

# Endothelium-restricted endothelin-1 overexpression in type 1 diabetes worsens atherosclerosis and immune cell infiltration via NOX1

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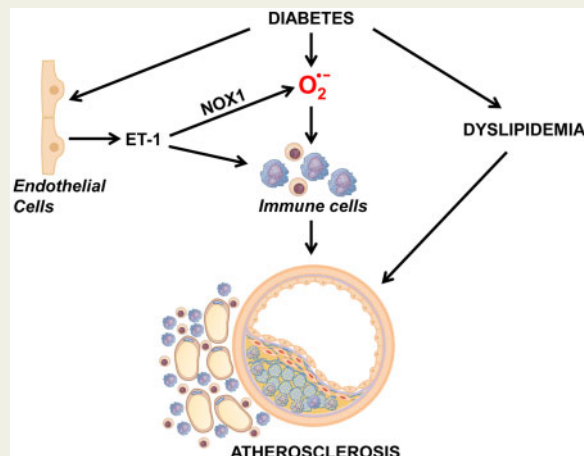
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**Aims** NADPH oxidase (NOX) 1 but not NOX4-dependent oxidative stress plays a role in diabetic vascular disease, including atherosclerosis. Endothelin (ET)-1 has been implicated in diabetes-induced vascular complications. We showed that crossing mice overexpressing human ET-1 selectively in endothelium (eET-1) with apolipoprotein E knockout (*Apoe*<sup>-/-</sup>) mice enhanced high-fat diet-induced atherosclerosis in part by increasing oxidative stress. We tested the hypothesis that ET-1 overexpression in the endothelium would worsen atherosclerosis in type 1 diabetes through a mechanism involving NOX1 but not NOX4.

**Methods and results** Six-week-old male *Apoe*<sup>-/-</sup> and eET-1/*Apoe*<sup>-/-</sup> mice with or without *Nox1* (*Nox1*<sup>-/-</sup>) or *Nox4* knockout (*Nox4*<sup>-/-</sup>) were injected intraperitoneally with either vehicle or streptozotocin (55 mg/kg/day) for 5 days to induce type 1 diabetes and were studied 14 weeks later. ET-1 overexpression increased 2.5-fold and five-fold the atherosclerotic lesion area in the aortic sinus and arch of diabetic *Apoe*<sup>-/-</sup> mice, respectively. Deletion of *Nox1* reduced aortic arch plaque size by 60%; in contrast, *Nox4* knockout increased lesion size by 1.5-fold. ET-1 overexpression decreased aortic sinus and arch plaque alpha smooth muscle cell content by ~35% and ~50%, respectively, which was blunted by *Nox1* but not *Nox4* knockout. Reactive oxygen species production was increased two-fold in aortic arch perivascular fat of diabetic eET-1/*Apoe*<sup>-/-</sup> and eET-1/*Apoe*<sup>-/-</sup>/*Nox4*<sup>-/-</sup> mice but not eET-1/*Apoe*<sup>-/-</sup>/*Nox1*<sup>-/-</sup> mice. ET-1 overexpression enhanced monocyte/macrophage and CD3<sup>+</sup> T-cell infiltration ~2.7-fold in the aortic arch perivascular fat of diabetic *Apoe*<sup>-/-</sup> mice. Both *Nox1* and *Nox4* knockout blunted CD3<sup>+</sup> T-cell infiltration whereas only *Nox1* knockout prevented the monocyte/macrophage infiltration in diabetic eET-1/*Apoe*<sup>-/-</sup> mice.

**Conclusion** Endothelium ET-1 overexpression enhances the progression of atherosclerosis in type 1 diabetes, perivascular oxidative stress, and inflammation through NOX1.

## Graphical Abstract



## Keywords

Atherosclerosis • Diabetes • Endothelin-1 • NADPH oxidases

## 1. Introduction

The major cause of mortality and disability in patients with diabetes mellitus is vascular disease. This includes macrovascular complications such as atherosclerosis and microvascular complications such as diabetic nephropathy.<sup>1</sup> Although the mechanism linking diabetes and vascular disease is complex, overproduction of reactive oxygen species (ROS) seems to play an important role.<sup>2</sup> The major source of ROS in the vasculature is the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase family of enzymes. Four isoforms of NADPH oxidase (NOX) have been described in the vasculature and each is characterized by its catalytic subunit: NOX1, NOX2, NOX4, and NOX5. Unlike NOX2, NOX1, and NOX4 do not participate in pathogen clearance and may therefore represent safer pharmacological targets in the prevention of diabetes-related vascular disease. NOX5 is not present in rodents.<sup>3</sup>

Several groups have investigated the role of NOX isoforms in diabetic cardiovascular disease. Using the murine model of streptozotocin (STZ)-induced type 1 diabetes, Youn *et al.*<sup>4</sup> found that diabetes-associated endothelial dysfunction, endothelial nitric oxide synthase uncoupling, and vascular ROS production was blunted in NOX1-deficient mice. More recently, deletion of *Nox1* has been shown to markedly reduce aortic atherosclerosis, ROS production, and inflammation in diabetes induced with STZ in apolipoprotein E knockout (*Apoe*<sup>-/-</sup>) mice.<sup>5</sup> In contrast to NOX1, the role of NOX4 in vascular disease is more controversial. Deletion of *Nox4* in rodent models of diet-induced atherosclerosis caused endothelial dysfunction and/or worsened atherosclerosis in the aorta.<sup>6-8</sup> Deletion of *Nox4* increased aortic atherosclerosis after 20 weeks of diabetes mellitus,<sup>9</sup> whereas it had no effect on plaque formation after 10 weeks.<sup>5</sup> Yet, deletion of *Nox4* prevented diabetic nephropathy in STZ-treated *Apoe*<sup>-/-</sup> mice<sup>10</sup> and flagellin-induced atherosclerosis in *Apoe*<sup>-/-</sup> mice fed a high-fat diet (HFD).<sup>11</sup>

Endothelin (ET)-1 is a proinflammatory and potent vasoconstrictor peptide produced largely by endothelial cells.<sup>12</sup> Plasma ET-1 levels are

increased in patients with diabetes mellitus<sup>13-15</sup> and correlate with severity of atherosclerosis in humans.<sup>16</sup> In the vasculature, ET-1 stimulates proliferation, fibrosis, and inflammation through pro-oxidant pathways.<sup>17</sup> ET-1 was shown to induce expression of NOX2 in human umbilical vein endothelial cells<sup>18</sup> and NOX1, NOX2, and NOX4 in rat mesenteric artery smooth muscle cells (SMCs).<sup>19</sup> Transgenic mice with endothelium-restricted human ET-1 overexpression (eET-1) present mesenteric artery endothelial dysfunction and vascular remodeling that are associated with increased NOX activity and gp91Phox (NOX2) protein levels.<sup>20</sup> Likewise, ET-1 overexpression worsened mesenteric artery endothelial dysfunction and oxidative stress in STZ-treated mice.<sup>21</sup> ET type A (ET<sub>A</sub>) receptor blockade with avosentan reduced aortic atherosclerosis and nephropathy in diabetic STZ-treated *Apoe*<sup>-/-</sup> mice.<sup>22</sup> Furthermore, human ET-1 overexpression worsened HFD-induced atherosclerosis and was associated with increased oxidative stress in *Apoe*<sup>-/-</sup> mice.<sup>23</sup> However, it remains unknown whether human ET-1 overexpression enhances atherosclerosis in diabetes through NOX1 but not NOX4.

We hypothesized that endothelium-restricted ET-1 overexpression would worsen atherosclerosis in diabetes through a mechanism involving NOX1 but not NOX4. To test this hypothesis, we first crossed eET-1 with *Apoe*<sup>-/-</sup> mice and evaluated whether ET-1 overexpression accelerated the progression of atherosclerosis in STZ-induced diabetes. Thereafter, we evaluated whether this enhancement of atherosclerosis would be prevented in eET-1/*Apoe*<sup>-/-</sup> deficient in *Nox1* but not *Nox4*. We also evaluated aortic perivascular oxidative stress and inflammation, as these processes are known to contribute to vascular damage and atherosclerosis.

## 2. Methods

Expanded Methods are provided in the [Supplementary material online](#).

## 2.1 Experimental 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.<sup>24</sup> C57BL/6 *Apoe*<sup>-/-</sup> mice were obtained from Jackson Laboratory (B6.129P2-*Apoe*<sup>tm1Unc</sup>/J, Bar Harbor, ME, USA). *Apoe*<sup>-/-</sup>/*Nox1*<sup>-/-</sup> and *Apoe*<sup>-/-</sup>/*Nox4*<sup>-/-</sup> mice were generated by backcrossing *Nox1*<sup>-/-</sup> and *Nox4*<sup>-/-</sup> mice with *Apoe*<sup>-/-</sup>/*Nox4*<sup>-/-</sup> mice (Animal Resources Centre, Canning Vale, WA, Australia) at the Baker IDI Heart & Diabetes Research Institute (Melbourne, Australia).<sup>5</sup> *Nox1*<sup>-/-</sup> and *Nox4*<sup>-/-</sup> mice have been described previously.<sup>25,26</sup> The eET-1/*Apoe*<sup>-/-</sup> mice were generated at the Lady Davis Institute for Medical Research (Montreal, QC, Canada) by crossing eET-1 mice with *Apoe*<sup>-/-</sup> mice obtained from Jackson Laboratories (Bar Harbor, ME, USA). eET-1/*Apoe*<sup>-/-</sup>/*Nox1*<sup>-/-</sup> and eET-1/*Apoe*<sup>-/-</sup>/*Nox4*<sup>-/-</sup> mice were generated at the Lady Davis Institute for Medical Research by crossing eET-1/*Apoe*<sup>-/-</sup> mice with *Apoe*<sup>-/-</sup>/*Nox1*<sup>-/-</sup> and *Apoe*<sup>-/-</sup>/*Nox4*<sup>-/-</sup> mice, respectively.

Six-week-old male *Apoe*<sup>-/-</sup>, *Apoe*<sup>-/-</sup>/*Nox1*<sup>-/-</sup>, *Apoe*<sup>-/-</sup>/*Nox4*<sup>-/-</sup>, eET-1/*Apoe*<sup>-/-</sup>, eET-1/*Apoe*<sup>-/-</sup>/*Nox1*<sup>-/-</sup>, and eET-1/*Apoe*<sup>-/-</sup>/*Nox4*<sup>-/-</sup> mice were injected IP with either vehicle (sodium citrate buffer) or with STZ (55 mg/kg/day) for five consecutive days to induce type 1 diabetes mellitus.<sup>5</sup> Ten days after the last injection of STZ, only mice presenting a blood glucose level  $\geq 15$  mmol/L after 4–6 h of fasting time were considered diabetic and included in the study. Mice were studied 14 weeks after the last IP injection of either vehicle or STZ. At the end of the protocol, blood glucose level was evaluated after 4–6 h of fasting. The following day, mice were euthanized by anaesthesia followed by blood and tissue collection as follows. Mice were weighed and anaesthetized with 3% isoflurane mixed with O<sub>2</sub> at 1 L/min and the depth of anaesthesia was confirmed by rear foot squeezing. Blood was collected by cardiac puncture for plasma ET-1 determination and measurement of plasma cholesterol, high-density lipoprotein (HDL) and triglycerides. Tissues were harvested in ice-cold phosphate buffered saline and weighed, and tibia length was determined. The base of the heart (which comprises the aortic sinus) and the aortic arch with perivascular adipose tissue (PVAT) were dissected and embedded in VWR Clear Frozen Section Compound (VWR international, Edmonton, AL, Canada) for characterization of atherosclerotic plaque, ROS production, alpha-smooth muscle actin ( $\alpha$ -SMA) expression, collagen content, buried fibrous caps, and monocyte/macrophage and CD3<sup>+</sup> T-cell infiltration. The remaining tissues were frozen in liquid nitrogen and stored at -80°C until used.

## 2.2 Data analysis

Results are presented as means  $\pm$  SEM. Data were compared with one-way analysis of variance (ANOVA) followed by a Student–Newman–Keuls *post hoc* test. In absence of normal distribution, a Kruskal–Wallis one-way ANOVA followed by a Dunn's multiple comparison test was used to compare data.  $P < 0.05$  was considered statistically significant.

## 3. Results

### 3.1 Fasting glycaemia, plasma ET-1, and lipaemia

STZ treatment induced type 1 diabetes efficiently in all the groups. Fasting glycaemia was increased three-fold in diabetic *Apoe*<sup>-/-</sup> mice, which was unaffected by ET-1 overexpression, *Nox1* or *Nox4* knockout (Supplementary material online, Figure S1). eET-1/*Apoe*<sup>-/-</sup> mice

presented an eight-fold higher plasma ET-1 level compared to *Apoe*<sup>-/-</sup> mice (Supplementary material online, Figure S1). Plasma ET-1 was unaffected by diabetes and *Nox1* or *Nox4* knockout in *Apoe*<sup>-/-</sup> mice. Plasma ET-1 tended to be decreased in diabetic eET-1/*Apoe*<sup>-/-</sup> mice, but remained five-fold higher, which was unaffected by *Nox1* or *Nox4* knockout. Plasma cholesterol levels were increased to a similar extent in diabetic *Apoe*<sup>-/-</sup> and eET-1/*Apoe*<sup>-/-</sup> mice, which was unaffected by *Nox1* or *Nox4* knockout (Supplementary material online, Figure S2). Diabetes tended to increase plasma HDL levels in *Apoe*<sup>-/-</sup> but not eET-1/*Apoe*<sup>-/-</sup> mice, which was unaffected by *Nox1* or *Nox4* knockout. Plasma triglycerides were increased three-fold in diabetic *Apoe*<sup>-/-</sup> mice and 1.7-fold in diabetic eET-1/*Apoe*<sup>-/-</sup> mice, which was blunted by *Nox1* and *Nox4* knockout in diabetic *Apoe*<sup>-/-</sup> mice and tended to be decreased in diabetic eET-1/*Apoe*<sup>-/-</sup> mice (Supplementary material online, Figure S2).

### 3.2 ET-1 overexpression worsened atherosclerosis in diabetes through NOX1

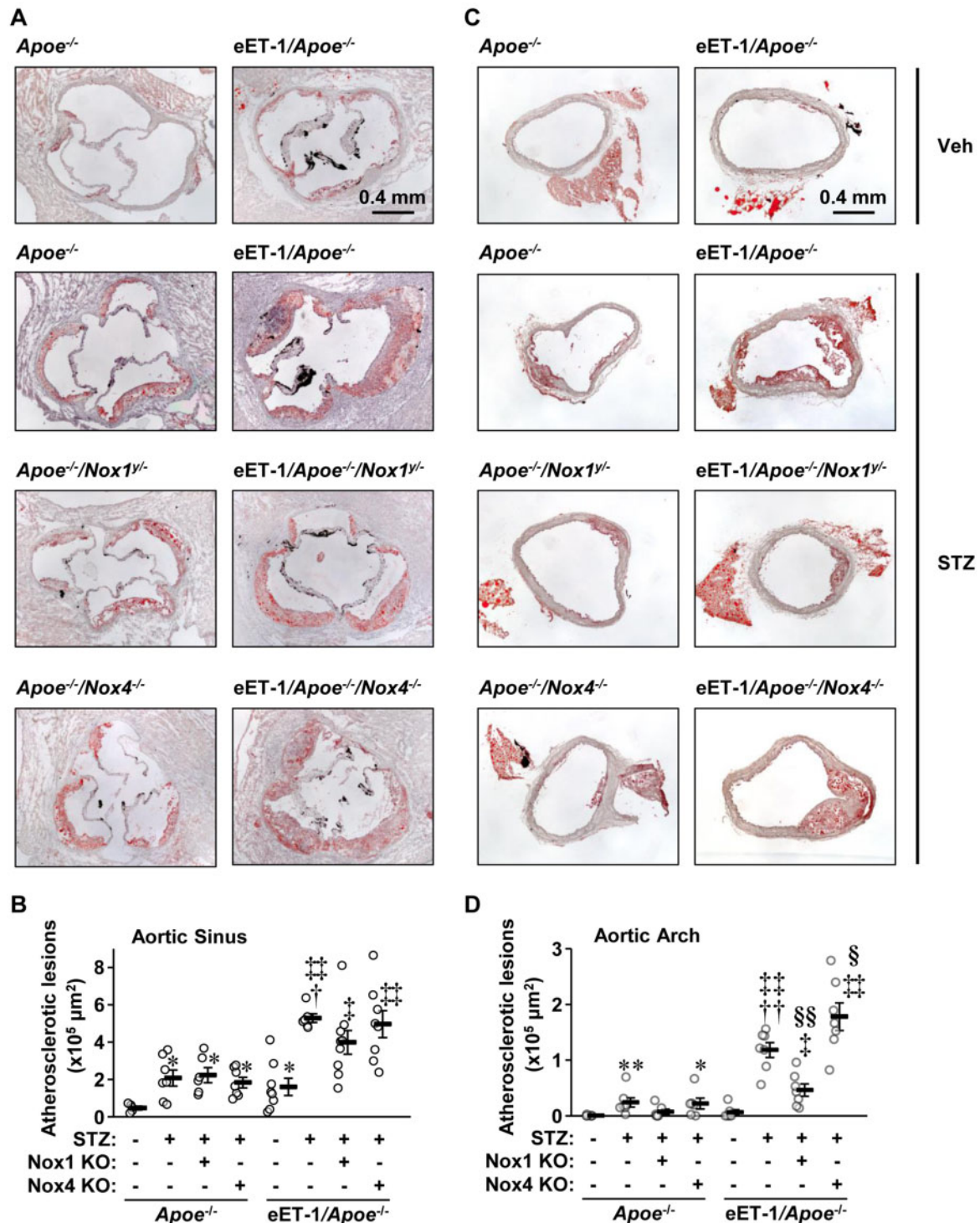
Atherosclerotic lesions were characterized in 8  $\mu$ m cryosections of aortic sinus and aortic arch obtained at 90- $\mu$ m intervals. In the aortic sinus, diabetes increased plaque size 4.4-fold in *Apoe*<sup>-/-</sup> mice, which was unaffected by *Nox1* or *Nox4* deletion (Figure 1A and B). ET-1 overexpression further enhanced plaque size 2.5-fold, which tended to be reduced by *Nox1* but not *Nox4* knockout. In the aortic arch, no plaques were detected in vehicle-treated *Apoe*<sup>-/-</sup> mice whereas they were present in diabetic *Apoe*<sup>-/-</sup> mice (Figure 1C and D). *Nox1* but not *Nox4* knockout tended to decrease diabetes-induced atherosclerosis in *Apoe*<sup>-/-</sup> mice. ET-1 overexpression augmented lesion size five-fold in diabetic *Apoe*<sup>-/-</sup> mice, which was reduced 60% by *Nox1* knockout and was further increased 1.5-fold by *Nox4* knockout.

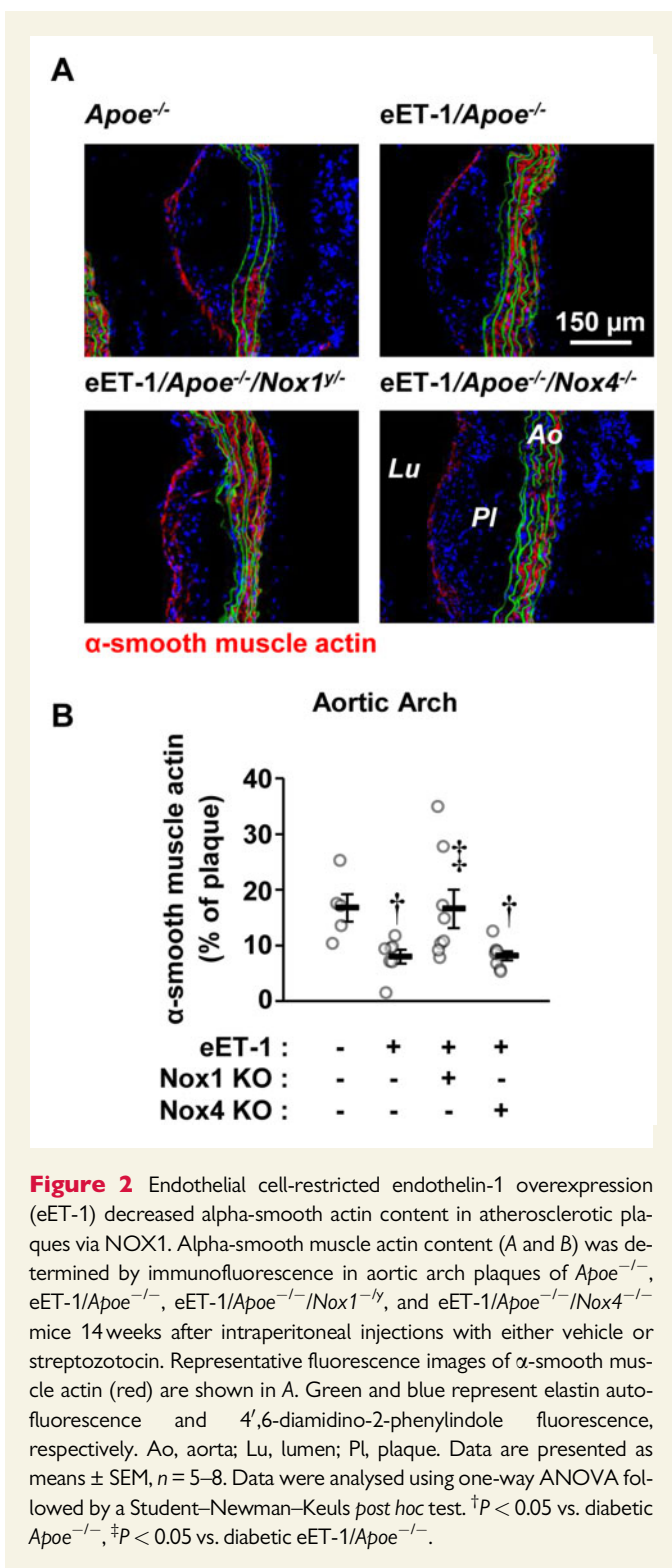
### 3.3 ET-1 overexpression decreased plaque $\alpha$ -SMA content through NOX1

$\alpha$ -SMA expression, a marker of plaque stability, was assessed in aortic sinus and arch lesions. In the aortic sinus, ET-1 overexpression decreased  $\alpha$ -SMA content per lesion area by  $\sim 35\%$ , which was blunted by *Nox1* but not *Nox4* knockout (Supplementary material online, Figure S4). *Nox1* knockout also tended to exhibit increased  $\alpha$ -SMA plaque content in diabetic *Apoe* knockout mice. In the aortic arch, ET-1 overexpression decreased  $\alpha$ -SMA content per lesion area by  $\sim 50\%$ , which was also blunted by *Nox1* but not *Nox4* knockout (Figure 2). Aortic sinus and arch plaque collagen content was unaffected by ET-1 overexpression, or lack of *Nox4* (Supplementary material online, Figures S5 and S6). However, mice deficient in *Nox1* tended to present more plaque collagen content in the aortic sinus but not the aortic arch. Buried fibrous caps that have been proposed as evidence of prior plaque rupture, tended to be more numerous in aortic sinus and arch plaques, and were unaffected by *Nox1* nor *Nox4* knockout.

### 3.4 ET-1 overexpression produced oxidative stress and PVAT immune cell infiltration through NOX1 or NOX4

Oxidative stress assessed by dihydroethidium (DHE) fluorescence in the aortic arch media and plaque was similar between all groups (Supplementary material online, Figure S3). ET-1 overexpression increased ROS production two-fold in aortic PVAT of diabetic *Apoe*<sup>-/-</sup> mice, which was blunted by *Nox1* but not *Nox4* knockout (Figure 3). *Nox1*, *Nox2*, and *Nox4* mRNA expressions were examined by reverse transcription-quantitative PCR in two highly vascularized tissues, the





unaltered by high ET-1 levels in diabetic  $Apoe^{-/-}$  mice.  $Nox1$  and  $Nox4$  mRNAs were undetectable in  $Nox1^{-/-}$  and  $Nox4^{-/-}$  mice, respectively.

ET-1 overexpression decreased by 35% monocyte/macrophage infiltration per lesion area in aortic sinus but not in aortic arch of  $Apoe^{-/-}$  mice (Supplementary material online, Figure S7). Aortic sinus and arch plaque monocyte/macrophage infiltration was unaffected by  $Nox1$  or  $Nox4$  knockout. ET-1 overexpression increased 2.7-fold aortic PVAT monocyte/macrophage infiltration in diabetic  $Apoe^{-/-}$  mice, which was prevented by  $Nox1$  but not  $Nox4$  knockout (Figure 4A and B). Diabetes tended to increase aortic PVAT CD3<sup>+</sup> T-cell infiltration in  $Apoe^{-/-}$  and  $Apoe^{-/-}/Nox4^{-/-}$  mice but not in  $Apoe^{-/-}/Nox1^{-/-}$  mice (Figure 4C and D). ET-1 overexpression increased CD3<sup>+</sup> T-cell infiltration 2.6-fold in diabetic  $Apoe^{-/-}$  mice, which was abrogated by  $Nox1$  knockout and reduced by  $Nox4$  knockout (Figure 4).

## 4. Discussion

It was previously demonstrated that the ET system plays a role in diabetes mellitus-induced development of aortic atherosclerotic plaques, oxidative stress, and inflammation in  $Apoe^{-/-}$  mice, since they were reduced by ET<sub>A</sub> receptor blockade with avosentan.<sup>22</sup> The present study demonstrates that endothelial human ET-1 overexpression worsens atherosclerosis, perivascular oxidative stress, and inflammation in diabetes, extending our previous findings to a model of type 1 diabetes mellitus.<sup>23</sup> It was also shown using gene deletion that two sources of ROS, NOX1 and NOX4, producing respectively superoxide and hydrogen peroxide, have opposite action, promoting and counteracting atherosclerotic plaque development and inflammation in diabetes mellitus.<sup>5,9</sup> In this study, we show that ET-1 overexpression induced an enhancement of atherosclerosis in type 1 diabetes that is mediated through NOX1 and worsened by the loss of NOX4, providing additional evidence that increased NOX isoforms play differential roles in atherosclerotic plaque progression.

It is well-established that diabetes mellitus increases the risk of developing atherosclerosis and other vascular complications.<sup>27</sup> Increased ET-1 expression may play a role in diabetes-associated atherosclerosis. Plasma ET-1 levels are increased in patients with diabetes<sup>13-15</sup> and correlate with severity of atherosclerosis in humans<sup>16</sup> and inhibition of the ET<sub>A</sub> receptors with avosentan reduced aortic atherosclerosis in diabetic  $Apoe^{-/-}$  mice.<sup>22</sup> To investigate the role of ET-1 overexpression in atherosclerosis in diabetes, we crossed eET-1 transgenic mice with atherosclerosis-prone  $Apoe^{-/-}$  mice and induced type 1 diabetes by administration of STZ. Consistent with our previous findings,  $eET-1/Apoe^{-/-}$  mice presented an eight-fold higher plasma ET-1 level compared to  $Apoe^{-/-}$  mice.<sup>23</sup> Plasma ET-1 levels in  $eET-1/Apoe^{-/-}$  mice were not statistically altered by diabetes induction,  $Nox1$  or  $Nox4$  knockout, suggesting that alterations in plasma ET-1 are unlikely to explain the enhanced atherosclerosis. Fasting blood glucose and total cholesterol levels were also increased to a similar extent in all diabetic mice at the end of the study groups.

In the current study, we found that endothelial ET-1 overexpression worsened atherosclerosis in diabetic mice in both the aortic arch and sinus. These findings extend our previous results showing that increased ET-1 worsens atherosclerosis caused by HFD and demonstrate that ET-1's modulation of atherogenesis is not limited to a single aetiology.<sup>23</sup> This is particularly important for application in humans, where the causes of atherosclerosis are often heterogeneous and multifactorial. However, ET-1 overexpression alone is not sufficient to consistently promote

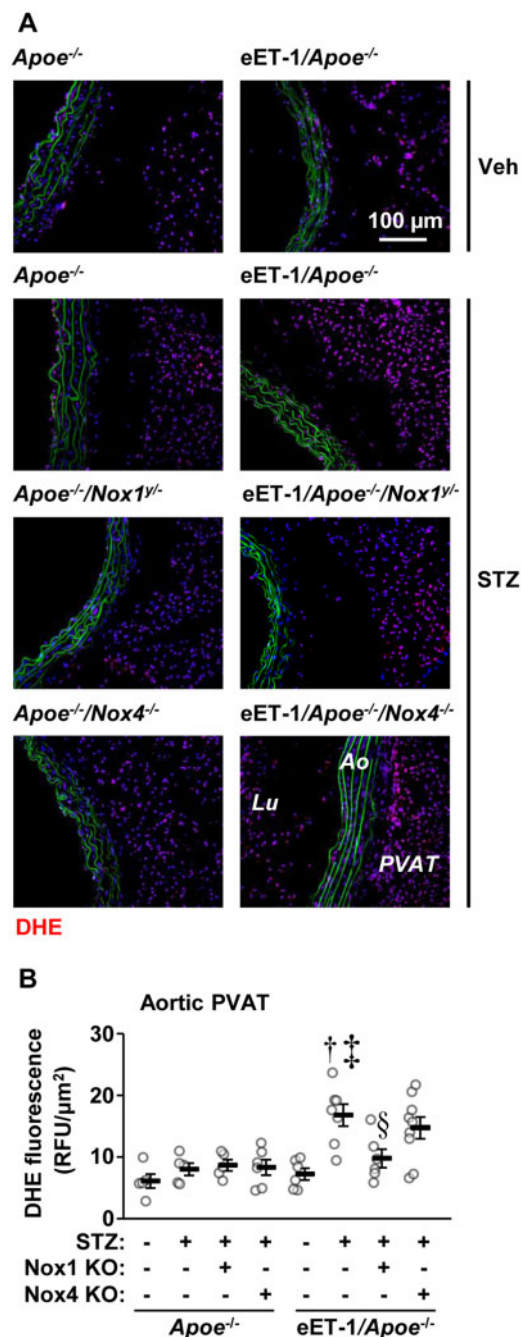
renal cortex and lung. Since the number of samples per group was less than 5 in some groups, the data were only analysed qualitatively. The results showed that the mRNA expression of  $Nox1$  and  $Nox2$  but not  $Nox4$  was increased in renal cortex and lung of diabetic  $Apoe^{-/-}$  compared to non-diabetic  $Apoe^{-/-}$  mice (Supplementary material online, Figure S8). The mRNA expression of  $Nox1$  and  $Nox2$  was not further increased by ET-1 overexpression, and  $Nox4$  mRNA expression was

atherosclerosis, as non-diabetic eET-1/*Apoe*<sup>-/-</sup> mice presented increased plaque size in the aortic sinus but not the arch. This suggests that increased ET-1 accelerates the progression of atherosclerosis in predisposed conditions such as diabetes and hypercholesterolaemia and it is therefore likely that patients with such conditions would benefit most from ET-1 blockade.

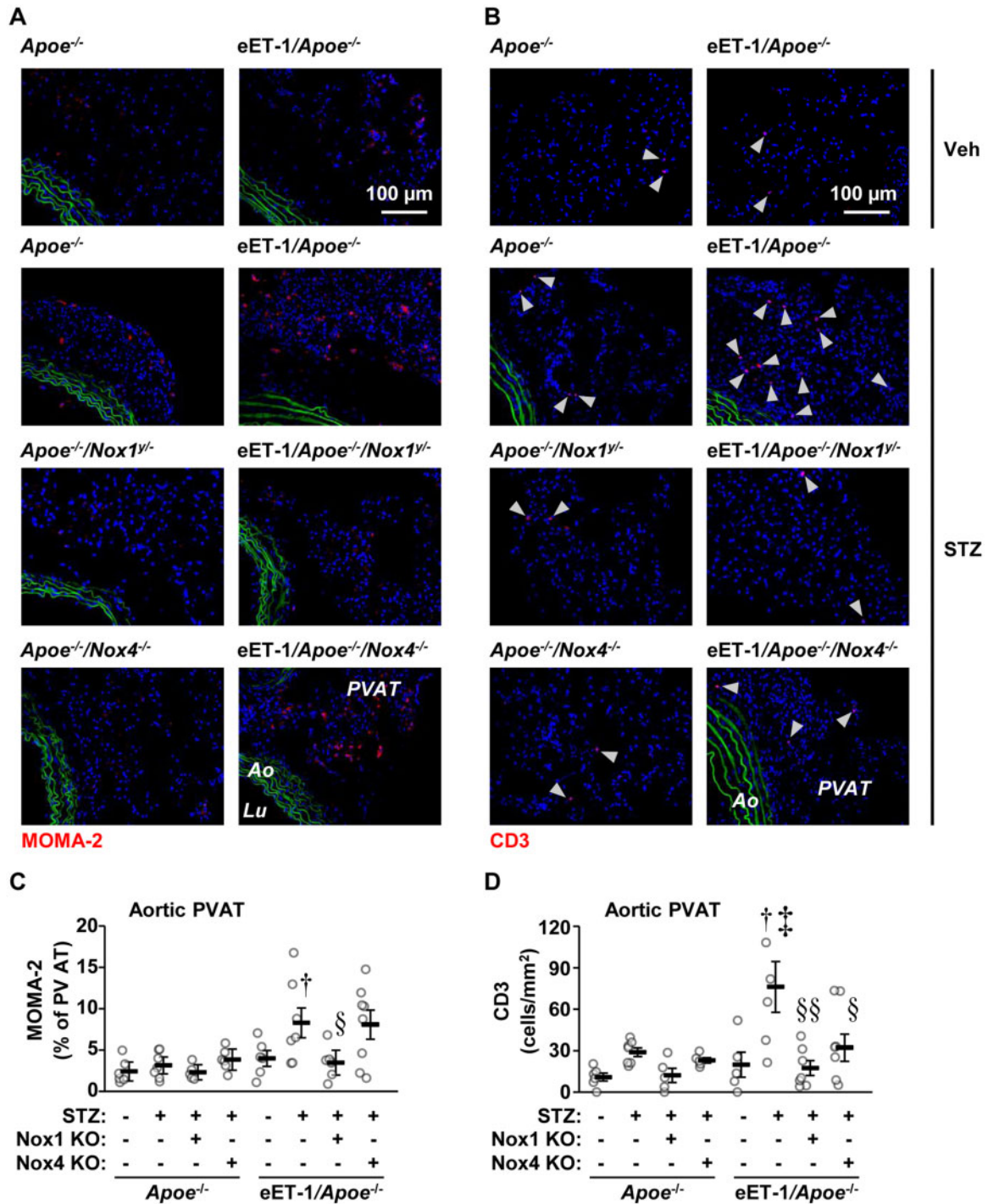
Increased ROS generation has been implicated in the development and progression of vascular complications in diabetes.<sup>2</sup> In this study, we observed that ET-1 overexpression induced enhanced progression of atherosclerosis in diabetic mice that was associated with an increase in ROS generation in aortic arch PVAT. We made a similar observation in HFD-induced atherosclerosis.<sup>23</sup> The increase in ROS generation could be a direct consequence of ET-1 overexpression. ET-1 was shown to induce NOX activity or ROS generation *in vitro* in vascular endothelial<sup>18</sup> and SMCs<sup>19,28,29</sup> and *ex vivo* in carotid arteries<sup>30</sup> and aortic rings.<sup>31</sup> One study has shown that ET-1 inhibits NOX activity in aortic endothelial cells.<sup>32</sup> Interestingly, ET-1 was revealed to mediate the action of angiotensin II in aortic SMCs. Six-hour angiotensin II stimulation-induced NOX activity and NOX1 and NOX4 protein expression were prevented by ET receptor blockers.<sup>28</sup> Furthermore, it has been reported that ET-1 increases the mRNA expression of *Nox2* (identified as gp91<sup>Phox</sup> in the publication) in endothelial cells<sup>18</sup> and protein levels of NOX1, NOX2 and NOX4 in SMCs.<sup>19</sup> In the latter study, the investigators demonstrated that knockdown of *Nox1* or *Nox2* with small interfering RNA prevented activation of NOX by 4-hour ET-1 stimulation.<sup>19</sup> However, it has not been determined whether *Nox4* knockdown affects ET-1 response. We previously showed that NOX activity and NOX2 protein levels were increased in mesenteric arteries of 10-week-old male eET-1 mice overexpressing ET-1 in endothelial cells.<sup>20</sup> Furthermore, we have observed that 14-week diabetes-induced ROS generation in mesenteric arteries was enhanced by ET-1 overexpression.<sup>21</sup> However, it has never been determined whether NOX1 or NOX4 plays a role in endothelial ET-1 overexpression-induced ROS generation and atherosclerosis in diabetes. NOX5 is expressed in human vascular cells and is not expressed in rodents. ET-1 has been shown to increase NOX5 protein levels in human aortic SMCs and microvascular endothelial cells.<sup>33,34</sup> It would be interesting to determine whether NOX5 plays a role in atherosclerosis in humans with diabetes mellitus.

Gray *et al.*<sup>9</sup> have previously shown that increased ROS generation in diabetic mice was associated with increased *Nox1* and *Nox2* mRNA expression levels in aorta. Increased ET-1 may have augmented ROS generation in diabetic mice by enhancing the increase in *Nox1* and *Nox2* mRNA expression levels. Qualitative investigation of two highly vascularized tissues, renal cortex and lungs, revealed that diabetes-induced elevation in *Nox1* and *Nox2* mRNA expression levels was unaffected by ET-1 overexpression. Furthermore, *Nox4* mRNA expression was unaltered by diabetes or high ET-1 levels. This study demonstrated that NOX1 is required for the enhancement of atherosclerosis and immune cell infiltration caused by high ET-1 expression, but the mechanisms remain unclear.

Several studies have reported a role for NOX1 in the development of atherosclerosis in *Apoe*<sup>-/-</sup> mice.<sup>5,35,36</sup> In our study, *Nox1* knockout blunted the atherosclerosis enhancement caused by ET-1 overexpression in the aortic arch which, to our knowledge, is the first time that ET-1 has been shown to mediate its proatherogenic actions through NOX1. In the aortic sinus of diabetic eET-1/*Apoe*<sup>-/-</sup> mice, *Nox1* knockout tended to reduce plaque size although this did not achieve statistical significance. It is possible that *Nox1* knockout only slows plaque progression and that the absence of significant difference in the aortic sinus



**Figure 3** Endothelial cell-restricted endothelin-1 overexpression (eET-1) produced perivascular adipose tissue oxidative stress through NOX1. Reactive oxygen species generation was determined by dihydroethidium (DHE) staining in aortic arch perivascular adipose tissue of *Apoe*<sup>-/-</sup>, eET-1/*Apoe*<sup>-/-</sup>, eET-1/*Apoe*<sup>-/-</sup>/*Nox1*<sup>-/-</sup>, and eET-1/*Apoe*<sup>-/-</sup>/*Nox4*<sup>-/-</sup> mice 14 weeks after intraperitoneal injections with either vehicle (Veh) or streptozotocin (STZ). Representative images of DHE-stained sections are shown in A. Red, green, and blue represent DHE fluorescence, elastin autofluorescence, and 4',6-diamidino-2-phenylindole fluorescence, respectively. Ao, aorta; Lu, lumen; PVAT, perivascular adipose tissue; RFU, relative fluorescent units. Data are presented as means ± SEM, *n* = 5–9. Data were analysed using one-way ANOVA followed by a Student–Newman–Keuls *post hoc* test. †*P* < 0.001 vs. diabetic *Apoe*<sup>-/-</sup>, ‡*P* < 0.01 vs. non-diabetic eET-1/*Apoe*<sup>-/-</sup>, §*P* < 0.05 vs. diabetic eET-1/*Apoe*<sup>-/-</sup>.



**Figure 4** Endothelial cell-restricted endothelin (eET)-1 overexpression caused perivascular adipose tissue immune cell infiltration through NOX1 or NOX4. Infiltration of monocytes/macrophages (A and C) and CD3+ T cells (B and D) was determined by immunofluorescence in aortic arch perivascular adipose tissue of *Apoe*<sup>-/-</sup>, *eET-1/Apoe*<sup>-/-</sup>, *eET-1/Apoe*<sup>-/-</sup>/*Nox1*<sup>+/+</sup>, and *eET-1/Apoe*<sup>-/-</sup>/*Nox4*<sup>-/-</sup> mice 14 weeks after intraperitoneal injections with either vehicle (Veh) or streptozotocin (STZ). Representative images of MOMA-2 (monocyte/macrophages, red) and CD3 (red) immunofluorescence are shown in A and B, respectively. Green and blue represent elastin autofluorescence and 4',6-diamidino-2-phenylindole (DAPI) fluorescence, respectively. Ao, aorta; Lu, lumen; PVAT, perivascular adipose tissue. Data are presented as means ± SEM, n = 5–8. Data were analysed using one-way ANOVA followed by a Student–Newman–Keuls *post hoc* test. †P < 0.05 vs. diabetic *Apoe*<sup>-/-</sup>, ‡P < 0.01 vs. non-diabetic *eET-1/Apoe*<sup>-/-</sup>, §P < 0.05 and §§P < 0.01 vs. diabetic *eET-1/Apoe*<sup>-/-</sup>.

reflects the fact that plaques are already near-maximal in size. Indeed, animals were studied 14 weeks after induction of diabetes and plaques were larger in the aortic sinus than in the aortic arch. In the current study, *Nox4* knockout further worsened aortic arch atherosclerosis in diabetic eET-1/*Apoe*<sup>-/-</sup> mice but not *Apoe*<sup>-/-</sup> mice and had no effect in the aortic sinus. Several studies using *Apoe*<sup>-/-</sup> or *Ldlr*<sup>-/-</sup> mice fed a HFD have reported that *Nox4* knockout enhances HFD-induced atherosclerosis.<sup>6-8</sup> On the other hand, studies using STZ-treated *Apoe*<sup>-/-</sup>/*Nox4*<sup>-/-</sup> mice have found no differences 10 weeks after induction of diabetes<sup>5</sup> and enhanced atherosclerosis after 20 weeks.<sup>9</sup> Our findings therefore seem consistent with the notion that NOX4 has an atheroprotective role, at least in certain vascular territories. The qualitative difference of actions between NOX1 and NOX4 is best explained by the type of ROS they produce, with NOX1-derived superoxide being deleterious to vascular health and NOX4-derived hydrogen peroxide being beneficial.<sup>37</sup>

Plaque stability, which depends on the thickness of the fibrous cap and the degree of cap inflammation, is particularly important in human atherosclerosis as it determines which plaques will rupture and ultimately cause myocardial infarction or stroke. While this is true in humans, traditional mouse models of atherosclerosis are particularly resistant to extensive plaque destabilization and have only been reported by a few investigators to exhibit spontaneous plaque rupture and secondary thrombosis.<sup>38-41</sup> Less severe plaque injury such as endothelial denudation and plaque fissure without necrotic core exposure still do occur in mice, and terms such as plaque disruption have been suggested to describe these in order to distinguish them from overt plaque rupture.<sup>42</sup> Nevertheless, features of plaque instability found in human atherosclerosis have widely been investigated in rodents,<sup>43,44</sup> as they may still provide insights into the biology of plaque remodelling. We found in this study that ET-1 overexpression decreased  $\alpha$ -SMA content, a marker of SMCs, which are a component of the fibrous cap,<sup>45</sup> which has been suggested by some investigators to indicate previous silent plaque disruption.<sup>43,46</sup> Noteworthy, it has been shown in humans that coronary plaque ET-1 immunoreactivity is greater in patients with unstable angina,<sup>47</sup> who have decreased plaque SMCs due to apoptosis than in those with stable angina.<sup>48</sup> Interestingly, the change in  $\alpha$ -SMA content but not in buried fibrous caps was blunted by *Nox1* but not *Nox4* knockout in both the aortic sinus and arch. Other studies have also implicated oxidative stress in plaque instability in humans. Indeed, ROS production and NOX subunit expression are both high in the shoulder region of plaques,<sup>49</sup> and their levels are higher in patients with unstable angina compared to stable angina independently of the degree of vessel stenosis.<sup>50</sup> It is therefore possible that ET-1 modulated SMC loss by stimulating NOX1-dependent superoxide production and triggering redox-sensitive pathways such as those involving nuclear factor  $\kappa$ B and activating protein-1. However, whether ET-1 overexpression plays a role in atherosclerotic plaque instability and rupture needs to be demonstrated using an appropriate model of plaque rupture.

In the current study, we found that ET-1 overexpression increased ROS production in the aortic PVAT of diabetic *Apoe*<sup>-/-</sup> mice through NOX1. Although previous studies using the non-selective NOX inhibitor apocynin have implicated a role for NOX in ET-1-induced vascular damage,<sup>20,30</sup> our findings show for the first time that ET-1 produces oxidative stress through the specific NOX1 isoform. We did not find that *Nox4* knockout reduced ROS production, although this may have been a result of concomitant up-regulation of NOX1 and/or NOX2. Indeed, Gray *et al.* showed a reduction in aortic hydrogen peroxide but not total ROS generation in diabetic *Apoe*<sup>-/-</sup>/*Nox4*<sup>-/-</sup> mice, the latter ascribed to

increased expression of NOX1, NOX2 and p47phox in these mice.<sup>9</sup> Increase in other sources of ROS such as xanthine oxidase in peroxisomes, and NADH dehydrogenase and ubiquinone cytochrome c reductase in mitochondria may also explain the lack of effect of *Nox4* deficiency on ROS generation.

Interestingly, ROS production in aortic arch plaque was similar between groups and is therefore unlikely to have contributed to the enhanced atherosclerosis seen in diabetic eET-1/*Apoe*<sup>-/-</sup> mice. Instead, it seems that NOX1-dependent PVAT oxidative stress is playing a more important role here. There is accumulating evidence that PVAT modulates the development of vascular disease through so-called 'outside-in' mechanisms. Transplant of proinflammatory visceral PVAT to the mid-perivascular area of the common carotid arteries, a site usually devoid of atherosclerosis, impairs vascular relaxation and causes atherosclerotic lesions to develop in that area.<sup>51</sup> In obese mice, PVAT-derived ROS production and p67phox expression are increased and removal of such prooxidant PVAT restores aortic endothelium-dependent relaxation.<sup>52</sup> Thus, it is possible that abluminal secretion of endothelium-derived ET-1 causes PVAT oxidative stress, which creates a pro-inflammatory milieu that in turn promotes endothelial dysfunction and atherosclerosis.

It is widely accepted that inflammatory processes play an important role in the pathogenesis of atherosclerosis.<sup>53,54</sup> These include innate responses involving monocytes/macrophages<sup>55</sup> and adaptive immunity involving T cells.<sup>56</sup> In the present study, we found that ET-1 overexpression increased aortic PVAT infiltration of both monocyte/macrophage and CD3<sup>+</sup> T cells in diabetic *Apoe*<sup>-/-</sup> mice. This observation is consistent with our previous findings of enhanced PVAT inflammation in eET-1/*Apoe*<sup>-/-</sup> mice fed a HFD.<sup>23</sup> Studies in *Apoe*<sup>-/-</sup> mice have reported that *Nox1* knockout reduces diabetes-induced aortic macrophage infiltration<sup>5</sup> and HFD-induced atherosclerotic plaque macrophage infiltration.<sup>35</sup> Here, *Nox1* knockout blunted ET-1-induced monocyte/macrophage and T-cell infiltration but did not statistically reduce inflammation in *Apoe*<sup>-/-</sup> mice. This suggests that NOX1 participates in ET-1-induced immune cell recruitment through activation by pro-inflammatory stimuli such as chemokines and cytokines. It is also interesting to note that enhanced inflammation occurred only in aortic PVAT where ROS production was also increased, possibly because one induces the other or because of reciprocal induction.

In contrast to NOX1, the role of NOX4 in inflammation is less clear. Studies in diabetic *Apoe*<sup>-/-</sup>/*Nox4*<sup>-/-</sup> mice have reported increased aortic macrophage infiltration 20 weeks after induction of diabetes<sup>9</sup> but no differences 10 weeks post-induction.<sup>5</sup> Inducible deletion of *Nox4* increased plaque macrophage infiltration,<sup>8</sup> whereas endothelial overexpression of NOX4 had no effect on it.<sup>6</sup> Here, we found that *Nox4* knockout blunted ET-1-induced T-cell infiltration but not macrophage infiltration. It is unclear to what extent aortic T-cell infiltration contributes to the progression of aortic atherosclerosis seen in diabetic eET-1/*Apoe*<sup>-/-</sup> mice, since T-cell infiltration was prevented by both *Nox1* and *Nox4* deletion.

#### 4.1 Limitations

A limitation of the present study is that DHE staining did not reveal changes in ROS generation in the aortic wall of diabetic *Apoe*<sup>-/-</sup> and eET-1/*Apoe*<sup>-/-</sup> mice and in PVAT of diabetic *Apoe*<sup>-/-</sup> mice. The reasons for this are unclear, since this technique has been previously demonstrated to detect changes in ROS generation in aorta and PVAT of HFD fed *Apoe*<sup>-/-</sup> and eET-1/*Apoe*<sup>-/-</sup> mice.<sup>23</sup> It is likely that many factors in our experimental design [environment (microbiota), genetic models used, method of ROS detection and operators] may have contributed to



the differences between our findings and others. Another significant limitation is that only one method of ROS generation, DHE staining, was used. Although DHE staining can accurately measure ROS generation as noted by a scientific statement from the American Heart Association,<sup>57</sup> it is far from being perfect, and there is a need to validate ROS generation by other methods. An additional limitation is that plaque rupture is largely absent in the atherosclerotic Apoe knockout mouse model. Nevertheless, this model presents features of plaque instability found in human atherosclerosis, which may still provide insights into the biology of plaque remodelling. There is a need for a murine model with plaque rupture. An important limitation is that while we found that endothelial cell-restricted ET-1 overexpression worsens atherosclerosis and immune cell infiltration *in vivo* through NOX1, we were unable to determine the mechanism whereby ET-1 interacts with NOX1. Our qualitative data suggest that *Nox1* mRNA is not up-regulated by ET-1 overexpression, but more in-depth future studies are required to clarify the mechanisms.

## 5. Conclusion

Taken together, our results demonstrate for the first time that endothelium-restricted ET-1 overexpression worsens atherosclerosis in type 1 diabetes through NOX1. Enhanced perivascular oxidative stress and/or inflammation may be responsible for this effect, as they represent key steps in atherogenesis and were also blunted by deletion of *Nox1*. The finding that *Nox4* knockout further augmented atherosclerotic burden supports the hypothesis that NOX1 and NOX4 have somewhat opposing roles within the vasculature. Clinically, this study provides a rationale for using ET receptor antagonists to slow down the progression of atherosclerosis in predisposed patients such as those with diabetes mellitus. Our results also suggest that ET receptor blockade may improve plaque stability in humans and therefore reduce the risk of major cardiovascular events such as myocardial infarction in diabetic subjects.

## Data availability

The data underlying this article are available in the article and in its online supplemental material.

## Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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## Translational perspective

We demonstrate that endothelial cell-restricted human ET-1 overexpression worsens atherosclerosis in diabetes and causes perivascular oxidative stress and inflammation, extending our previous findings to a model of type 1 diabetes mellitus. We also show that these effects are mediated through NOX1 and that atherosclerosis is worsened by loss of NOX4, providing evidence that NADPH oxidase isoforms play differential roles in plaque progression. These results offer new approaches to prevent the progression of atherosclerosis in diabetes, which is associated with significantly increased risk of cardiovascular events.