. *\(P<0.001\) vs wild type (WT), †\(P<0.001\) vs transgenic mice overexpressing ET-1 selectively in endothelium (eET-1), and ‡\(P<0.001\) vs \textit{Apoe}^{−/−}.

Endothelin (ET-1) overexpression exacerbated high-fat diet–induced reactive oxygen species (ROS) production in ascending and abdominal suprarenal aorta of \textit{Apoe}^{−/−} mice. ROS were determined using dihydroethidium (DHE) staining in ascending (A) and abdominal suprarenal (B) aortic sections of the same groups as in Figure 1. Representative images of DHE-stained ascending (A) and abdominal suprarenal (B) aortic sections with quantification of DHE fluorescence in media, perivascular fat, and atherosclerotic plaques are shown. Red fluorescence indicates DHE fluorescence, and green fluorescence represents the autofluorescence of elastin. The lumen (Lu), plaque (Pl), and perivascular fat (Fat) are indicated. Values are mean±SEM, n=4 to 6 for A and 4 to 5 for B. *\(P<0.05\) and **\(P<0.01\) vs wild type (WT), †\(P<0.05\) vs transgenic mice overexpressing ET-1 selectively in endothelium (eET-1), and ‡\(P<0.05\) and ‡‡\(P<0.01\) vs \textit{Apoe}^{−/−}.
of atherosclerosis. The increase in plasma ET-1, however, did not reach the levels observed in previous pig and human studies. Consistent with our previous reports, both eET-1 and eET-1/Apo<sup>e<sup>-/-</sup> mice demonstrated 10-fold elevation in plasma ET-1. However, only eET-1/Apo<sup>e<sup>-/-</sup> mice presented advanced atherosclerosis. This suggests that increased ET-1 levels exaggerate the progression of atherosclerosis only in conditions predisposed toward the development of atherosclerosis. In agreement with this, it has been shown that ET-1 infusion inducing a similar increase in plasma levels caused worsening of the intimal hyperplastic response after mechanical injury in rats. Interestingly, plasma ET immunoreactivity is higher in patients with AAAs and correlates with AAA size, suggesting a role for ET-1 in AAA pathogenesis. In this study, exaggeration of atherosclerosis in HFD-fed eET-1/Apo<sup>e<sup>-/-</sup> mice was accompanied by increased incidence of AAAs, indicating that elevated ET-1 levels could contribute to the development of AAAs in an atherosclerotic setting.

We found a minor increase in total plasma cholesterol in HFD-fed WT and eET-1 mice. A similar increase in cholesterol levels was previously reported in WT C57BL/6 mice fed the same HFD as in this study for 12 or 18 weeks. It has also been shown that the increase in plasma cholesterol reaches a plateau after ≈2 weeks of HFD feeding. Therefore, it is not surprising that the plasma cholesterol levels were increased in HFD-fed WT and eET-1 mice, which are on a C57BL/6 background. A large increase in plasma cholesterol was observed in HFD-fed Apo<sup>e<sup>-/-</sup> and eET-1/Apo<sup>e<sup>-/-</sup> mice. However, aggravation of atherosclerotic lesions and formation of AAAs occurred only in eET-1/Apo<sup>e<sup>-/-</sup> mice. This was accompanied by a decrease in plasma HDL. HDL levels are inversely correlated with the risk for coronary vascular disease. HDL mediates the reverse transport of cholesterol from the vasculature to the liver. It has also been shown that, in addition to apolipoprotein A-I, HDL carry several proteins including α1-antitrypsin with anti-elastase activity, which can counteract the development of AAAs. Therefore, it is plausible that ET-1 overexpression–induced decrease in HDL contributed to the progression of atherosclerosis in eET-1/Apo<sup>e<sup>-/-</sup> mice.

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CD<sup>4</sup> cell infiltration was determined in 16-week-old wild-type (WT), transgenic mice overexpressing endothelin (ET)-1 selectively in endothelium (eET-1), Apo<sup>e<sup>-/-</sup> and eET1/Apo<sup>e<sup>-/-</sup> mice fed a high-fat diet for 8 weeks. Values are ratios of number of animals with CD4<sup>+</sup> cells over total number of mice studied. NP indicates no plaques. *Greater CD4<sup>+</sup> cell infiltration in eET-1/Apo<sup>e<sup>-/-</sup>, P<0.05 by χ<sup>2</sup>.
to the enhanced damaging effects of increased plasma cholesterol on the induction of atherosclerosis and triggering of AAAs, as observed in HFD-fed eET-1/Apoe−/− mice. Further studies are required to determine the mechanisms by which ET-1 overexpression decreases plasma HDL.

Previous studies have shown that the development of atherosclerosis in Apoe−/− mice can be accelerated by Ang II infusion or deoxycorticosterone acetate–salt treatment. Although Weiss et al and others have demonstrated that BP elevation by norepinephrine (40 mm Hg) does not accelerate the development of atherosclerosis to the same extent as Ang II (40–60 mm Hg), elevated BP may contribute to development of atherosclerotic lesions. In the present study, BP was unaltered by ET-1 overexpression, Apoe knockout, the combination of both, or by HFD feeding. A 10 to 15 mm Hg BP rise was found in eET-1, Apoe−/−, and eET-1/Apoe−/− mice compared with WT mice only when regular chow and HFD data were combined. Thus, exacerbation of atherosclerosis and triggering of AAAs found in HFD-fed eET-1/Apoe−/− mice occurred in absence of significant BP elevation. This new animal model combining genetically modified mice overexpressing human ET-1 on a genetic background of atherosclerosis-prone Apoe−/− provides novel evidence that pathological effects of ET-1 on atherosclerosis and AAA development are BP-independent.

AAAs were observed in 40% of the eET-1/Apoe−/− mice fed a HFD, whereas none were detected in the other groups of mice. AAAs were observed at the suprarenal aortic level, as reported previously for Ang II–infused Apoe−/− mice. As observed in humans, AAAs in HFD-fed eET-1/Apoe−/− mice were characterized by elastin breaks and flattening, and decreased media collagen fraction. However, a decrease in media collagen fraction was also observed in HFD-fed Apoe−/− mice. Consequently, although collagen degradation lowers aortic tensile strength and therefore weakens the aortic wall, it predisposes to but is not sufficient for AAA development in Apoe−/− mice. Incidentally, ET-1 overexpression in Apoe−/− mice caused a decrease in media fibronectin expression that could contribute to the development of AAAs by further weakening the aortic wall.

Recently, Suen et al reported that treatment with a mixed ETa and ETb receptor antagonist reduced Ang II–induced atherosclerosis in young (4 weeks) but not in old (6 months) Apoe−/− mice fed a Western-type diet, and failed to protect against Ang II–triggered AAAs. It is possible that blocking ET receptors only partially inhibits Ang II–mediated vascular inflammation and signaling pathways involved in the development of atherosclerosis and aneurysms. It is also possible that the increase in ET-1 expression might be suboptimal to induce AAAs in this model because Ang II did not cause a significant increase in plasma ET-1. Therefore, our findings suggest a novel mechanism of AAAs mediated by ET-1. The exact mechanism for aneurysm formation remains to be established. However, it is likely that oxidative stress and low-grade inflammation triggered by ET-1 overexpression aggravate oxidative stress and inflammation already present in Apoe−/− mice, thereby playing a role in development of aneurysms.

We have previously reported that blood vessels in eET-1 mice are characterized by increased oxidative stress compared with WT mice. Our current data show that ET-1 overexpression exaggerates ROS generation not only in the vascular wall but also in atherosclerotic plaques and PVAT in ascending aorta and AAAs of eET-1/Apoe−/− mice. This enhancement of oxidative stress could be attributable to increased activity of reduced nicotinamide adenine dinucleotide oxidase or uncoupling of endothelial NO synthase, or both. Kuhlencordt et al previously showed that double Apoe and Nos3 knockout mice on a Western style diet exhibit increases in atherosclerotic lesions compared with Apoe−/− mice. In addition, Gao et al demonstrated a role for tetrahydrobiopterin deficiency–induced endothelial NO synthase uncoupling causing •O2− production and reduced NO availability in AAA formation. Ang II–induced AAAs are increased in hyperphenylalaninemia (hph)-1 mice deficient in endothelial NO synthase cofactor tetrahydrobiopterin biosynthetic enzyme guanosine triphosphate cyclohydrolase 1, which can be prevented by restoring tetrahydrobiopterin levels by treatment with folic acid or endothelium-targeted dihydrofolate reductase gene therapy. These data are consistent with our findings, suggesting a key role of ET-1–induced oxidative stress in the development of atherosclerosis and AAA formation. In addition, our results suggest a participation of ET-1–induced ROS production by PVAT in atherosclerosis and AAA development.

The concept that atherosclerosis is an inflammatory disease is now well accepted. Monocyte/macrophage and T cell infiltration play roles in the pathogenesis of atherosclerosis and AAA formation. ET-1 can also be produced by macrophages, which could further contribute to inflammation. Heavy ET-1 staining has been reported in foam cells within atherosclerotic lesions, and in vascular smooth muscle cells of the intima and media. Here, ET-1 overexpression exaggerated monocyte/macrophage infiltration primarily in the PVAT of ascending aorta and AAAs in eET-1/Apoe−/− compared with Apoe−/− mice. However, monocyte/macrophage infiltration was equally high in plaques from eET-1/Apoe−/− and Apoe−/− mice. Because atherosclerotic plaques are larger in eET-1/Apoe−/− compared with Apoe−/− mice, and the cell infiltration is expressed as percentage of total surface, these data can also be interpreted as eET-1/Apoe−/− mice having more atherosclerotic plaque monocytes/macrophages. Interestingly, ET-1 overexpression exaggerated the increase in the percentage of spleen proinflammatory Ly-6Cm monocyes in Apoe−/− mice, suggesting that there is an increase in circulating proinflammatory Ly-6Cm monocytes that can contribute to the increase in monocyte/macrophage infiltration in the PVAT and, hence, to the development of atherosclerotic plaques. It should be noted that the monocyte subtype profile was unchanged by ET-1 overexpression in absence of Apoe knockout. MMP2, which could be secreted by macrophages, was higher in plaques from eET-1/Apoe−/− than from Apoe−/− mice. The increase in MMP2 could contribute to plaque development and rupture, and AAA formation. However, the mechanisms by which ET-1 overexpression increases MMP2 expression in the plaques are unknown and could be mediated in part by monocytes and macrophages. It has been shown that the severity of atherosclerosis is reduced in Apoe−/− mice.
that are lacking functional monocyte/macrophages or are deficient in MMP2. Recently, we reported that ET-1 overexpression–induced vascular remodeling and oxidative stress, which is associated with monocyte/macrophage infiltration in the adventitia and PVAT, is prevented by crossing eET-1 with mice carrying an osteopetrotic mutation in the macrophage colony–stimulating factor (Csf1op) gene. Increased expression of ET-1 in endothelial cells of eET-1/ApoE−/− mice could act in paracrine fashion to stimulate the production of proinflammatory monocytes in the periphery, increase the recruitment of monocytes to atherosclerotic plaques, and promote the differentiation of monocytes into macrophages. Whether ET-1 overexpression directly increases the expression of MMP2 in macrophages remains to be determined.

In this study, the eET-1 mice did not present signs of systemic inflammation because plasma cytokines and spleen T cells and monocyte subtype profiles were unaltered. Apoe−/− mice fed a HFD for 8 weeks presented low levels of systemic inflammatory mediators. Plasma tumor necrosis factor-α, IL-6, monocyte chemotactic protein-1, and IL-10 levels were unchanged, and the percentage of spleen-activated CD4+CD69+, CD8+CD69+ T cells, and CD4+CD25+FOXP3+ T regulatory cells, which are suppressor T cells, were elevated. The increase in T regulatory cells could probably be a compensatory mechanism counteracting the increase in activated T cells. ET-1 overexpression did not change the plasma cytokine levels and the T cell profile and, therefore, did not alter the systemic inflammation in Apoe−/− mice. CD4+ T cell infiltration was barely detectable in atherosclerotic plaques of Apoe−/− mice. ET-1 overexpression induced CD4+ T cell infiltration in atherosclerotic plaques and PVAT in ascending aorta, and in plaques in AAs of eET-1/ApoE−/− mice on HFD. CD8+ T cells could not be detected in media, atherosclerotic plaque or PVAT of Apoe−/− or eET-1/ApoE−/− mice (data not shown). Interestingly, crossing Apoe−/− mice with severe combined immunodeficiency (Scid) mice lacking T and B cells has been shown to reduce atherosclerosis. Adoptive transfer of CD4+ T cells abolished this protection and increased atherosclerotic lesions in immunodeficient Apoe−/− mice. Therefore, previous data are consistent with our findings and suggest an important role for ET-1–induced infiltration of monocyte/macrophages and CD4+ T cells in development of atherosclerosis and AAA formation. In addition, our results indicate an important role for ET-1–induced inflammatory cell infiltration in PVAT for both atherosclerosis and AAA development. However, the mechanisms of ET-1–induced T cell infiltration remain to be determined.

There is evidence that PVAT plays a role in atherosclerosis. In humans and mice, PVAT presents a higher inflammatory state compared with subcutaneous and epididymal fat. HFD induced ROS production in PVAT, which caused endothelial dysfunction. PVAT is a source of vascular inflammatory cells that play a role in hypertension and vascular damage. PVAT surrounding atherosclerotic aorta has chemotactic properties through secretion of cytokines, thereby attracting macrophages and T cells. Accumulation of macrophages and T cells at the interface between PVAT and adventitia of human atherosclerotic aorta has been previously reported. In Apoe−/− mice fed HFD, PVAT transplantation next to the common carotid artery, a site normally devoid of atherosclerotic lesions, impairs vascular relaxation and causes local formation of an atherosclerotic plaque. So far, there has been no evidence of a role of PVAT in AAA formation. However, the previously cited results are consistent with our finding that increased ET-1 enhances ROS production and monocyte/macrophage and CD4+ T cell infiltration in PVAT, which then plays a role in progression of atherosclerosis and in AAA formation.

Recently, we showed that there was an increase in expression of genes associated with lipid biogenesis, including elongation of very long chain fatty acids family member 6 (Elovl6) that is responsible for the final step in saturated fatty acid synthesis, which could lead to excess accumulation of lipids within the vascular wall and, hence, contribute to vascular remodeling, endothelial dysfunction, and development of atherosclerosis. Saito et al have suggested a role for ELOVL6-induced elongation of saturated and monounsaturated fatty acids in foam cell formation and atherosclerosis progression. In their study, Western diet–induced atherosclerotic lesions and monocyte/macrophage infiltration were reduced in irradiated Ldlr−/− mice transplanted with bone marrow cells from Elovl6−/− versus cells from WT mice. It is possible that higher levels of ET-1 might increase the expression of Elovl6 in macrophages resulting in exaggeration of foam cell formation, progression of atherosclerosis, and development of AAA formation.

A limitation of the present study is that the eET-1 mice presented a greater increase in plasma levels of ET-1 than that observed in humans with atherosclerosis or aneurysms. However, by achieving greater increase in ET-1 expression, and thereby exaggerating the activity of a biological system such as ET-1, we were able to reveal the underlying pathophysiological mechanisms by which ET-1 contributes to the development of atherosclerosis and aneurysms. Nevertheless, additional studies might be necessary to confirm the importance for humans of the present findings.

Conclusions and Perspectives

We have demonstrated that ET-1 overexpression exerts a potent proatherogenic effect and triggers AAA formation in Apoe−/− mice on a HFD, possibly through pro-oxidant and inflammatory mechanisms and decrease in HDL. We also suggest that PVAT plays a prominent role. Clinically, our results underscore the importance of ET-1 in human atherosclerosis and AAA formation. ETα or ETβ receptor blockers and ET converting enzyme inhibitors could be potential pharmacological candidates for treatment of atherosclerosis and prevention of growth of AAAs. In support, Yoon et al have recently showed that long-term ETα receptor blocker treatment attenuates the progression of coronary plaques in patients with early atherosclerosis.

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We are grateful to Marie-Ève Deschênes, Adriana Cristina Ene, Nourredine Idris-Khodja, Christian Young (Flow cytometry facility), and Lilian Canetti (Research Pathology Facility) for excellent technical assistance.
A role for the vasoconstrictor peptide, endothelin-1, has been suggested in the development atherosclerosis and aneurysms in humans and animal models. In this study, we show that overexpressing endothelin-1 in the endothelium of apolipoprotein E knockout (ApoE−/−) mice fed a high-fat diet exaggerated the development of atherosclerosis and triggered abdominal aortic aneurysms. This was associated with increased vascular oxidative stress and immune cell infiltration, mostly monocytes/macrophages, which could be mediated in large part by perivascular fat. An exaggerated increase in spleen proinflammatory Ly-6C+ monocytes was also present, which could contribute to the monocyte/macrophage infiltration in perivascular fat and ultimately in the atherosclerotic plaque. A decrease in plasma high-density lipoprotein, which is a risk factor for coronary vascular disease, also accompanied endothelin-1–induced exaggeration of atherosclerosis and abdominal aortic aneurysms. This could contribute to atherosclerotic plaque enlargement and abdominal aortic aneurysms development. These observations establish the mechanisms whereby endothelin-1 could contribute to atherosclerosis and abdominal aortic aneurysms in humans.
Endothelin-1 Overexpression Exacerbates Atherosclerosis and Induces Aortic Aneurysms in Apolipoprotein E Knockout Mice
Melissa W. Li, Muhammad Oneeb Rehman Mian, Tili Barhoumi, Asia Rehman, Koren Mann, Pierre Paradis and Ernesto L. Schiffrin

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Materials and Methods

ENDOTHELIN-1 OVEREXPRESSION EXACERBATES ATHEROSCLEROSIS AND INDUCES AORTIC ANEURYSMS IN APOLIPOPROTEIN E KNOCKOUT MICE

Melissa W. Li1*, Muhammad Oneeb Rehman Mian1*, Tlili Barhoumi1, Asia Rehman1, Koren Mann1,2, Pierre Paradis, Ernesto L. Schiffrin1,3

1Lady Davis Institute for Medical Research, and 2Department of Medicine and 3Department of Oncology, Sir Mortimer B. Davis-Jewish General Hospital, McGill University, Montréal, Québec, Canada.

*These authors contributed equally.

Li et al: Endothelin-1, atherosclerosis and aneurysms

Corresponding author:
Ernesto L. Schiffrin, CM, MD, PhD, FRSC, FRCPC
Sir Mortimer B. Davis-Jewish General Hospital, #B-127,
3755 Côte-Ste-Catherine Rd.,
Montreal, PQ, Canada H3T 1E2
Fax: 514-340-7539
Ph: 514-340-7538
E-mail: ernesto.schiffrin@mcgill.ca
http://ladydavis.ca/en/ernestoschiffrin
**Generation of eET-1/Apoε⁻/⁻ mice and study design**

The study was approved by the Animal Care Committee of the Lady Davis Institute for Medical Research and McGill University, followed recommendations of the Canadian Council for Animal Care and was in agreement with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health. C57BL/6 transgenic mice overexpressing the human ET-1 (eET-1) driven by the *Tie2* promoter conferring endothelial-specific expression were described previously. It should be noted that the human ET-1 signal peptide is very well conserved and the peptide processing sites necessary for the maturation of the proET-1 and the mature ET-1 peptide are identical to those found in mouse ET-1 (Figure I in the online-only Data Supplement). C57BL/6 *Apoε⁻/⁻* mice were obtained from The Jackson Laboratory (B6.129P2-Apoε^tm1Unc/J, Bar Harbor, ME, USA). The eET-1/Apoε⁻/⁻ mice were generated by crossing eET-1 mice with Apoε⁻/⁻ mice and then eET-1/Apoε⁺/⁻ mice with eET-1/Apoε⁻/⁻ mice. Eight-week old male wild-type (WT), eET-1, Apoε⁻/⁻, and eET-1/Apoε⁻/⁻ mice were fed a high-fat, cholesterol-rich diet (35% fat, 1.25% cholesterol, D12336, Research Diets Inc., New Brunswick, NJ, US) (n = 11-15) or regular chow (n = 9-13) for 8 weeks. At the end of the protocol, systolic blood pressure was measured by the tail-cuff method using a MC4000 blood pressure analysis system (Hatteras Instruments, Cary, NC, USA). Body weight was measured and mice anesthetized with 3% isoflurane (mixed with O₂ at 1 L/mL, depth of anesthesia confirmed by rear foot squeezing). One mL of blood was collected by cardiac puncture, on heparin for measurement of plasma cholesterol, high-density lipoprotein (HDL) and triglycerides or on EDTA for ET-1 and cytokines. In one group of animals, the whole aorta was excised from the root to the iliac bifurcation for study of abdominal aortic aneurysms (AAA), and heart, kidneys and spleen were harvested and weighed. Tibia length was measured. In another group of mice, the top part of the heart and thoracic aorta with perivascular fat (PVAT) were dissected to characterize atherosclerotic plaques, and the abdominal suprarenal aorta with PVAT was collected to study AAA. The spleen was used to determine T cells and monocyte subtype profiles. Atherosclerotic plaques, reactive oxygen species (ROS) production, and monocyte/macrophage and CD4⁺ cell infiltration were determined in cryosections of aortic sinus and ascending aorta. Matrix metalloproteinase-2 (MMP2) expression was determined in cryosections of ascending aorta. Collagen content was examined in paraffin sections of aortic arch and abdominal suprarenal aorta. Elastin structure and fibronectin expression were determined in paraffin sections of the abdominal suprarenal aorta.

**Quantification of atherosclerosis and abdominal aortic aneurysm**

To study atherosclerotic plaques in the aortic sinus and ascending aorta, the top part of the heart and a section of ascending aorta were embedded in VWR Clear Frozen Section Compound (VWR International, West Chester, PA, USA). Five µm cryosections of aortic sinus and four cryosections at 80-µm intervals of ascending aorta were air-dried for 30 min and fixed 5 min with 4% paraformaldehyde (PFA) solution in phosphate buffered saline (PBS). Sections were rinsed in three changes of distilled water, immersed in 100% propylene glycol for 5 min, and then stained with pre-warmed Oil Red O solution (Electron Microscopy Sciences, Hatfield, PA, USA) for 10 min at 60 °C. Excess stain was removed with 85% propylene glycol for 5 min, after which the sections were rinsed with two changes of distilled water and then counterstained with Mayer’s hematoxylin (Sigma-Aldrich, St Louis, MO, USA) for 10 min. The sections were washed thoroughly in running tap water, adjusted to RT, for 3 min, and then mounted using aqueous mounting medium (Thermo Scientific, Pittsburgh, PA, USA).
To study abdominal aortic aneurysms, a section of the abdominal suprarenal aorta was embedded in Tissue-Tek OCT (Sakura, Torrance, CA, USA). Five µm cryosections were fixed for 5 min in 4% PFA followed by rinsing with water. The sections were stained with 0.5% oil red O working solution for 30 min. The sections were rinsed 3 times for 5 min with water and mounted with Immuno-Mount mounting solution (Thermo Scientific, Pittsburgh, PA). In a second set of samples, sections of the abdominal suprarenal aorta were fixed in 4% PFA at 4°C for 48 h and embedded in paraffin. Sections were stained with Sirius red as indicated below.

Images were captured with Infinity capture imaging software (Luminera Corp., Ottawa, ON, Canada). Atherosclerotic plaques and abdominal suprarenal aorta circumference at abdominal aortic aneurysm level were quantified using ImageJ. The atherosclerotic plaques were expressed in µm² for aortic sinus and in mean µm² of the four sections at 80-µm intervals for ascending aorta.

**Reactive oxygen species production**

Dihydroethidium (DHE) was used to evaluate the in situ production of reactive oxygen species (ROS) as previously described. Ascending and abdominal suprarenal aortas containing the perivascular fat were embedded in Tissue-Tek OCT. Five µm sections of unfixed frozen ascending and abdominal suprarenal aorta were thawed, air dried for 30 min at RT and incubated in DHE (2 µmol/l) at RT for 5 min. Fluorescent images were captured as above using a CY3 filter. The fluorescence was quantified in media, perivascular fat and plaque separately using ImageJ. DHE fluorescence per unit of surface was normalized to fluorescence in the media of wild-type (WT) mice acquired on the same day and expressed as % of WT media. This was done to eliminate any variation due to changes in the oxidative environment on any given day.

**Monocyte/macrophage and CD4⁺ cell infiltration and fibronectin and matrix metalloproteinase-2 expression**

Infiltration of monocyte/macrophage (MOMA-2) and CD4⁺ cells (CD4) in ascending aorta and abdominal suprarenal aorta, and the expression of MMP2 in the ascending aorta were determined by immunofluorescence microscopy on 5 µm cryostat sections. Fibronectin expression in abdominal suprarenal aorta was determined in 5 µm paraffin sections. All the aortic sections contained perivascular fat. Tissue cryosections were air-dried for 30 min and then fixed in 4% paraformaldehyde for 20 min at RT (for MOMA-2, and MMP2) or in ice cold acetone for 10 min at RT (for CD4). Thereafter, sections were washed with PBS twice for 5 min. Sections were blocked for 1 h at RT with TBS (50 mM Tris pH 7.4, 150 mM NaCl) containing 1% bovine serum albumin, 0.4% Triton X-100 and 20% fetal bovine serum for MOMA-2 and CD4 or PBS containing 10% normal donkey serum (Jackson ImmunoResearch Laboratories, West Grove, PA) for MMP2. Sections were incubated overnight at 4°C with a rat anti-monocyte/macrophage-specific antigen MOMA-2 (1:50, Abcam, Cambridge, MA) or a rat anti-CD4 (1:20, BD Biosciences, Mississauga, ON, Canada) antibody in TBS blocking solution, or with a goat anti-MMP2 antibody (1:50, Santa Cruz Biotechnology, Santa Cruz, CA), or normal goat IgG as negative control for MMP2 immunofluorescence in PBS. The sections were washed 3 times with TBS containing 0.1% Tween-20 (TBST) for MOMA-2 and CD4 or with PBS for MMP2. Sections were incubated with Alexa Fluor® 555 goat anti-rat (1/200) (Invitrogen Corp., Carlsbad, CA, USA), Alexa Fluor® 568 goat anti-rat (1/400) or Alexa Fluor® 555 donkey anti-
goat (1:200) antibody for 1 h at RT, and then washed 3 times with TBST for MOMA-2 and CD4 or with PBS for MMP2. Sections were mounted with Vectashield containing 4’,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA, USA) or stained with 3 µM DAPI (Invitrogen) in PBS, washed 3 times with PBS and mounted with Fluoromount (Sigma-Aldrich). Paraffin sections were deparaffinized with two 5 min xylene baths, rehydrated in successive 3 min baths of ethanol (100%, 100%, 95%, 90%, 80%, 70% and 50%) followed by 5 min incubation in PBS. Sections were blocked with 10% normal goat serum (NGS) for 1 h, and then incubated with rabbit anti-mouse fibronectin antibody (1:200, EMD Millipore, Billerica, MA, USA) overnight at 4 ºC. Sections were then washed three times using TBST and incubated with Alexa® Fluor 568 goat anti-rabbit antibody (1:200, Life Technologies, Burlington, ON, Canada) for 1 h at RT. After, sections were counterstained with DAPI and mounted as above. Images were captured using a fluorescent microscope Leica DM2000 (Leica Microsystems, Richmond Hill, ON, Canada) and analyzed with ImageJ. MOMA-2 expression and CD4+ cells were determined in the media, perivascular fat and atherosclerotic lesions separately. MOMA-2 was expressed as % of studied area and presence or absence of CD4+ cells determined. MMP2 was determined in atherosclerotic plaques, and expressed as the relative fluorescence unit (RFU) per µm² of atherosclerotic lesion.

Collagen content and elastin structure

Collagen content in abdominal suprarenal aortic media and in aortic arch atherosclerotic plaques was determined in 5 µm paraffin sections as follows. Paraffin sections were deparaffinized with two 5 min xylene baths, rehydrated in successive 3 min baths of ethanol (100%, 100%, 95%, 95% and 70%) followed by 5 min incubation in water. Sections were stained with 0.1% Sirius red solution (Sigma-Aldrich, St. Louis, MO, USA) prepared in saturated picric acid for 60 min at RT. Excess stain was removed by rinsing in 100 mM acetic acid for 3 min. Sections were then de-hydrated in successive 3 min baths of ethanol (95%, 100%, 100% and 100%) and immersed in two baths of xylene for 3 min. Sections were then mounted using Eukitt Mounting Medium (Electron Microscopy Sciences, Hatfield, PA, USA). Images were acquired by light microscopy using a Leica DM2000 microscope, and analyzed by color RGB thresholding using Northern Eclipse software (EMPIX Imaging, Mississauga, ON, Canada). Abdominal suprarenal aorta media collagen fraction was defined as the ratio of the media stained area to the total media area and expressed as a percentage.

Elastin structure in abdominal suprarenal aortic media was determined in 5 µm paraffin sections as follows. Paraffin sections were deparaffinized with two 2 min xylene baths, rehydrated in successive 1 min baths of ethanol (100%, 95%, 90% and 70%) followed by 3 min incubation in water. Sections were stained with freshly prepare Verhoeff’s solution for 30 min at RT. Verhoeff’s stain was made by mixing 2.5 part of of 0.17 M hematoxylin BSC (Fisher Scientific, Fair Lawn, NJ, USA) in ethanol, 1 part of 10% ferric chloride solution (Fisher, Fair Lawn, NJ, USA) and 1 part of Lugol solution (Sigma-Aldrich). Excess stain was differentiated using 2% ferric chloride solution for ~3 min. Sections were then washed in running tap water for 30 sec at RT, and dipped in 5% sodium hyposulphite solution (Fisher Scientific). Sections were then de-hydrated in successive 3 min baths of ethanol (70%, 90%, 95% and 100%) and, and cleared by immersing in three changes of xylene for 1 min each. Sections were then mounted and imaged as above.
Flow cytometry profiling of splenic T cells and monocytes
Profile of T cells and monocyte subtypes was determined by flow cytometry as follows. The technique of monocyte profiling has previously been described. Single splenocyte suspension was obtained by releasing the splenocytes by forcing pieces of spleen through a 70 µm nylon mesh cell strainer (BD Biosciences, Durham, NC, USA) pre-wet with PBS supplemented with 5% fetal bovine serum (FBS) with the back of a 3 mL syringe plunger. The cell strainer was washed with PBS/5% FBS to flush the cells through the nylon mesh. The two previous steps were repeated until only connective tissue remained in the cell strainer. Cells were centrifuged at 300 x g for 10 min at RT. Cells were resuspended in 5 mL of Red Blood Cell lysis buffer (Sigma-Aldrich) and incubated at RT for 3 min with occasional gentle mixing to eliminate red blood cells. The mixture was diluted with 30 mL of PBS/5% FBS, filtered through a 70 µm nylon mesh cell strainer, and centrifuged at 300 x g for 5 min at RT. Cells were resuspended in 2 mL of PBS/5% FBS and counted using a Z2 Coulter® Counter (Beckman-Coulter, Mississauga, ON, Canada). Two million cells were stained with a fixable viability dye eFluor® 506 (eBioscience, San Diego, CA, USA) in PBS, incubated with rat anti-mouse CD16/CD32 Fc receptor block (clone 2.4G2, BD Biosciences), and stained with specific antibodies or appropriate isotype control antibodies in PBS/5% FBS. Specific antibody-fluorochrome staining panel and example of the general gating procedure used for analysis are included in Table II and Figure VIII in the online-only Data Supplement for profiling of T cells and Table III and Figure IX in the online-only Data Supplement for profiling of monocytes. Flow cytometry was performed on the BD LSRFortessa cell analyzer (BD Biosciences). Fluorescence minus one controls were used to determine fluorescence background and positivity. Data analysis was performed using FlowJo software (Tree Star Inc., Ashland, OR, USA). Data are expressed as indicated in Figure. VIII and IX legends in the online-only Data Supplement.

Plasma determinations
Blood samples were centrifugation at 1,000 x g for 15 min at 4°C to remove blood cells followed by a centrifugation at 10,000 x g for 10 min at 4°C to remove platelets. Plasma samples were stored at -80°C until tested. Plasma cholesterol, triglycerides and HDL were measured using a J&J Vitros 250 chemistry analyzer by Diagnostic Research Support Services at the Comparative Medicine and Animal Resource Centre of McGill University. The concentration of ET-1 was determined in plasma on EDTA using a human ET-1 QuantiGlo ELISA Kit (R&D Systems Inc., Minneapolis, MN, USA). Plasma levels of interleukin (IL)-6, IL-10, monocyte chemotactic protein-1 (MCP-1) and tumor necrosis factor (TNF)-α were measured using micro-bead multiplex immunoassays on a Bio-Plex 200 (Bio-Rad Laboratories).

Statistical analysis
Data are shown as means ± SEM. Comparisons were made by one- or two-way ANOVA as appropriate, followed by a Student–Newman–Keuls or Dunnett's T3 post-hoc test, as appropriate. Comparisons between two groups were made using an unpaired t-test. Percent prevalence was compared using a χ² test or a Fisher Exact test, as appropriate. A value of P<0.05 was considered significant.
References


Supplemental Material

ENDOTHELIN-1 OVEREXPRESSION EXACERBATES ATHEROSCLEROSIS AND INDUCES AORTIC ANEURYSMS IN APOLIPOPROTEIN E KNOCKOUT MICE

Melissa W. Li¹*, Muhammad Oneeb Rehman Mian¹*, Tlili Barhoumi¹, Asia Rehman¹, Koren Mann¹², Pierre Paradis, Ernesto L. Schiffrin¹³

¹Lady Davis Institute for Medical Research, and ²Department of Medicine and ³Department of Oncology, Sir Mortimer B. Davis-Jewish General Hospital, McGill University, Montréal, Québec, Canada.

*These authors contributed equally.

Li et al: Endothelin-1, atherosclerosis and aneurysms

Corresponding author:
Ernesto L. Schiffrin, CM, MD, PhD, FRSC, FRCP C
Sir Mortimer B. Davis-Jewish General Hospital, #B-127,
3755 Côte-Ste-Catherine Rd.,
Montreal, PQ, Canada H3T 1E2
Fax: 514-340-7539
Ph: 514-340-7538
E-mail: ernesto.schiffrin@mcgill.ca
http://ladydavis.ca/en/ernestoschiffrin
**Table I.** Body and organ weights of animals

<table>
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<tr>
<th>Description</th>
<th>WT</th>
<th>HFD</th>
<th>eET-1</th>
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<th>HFD</th>
<th>Ctrl</th>
<th>eET-1/Apo&lt;sup&gt;e&lt;/sup&gt;/&lt;sup&gt;e&lt;/sup&gt;</th>
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<tr>
<td>Diet</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>n</td>
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<td>8</td>
<td>15</td>
<td>10</td>
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<td>7</td>
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<tr>
<td>BW (g)</td>
<td>30.3 ± 0.9</td>
<td>27.9 ± 0.6§</td>
<td>30.1 ± 1.1</td>
<td>27.4 ± 0.7§</td>
<td>30.8 ± 0.6</td>
<td>24.4 ± 1.2**,††,§§</td>
<td>30.0 ± 0.6</td>
</tr>
<tr>
<td>TL (mm)</td>
<td>18.2 ± 0.2</td>
<td>18.0 ± 0.2</td>
<td>17.8 ± 0.1</td>
<td>17.5 ± 0.2</td>
<td>17.8 ± 0.1</td>
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<tr>
<td>HW (mg)</td>
<td>129 ± 4</td>
<td>117 ± 3§</td>
<td>137 ± 5</td>
<td>121 ± 4§</td>
<td>138 ± 4</td>
<td>103 ± 2*,††,§§</td>
<td>155 ± 13*</td>
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<td>HW/TL (mg/mm)</td>
<td>7.1 ± 0.2</td>
<td>6.5 ± 0.1</td>
<td>7.7 ± 0.3</td>
<td>7.0 ± 0.2</td>
<td>7.7 ± 0.2</td>
<td>5.9 ± 0.1*,††,§§</td>
<td>8.5 ± 0.8*</td>
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<tr>
<td>KW (mg)</td>
<td>368 ± 11</td>
<td>366 ± 8</td>
<td>365 ± 15</td>
<td>353 ± 9</td>
<td>396 ± 13</td>
<td>269 ± 11**,††,§§</td>
<td>404 ± 18</td>
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<tr>
<td>KW/TL (mg/mm)</td>
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<td>20.4 ± 0.4</td>
<td>20.5 ± 0.9</td>
<td>20.5 ± 0.5</td>
<td>22.2 ± 0.7</td>
<td>15.4 ± 0.6**,††,§§</td>
<td>22.3 ± 1.1</td>
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<tr>
<td>SW (mg)</td>
<td>78 ± 12</td>
<td>85 ± 5</td>
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<td>92 ± 6§</td>
<td>98 ± 9</td>
<td>104 ± 5</td>
<td>107 ± 11</td>
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<tr>
<td>SW/TL (mg/mm)</td>
<td>4.3 ± 0.6</td>
<td>4.7 ± 0.2</td>
<td>3.8 ± 0.4</td>
<td>5.3 ± 0.4§</td>
<td>5.5 ± 0.5</td>
<td>6.0 ± 0.3*</td>
<td>5.9 ± 0.6</td>
</tr>
</tbody>
</table>

Body and organ weights were determined in 16-week old wild-type (WT), eET-1, Apoe<sup>e</sup>/<sup>e</sup> and eET-1/Apo<sup>e</sup>/<sup>e</sup> mice fed a regular chow (Ctrl) or a high-fat diet (HFD) for 8 weeks starting at 8 weeks of age. Values are means ± SEM, *P<0.05 and **P<0.01 vs. WT, †P<0.05 and ††P<0.01 vs. eET-1, ‡P<0.05 and ‡‡P<0.01 vs. Apoe<sup>e</sup>/<sup>e</sup>, $P<0.05 and §§P<0.01 vs. Ctrl. BW, body weight; TL, tibia length; HW, heart weight; KW, kidney weight; SW, spleen weight.
**Table II. Antibodies for flow cytometry profiling of T cells**

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Description</th>
<th>Clone, company</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3</td>
<td>A700-conjugated rat anti-mouse CD3 antibody</td>
<td>17A2, eBioscience</td>
</tr>
<tr>
<td>CD3 isotype</td>
<td>A700-conjugated rat IgG2b κ isotype control antibody</td>
<td>eB149/10H5, eBioscience</td>
</tr>
<tr>
<td>CD4</td>
<td>PerCP-e710-conjugated rat anti-mouse CD4 antibody</td>
<td>RM4-5, eBioscience</td>
</tr>
<tr>
<td>CD4 isotype</td>
<td>PerCP-e710-conjugated rat IgG2a κ isotype control antibody</td>
<td>eBR2a, eBioscience</td>
</tr>
<tr>
<td>CD8a</td>
<td>APC-e780-conjugated rat anti-mouse-CD8a antibody</td>
<td>53-6.7, eBioscience</td>
</tr>
<tr>
<td>CD8a isotype</td>
<td>APC-e780-conjugated rat IgG2a κ isotype control antibody</td>
<td>eBR2a, eBioscience</td>
</tr>
<tr>
<td>CD25</td>
<td>e450-conjugated rat anti-mouse-CD25 antibody</td>
<td>PC61.5, eBioscience</td>
</tr>
<tr>
<td>CD25 isotype</td>
<td>e450-conjugated rat IgG1 κ isotype control antibody</td>
<td>eBRG1, eBioscience</td>
</tr>
<tr>
<td>FOXP3</td>
<td>APC-conjugated rat anti-mouse-FOXP3 antibody</td>
<td>FJK-16s, eBioscience</td>
</tr>
<tr>
<td>FOXP3 isotype</td>
<td>APC-conjugated rat IgG2a κ isotype control antibody</td>
<td>eBR2a, eBioscience</td>
</tr>
<tr>
<td>CD69</td>
<td>PE-conjugated hamster anti-mouse CD69 antibody</td>
<td>H1.2F3, BD Biosciences</td>
</tr>
<tr>
<td>CD69 isotype</td>
<td>PE-conjugated hamster IgG1 2,1 isotype control antibody</td>
<td>G235-2356, BD Biosciences</td>
</tr>
</tbody>
</table>

A700, Alexa Fluor®, 700, APC, allophycocyanin, APC-e780, APC-eFluor® 780, e450, eFluor® 450, FOXP3, transcription factor X-linked forkhead/winged helix, PE, phycoerythrin, PerCP-e710, PerCP-eFluor® 710.
<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Description</th>
<th>Clone, company</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD90</td>
<td>PE-conjugated rat anti-mouse CD90.2 (Thy-1.2) antibody</td>
<td>53-2.1, eBioscience</td>
</tr>
<tr>
<td>CD90 isotype</td>
<td>PE-conjugated rat IgG2a κ isotype control antibody</td>
<td>eBR2a, eBioscience</td>
</tr>
<tr>
<td>B220</td>
<td>PE-conjugated rat anti-human/mouse CD45R (B220) antibody</td>
<td>RA3-6B2, eBioscience</td>
</tr>
<tr>
<td>B220 isotype</td>
<td>PE-conjugated rat IgG2a κ isotype control antibody</td>
<td>eBR2a, eBioscience</td>
</tr>
<tr>
<td>CD49b</td>
<td>PE-conjugated anti-mouse CD49b (Integrin alpha 2) antibody</td>
<td>DX5, eBioscience</td>
</tr>
<tr>
<td>CD49b isotype</td>
<td>PE-conjugated rat IgG2M κ isotype control antibody</td>
<td>eBRM, eBioscience</td>
</tr>
<tr>
<td>NK1.1</td>
<td>PE-conjugated mouse anti-mouse NK1.1 antibody</td>
<td>PK136, BD Biosciences</td>
</tr>
<tr>
<td>NK1.1 isotype</td>
<td>PE-conjugated mouse IgG2a κ isotype control antibody</td>
<td>G155-178, BD Biosciences</td>
</tr>
<tr>
<td>Ly-6G</td>
<td>PE-conjugated rat anti-Ly-6G (Gr-1) antibody</td>
<td>RB6-8C5, eBioscience</td>
</tr>
<tr>
<td>Ly-6G isotype</td>
<td>PE-conjugated rat IgG2b κ isotype control antibody</td>
<td>eB149/10H5, eBiosciences</td>
</tr>
<tr>
<td>CD11b</td>
<td>APC-conjugated rat anti-mouse CD11b antibody</td>
<td>M1/70, eBioscience</td>
</tr>
<tr>
<td>CD11b isotype</td>
<td>APC-conjugated rat IgG2b κ Isotype control</td>
<td>eB149/10H5, eBioscience</td>
</tr>
<tr>
<td>Ly-6C</td>
<td>eFluor® 450 conjugated anti-mouse Ly-6C antibody</td>
<td>HK1.4, eBioscience</td>
</tr>
<tr>
<td>Ly-6C isotype</td>
<td>Not available</td>
<td>eBioscience</td>
</tr>
</tbody>
</table>

APC, allophycocyanin, PE, phycoerythrin. No antibody was used in replacement of Ly-6C isotype control antibody since this latter was not available.
Figure I. Alignment of the human and mouse preproendothelin-1 amino acid sequence. Human and mouse amino acid sequences were obtained from UCSC Genome Bioinformatics web site (http://www.genome.ucsc.edu/) and the sequences were compared using the multiple sequence alignment software ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/#). An asterisk (*) indicates positions which have a single, fully conserved residue. A colon (:) indicates conservation between groups of strongly similar properties with a scoring > 0.5 in the Gonnet PAM 250 matrix. A period (.) indicates conservation between groups of weakly similar properties with a scoring ≤ 0.5 in the Gonnet PAM 250 matrix.
**Figure II.** Heart rate was determined in 16-week old wild-type (WT), eET-1, Apoe<sup>−/−</sup> and eET1/Apoe<sup>−/−</sup> mice fed a high-fat diet (HFD) or regular chow (Ctrl) for 8 weeks starting at age 8 weeks. Values are means ± SEM, n = 6 for WT, 9 for eET-1, 9 for Apoe<sup>−/−</sup> and 9 for eET1/Apoe<sup>−/−</sup> mice fed regular chow, and 6 for WT, 8 for eET-1, 10 for Apoe<sup>−/−</sup> and 6 for eET1/Apoe<sup>−/−</sup> mice fed HFD.
Figure III. Plasma triglyceride levels were determined in 16-week old wild-type (WT), eET-1, Apoe\textsuperscript{-/-} and eET-1/Apoe\textsuperscript{-/-} mice fed a regular chow (Ctrl) or a high-fat diet (HFD) for 8 weeks starting at 8 weeks of age. Values are means ± SEM, n = 7 for WT, 7 for eET-1, 9 for Apoe\textsuperscript{-/-} and 8 for eET1/Apoe\textsuperscript{-/-} mice fed a regular chow, and 9 for WT, 12 for eET-1, 8 for Apoe\textsuperscript{-/-} and 11 for eET1/Apoe\textsuperscript{-/-} mice fed a HFD. §P<0.05 and §§P<0.001 vs. Ctrl.
Figure IV. Representative images of oil red O-stained and Mayer’s hematoxylin counterstained aortic sinus of wild-type (WT) and eET-1 mice.
Figure V. Collagen content is decreased to the same extent in Apoe<sup>−/−</sup> and eET-1/ Apoe<sup>−/−</sup> mice. Collagen content was determined in the media of abdominal suprarenal aorta by Sirius red staining in 16-week old wild-type (WT), eET-1, Apoe<sup>−/−</sup> and eET-1/Apoe<sup>−/−</sup> mice fed a high-fat diet (HFD) for 8 weeks starting at 8 weeks of age. Representative RGB thresholded images of Sirius red-stained sections of abdominal suprarenal aorta. Collagen was quantified as the % of red staining contained in the aortic media in the RGB thresholded images. Values are means ± SEM, n = 4 for WT, 3 for eET-1, 5 for Apoe<sup>−/−</sup> and 6 for eET1/Apoe<sup>−/−</sup> mice. *P<0.05 and **P<0.01 vs. WT and †P<0.05 vs. eET-1.
Figure VI. Collagen content was determined in atherosclerosis plaques of aortic arch of 16-week old Apoe<sup>−/−</sup> and eET-1/Apo<sup>−/−</sup> mice fed a high-fat diet (HFD) for 8 weeks starting at 8 weeks of age. Representative bright field and RGB thresholded images of Sirius red stained sections are shown. Collagen is visualized as the red staining contained in the aortic media and the plaques in the RGB thresholded images. n = 7 for Apoe<sup>−/−</sup> and 8 for eET-1/Apo<sup>−/−</sup> mice.
Figure VII. ET-1 overexpression did not alter plasma cytokine levels in Apoε⁻/⁻ mice. Plasma levels of tumor necrosis factor (TNF)-α (A), interleukin (IL)-6 (B), monocyte chemotactic protein-1 (MCP-1, C) and IL-10 (D) were measured in 16-week old wild-type (WT), eET-1, Apoε⁻/⁻ and eET1/Apoε⁻/⁻ mice fed a high-fat diet for 8 weeks. Values are means ± SEM, n = 7 for WT, 8 for eET-1, 9 for Apoε⁻/⁻ and 12 for eET1/Apoε⁻/⁻ mice. *P<0.01 vs. WT and †P<0.01 vs. eET-1.
Figure VIII. Flow cytometry profiling of splenic T cells. The profile of CD3\(^+\), CD4\(^+\), CD8\(^+\), CD4\(^+\)CD25\(^+\)FOXP3\(^+\), CD4\(^+\)CD69\(^+\) and CD8\(^+\)CD69\(^+\) cells were determined by flow cytometry in the spleen of 16-week old wild-type (WT), eET-1, Apoe\(^{-/-}\) and eET1/Apoe\(^{-/-}\) mice fed a high-fat diet for 8 weeks. Splenocytes were stained with Fixable Viability Dye eFluor® 506 (e506), Alexa Fluor® 700 (A700)-conjugated anti-mouse CD3, PerCP-eFluor® 710 (PerCP-e710)-conjugated anti-mouse CD4, allophycocyanin (APC)-eFluor® 780 (APC-e780)-conjugated anti-mouse CD8a, eFluor® 450 (e450)-conjugated anti-mouse CD25, phycoerythrin (PE)-conjugated hamster anti-mouse CD69 and APC-conjugated anti-mouse Foxp3 antibodies, and analyzed by flow cytometry. Fluorophores were respectively excited and analyzed with appropriate laser and bandpass filter (BP) (e450: 405 nm with 450/50 BP, e506: 405 nm with 525/50 BP, A700: 640 nm with 730/45 BP, PerCP-eFluor® 710: 488 nm with 695/40 BP, APC-e780: 640 nm with 780/60 BP, APC: 640 nm with 670/14 BP, PE: 561 nm with 582/15 BP). Representative Flow
cytometry profile of splenocytes (A-H) with the gating strategy (I) and the % of cells are showed for an eET-1/Apoel-/- mouse. A. Lymphocytes were gated in the side scatter (SSC)/forward scatter (FSC) plot. B. Singlet lymphocytes were gated using FSC height (FSC-H) over FSC area (FSC-A). C. Live lymphocytes were gated in the viability dye/FSC-A plot. D. CD3+ cells were gated in the CD3/SSC-A plot. E. CD3+CD4+ and CD3+CD8+ were gated in the CD8/CD4 plot from the CD3+ cells population. F. Gated CD3+CD4+ cells were further characterized for CD25 and FOXP3 expression in the CD25/FOXP3 plot. Gated CD3+CD4+ and CD3+CD8+ cells were further examined for CD69 expression in CD4/CD69 (G) and CD8/CD69 (H) plots, respectively. The % CD3+ cells in lymphocytes (J), % of CD4+ (K) and CD8+ (L) cells in CD3+ cells, % of CD4+CD25+FOXP3+ cells in CD4+ cells (M), % of CD4+CD69+ cells in CD4+ cells (N) and CD8+CD69+ cells in CD8+ cells (O) are presented. Values are means ± SEM, n = 3 for WT, 4 for eET-1, 5 for Apoel-/- and 7 for eET1/Apoel-/- mice. *P<0.05 and **P<0.001 vs. WT and †P<0.05 and ††P<0.001 vs. eET-1.
**Figure IX.** Flow cytometry profiling of splenic monocytes. The profile of monocytes and monocyte subset Ly-6C$^{hi}$ in the spleen of 16-week old wild-type (WT), eET-1, Apoe$^{-/-}$ and eET1/Apoe$^{-/-}$ mice fed a high-fat diet for 8 weeks was determined by flow cytometry as previously described. Splenocytes were stained with Fixable Viability Dye eFluor® 506 (e506), phycoerythrin (PE)-conjugated rat anti-mouse CD90.2 (T cells), PE-conjugated rat human/mouse B220 (B cells), PE-conjugated anti-mouse CD49b (NK cells), PE-conjugated mouse anti-mouse NK1.1 (NK cells), PE-conjugated rat anti-Ly-6G (granulocytes), allophycocyanin (APC)-conjugated rat anti-mouse CD11b (myeloid cells) and eFluor® 450 conjugated anti-mouse Ly-6C (monocyte subsets) antibodies, and analyzed by flow cytometry. Fluorophores were respectively excited and analyzed with appropriate laser and bandpass filter (BP) (PE: 561 nm with 582/15 BP, APC-e780: 640 nm with 780/60 BP, e450: 405 nm with 450/50 BP, e506: 405 nm with 525/50 BP). As presented in Fig. S3, lymphocytes were gated in the side scatter (SSC)/forward scatter (FSC) plot, singlet lymphocytes were gated using FSC height (FSC-H) over FSC area (FSC-A) and live lymphocytes were gated in the viability dye/FSC-A plot. Monocytes (CD11b$^{hi}$CD90$^{lo}$B220$^{lo}$CD49b$^{lo}$NK1-1$^{lo}$Ly-6G$^{lo}$) were gated in CD90 B220 CD49b NK1-1 Ly-6G/CD11b plot (A), which was further divided into Ly-6C$^{lo}$ (B and D) and Ly-6C$^{hi}$ subsets (B and E). Representative dot plots (A) and histograms (B) for WT, eET-1, Apoe$^{-/-}$ and...
eET1/Apoε-/- mice fed a HFD are shown. Values are means ± SEM, n = 9 for WT, 5 for eET-1, 5 for Apoe-/- and 9 for eET1/Apoε-/- mice. *P<0.05 and **P<0.001 vs. WT, †P<0.05 and ††P<0.001 vs. eET-1 and ‡P<0.05 vs. Apoe-/- mice.
Figure X. ET-1 overexpression increased MMP2 expression in aortic atherosclerotic plaques of Apoe^-/- mice. MMP2 expression was determined by immunofluorescence in ascending aortic sections of 16-week old Apoe^-/- and eET1/Apoe^-/- mice fed a high-fat diet for 8 weeks. Representative images of MMP2-stained ascending aortic sections of Apoe^-/- and eET1/Apoe^-/- mice with quantification of relative fluorescence unit (RFU)/µm² of atherosclerotic plaque are presented. Blue, green and red fluorescence represent nuclear DAPI staining, autofluorescence of elastin and MMP2 staining, respectively. Values are means ± SEM, n = 5. ‡P<0.05 vs. Apoe^-/-.