NADPH Oxidase 1 Plays a Key Role in Diabetes Mellitus–Accelerated Atherosclerosis

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Background—In diabetes mellitus, vascular complications such as atherosclerosis are a major cause of death. The key underlying pathomechanisms are unclear. However, hyperglycemic oxidative stress derived from NADPH oxidase (Nox), the only known dedicated enzyme to generate reactive oxygen species appears to play a role. Here we identify the Nox1 isoform as playing a key and pharmacologically targetable role in the accelerated development of diabetic atherosclerosis.

Methods and Results—Human aortic endothelial cells exposed to hyperglycemic conditions showed increased expression of Nox1, oxidative stress, and proinflammatory markers in a Nox1-siRNA reversible manner. Similarly, the specific Nox inhibitor, GKT137831, prevented oxidative stress in response to hyperglycemia in human aortic endothelial cells. To examine these observations in vivo, we investigated the role of Nox1 on plaque development in apolipoprotein E–deficient mice 10 weeks after induction of diabetes mellitus. Deletion of Nox1, but not Nox4, had a profound antiatherosclerotic effect correlating with reduced reactive oxygen species formation, attenuation of chemokine expression, vascular adhesion of leukocytes, macrophage infiltration, and reduced expression of proinflammatory and profibrotic markers. Similarly, treatment of diabetic apolipoprotein E–deficient mice with GKT137831 attenuated atherosclerosis development.

Conclusions—These studies identify a major pathological role for Nox1 and suggest that Nox1-dependent oxidative stress is a promising target for diabetic vasculopathies, including atherosclerosis. (Circulation. 2013;127:1888-1902.)

Key Words: atherosclerosis ■ diabetes mellitus ■ NADPH oxidase ■ oxidative stress

In diabetes mellitus the risk for the development of atherosclerosis is enhanced, which results in an increased risk for stroke, myocardial infarction, and death.5,6 The exact mechanisms responsible for this accelerated development of atherosclerosis have remained elusive, but excess production of reactive oxygen species (ROS) appears to play a major role.3,4 Many sources of ROS contribute to increased oxidative stress, however NADPH oxidases (Nox) and their catalytic subunit are the only known enzyme family solely dedicated to producing ROS.5,6 Furthermore, Nox isoforms are upregulated in the presence of high glucose, making this enzyme family a prime candidate for treating diabetes mellitus–related cardiovascular disorders.7,8 Several Nox isoforms are present in the vasculature, Nox1, Nox2, Nox4, and Nox5. These isoforms have been proposed to play an important role in vascular pathobiology, inducing both inflammation and fibrosis.6,9-13

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In vascular cells, Nox1 mRNA expression is low under physiological conditions but induced in the presence of factors elevated in diabetes mellitus, such as platelet derived growth factor and angiotensin II (AngII), and other pathological conditions such as atherosclerosis and hypertension.16-19 Thus, Nox1 is a potential therapeutic target in diabetes mellitus–related vasculopathies. Similar roles in vascular disease have been suggested for Nox2,
Nox4 and Nox5. However, Nox2 plays an important role in the innate immune system and appears an inappropriate target in diabetes mellitus, a condition associated with increased susceptibility to infections. Nox4 in the vasculature plays a role in ischemia–reperfusion and angiogenesis. The role of the more recently discovered isofrom Nox5 in the vasculature has not been clearly determined, and this isofrom is not present in the mouse or rat.

Delineating the precise role of NADPH oxidases in any disease condition and proof-of-principle studies have been limited by the lack of specificity of available pharmacological inhibitors. GKT137831 now represents the first specific, orally and chronically active Nox inhibitor with a relative specificity for Nox1 and Nox4.

To delineate the contribution of Nox in promoting atherosclerosis in diabetes mellitus, we began with a human in vitro model of diabetes mellitus–associated oxidative stress using human aortic endothelial cells (HAEC) cultured under high glucose conditions. Production of reactive oxygen species as well as proinflammatory and profibrotic markers were assessed under high glucose conditions and after siRNA silencing as well as after treatment with GKT137831. The in vivo relevance of our findings was then examined in genetic mouse models of atherosclerosis (apolipoprotein E–deficient [ApoE−/−] mouse models of atherosclerosis (apolipoprotein E–deficient [ApoE−/−]) 10 weeks after induction of diabetes mellitus with streptozotocin in mice with and without deletion of various Nox isoforms or GKT137831 treatment. Our findings not only clearly identify Nox1 as the key source of ROS in the vasculature in diabetes mellitus but also provide evidence of a pathophysiologic role for this low-abundance Nox isofrom.

Methods

Refer to Methods in the online-only Data Supplement for greater detail.

Animal Models

Nox127 and Nox420 knockout mice were backcrossed onto ApoE−/− mice (ARC, WA, Australia) for 10 generations to generate double knockout animals (Transgenix, USA). Six-week-old Nox1−/−ApoE−/−, Nox4−/−ApoE−/−, Nox4+/−ApoE−/−, Nox1−/−ApoE−− and ApoE−− male mice were rendered diabetic by 5 daily IP injections of streptozotocin (Sigma-Aldrich, St Louis, MO) at a dose of 55 mg/kg. A subgroup of diabetic and nondiabetic ApoE−− mice were administered by 5 daily IP injections of streptozotocin (Sigma-Aldrich, St Louis, MO) at a dose of 55 mg/kg. A subgroup of diabetic and nondiabetic ApoE−− mice were administered the Nox inhibitor, GKT137831 (GKT) by daily gavage at a dose of 60 mg/kg/d.

Atherosclerotic Plaque Area Quantification

Assessment of plaque area was undertaken using en face analysis, after staining with Sudan IV-Herxheimer’s solution (BDH, Poole UK) as previously described.

Ex Vivo Vessel Chamber Studies

Isolated aortas were removed and mounted in a vessel chamber for the measurement of adherence of fluorescently labeled human leukocytes as previously described.

Quantitative RT-PCR

Total RNA was extracted after homogenising whole aorta (Polytron PT-MR2100; Kinematica, Littau/Lucerne, Switzerland) in TRizol reagent (Invitrogen Australia, Mt Waverly, Vic, Australia) as previously described. Gene expression were analyzed quantitatively as previously described.

Immunohistochemistry

Paraffin sections of aorta were used to stain for nitrotirosine (Millipore, Billerica, MA), F4/80 (Abcam, Cambridge, MA), monocyte chemottractant protein 1 (MCP-1; BioVision, CA) and 4-Hydroxynonenal (4-HNE) (Abcam, Cambridge MA) as previously described.

Measurement of H2O2 and Superoxide Production in Aortic Tissue

Aortic hydrogen peroxide generation was measured by Amplex red using a commercial kit (Molecular Probes, Eugene, OR). Aortic superoxide was measured using high pressure liquid chromatography calibrated to measure DHE by a previously established method.

Cell Culture Experiments

HAECs were obtained from Clonetics (Lonza) and grown in EGM-2 endothelial growth media at either normal (5 mmol/L) glucose or high glucose (25 mmol/L). Cells used for RT-PCR were plated and incubated for 2 days, then supplemented with GKT137831 and incubated for 24 hours before being harvested for RNA isolation. Cells that were used for ROS measurements were incubated with GKT137831 for 1 hour before harvest.

siRNA Knockdown of Nox1 and Nox4 In Vitro

The knockdown of Nox1 and Nox4 was performed in HAECs using MISSION siRNA expressing lentivirus vectors as described previously. The knockdown efficiency in the cells was verified by RT-PCR and was greater than 90% for Nox1 and 90% for Nox4.

Measurement of ROS In Vitro

ROS production in HAEC (± Glucose) was measured by DCFDA (Invitrogen Molecular Probes), L-012 (Wako Chemicals), Amplex Red (Molecular Probes), and DHE (dihydroethidium).

Statistical Analysis

Data were analyzed for normality using the Shapiro-Wilk test before being analysed either with a 1-way ANOVA or 2-way ANOVA using SPSS Statistics version 20 (IBM) with an LSD post hoc test for multiple comparison of the means. Effects of drug dose were assessed by ANCOVA with repeated measures. P <0.05 was considered significant. Results are expressed as mean±SEM, unless otherwise specified.

Results

Human In Vitro Studies

Gene Expression of Nox Isoforms and ROS Generation

HAECs grown in the presence of high glucose (25 mmol/L) showed increased gene expression of Nox1, Nox2, Nox4 and Nox5 (Table I in the online-only Data Supplement). In addition, high glucose increased production of ROS when assessed by 4 different approaches, L-012 (F=10.9, P<0.01), DCFDA (F=16.7, P<0.01), Amplex Red (F=39.7, P<0.01), and DHE (F=38.1, P<0.01) quantified by flow cytometry when compared with HAECs grown under normal glucose conditions (Figure 1). To identify the responsible Nox isofrom, different Nox siRNAs were tested. Transfection of
Figure 1. Glucose-induced increase in cellular reactive oxygen species was attenuated by transfection with siRNA targeted against Nox1 and by treatment with GKT137831. ROS as assessed by 4 different methods, L-012, DCFDA, Amplex Red, and DHE by FACS analysis in HAECs transfected with siRNA specific for Nox1 or Nox4 grown under normal glucose (5 mmol/L) or high glucose (25 mmol/L) conditions (A–H) in addition to HAECs grown under normal glucose (5 mmol/L) or high glucose (25 mmol/L) conditions with or without GKT137831 (GKT) treatment at either 0 μmol/L, 0.1 μmol/L, and 10 μmol/L (I–L). Data are mean±SEM (n=6/group). Groups were analyzed by ANOVA with a LSD post hoc test. Data presented in I–L were analyzed using ANCOVA with repeated measures. *P<0.05 compared with nontarget normal Glucose, #P<0.05 compared with nontarget high glucose. DCFDA indicates 2',7'-dichlorofluorescein diacetate; DHE, dihydroethidium; HAEC, human aortic endothelial cell; Nox, NADPH oxidase; and ROS, reactive oxygen species.
HAECs with Nox1 siRNA resulted in a 95% reduction of Nox1 mRNA (Table II in the online-only Data Supplement), and this was associated with a significant reduction in all four measures for ROS used (Figure 1A–1D). Specifically, siRNA to Nox1 reduced ROS, as assessed by 3 different methods, in the high but not normal glucose milieu (L-012, F=6.1, P<0.025; DCFDA, F=7.5, P<0.01; DHE, F16.6, P<0.001). In contrast, transfecting HAECs with siRNA for Nox4 did not result in significant attenuation of ROS generation when assessed by DHE and L-012. However, when ROS was measured using Amplex Red and DCFDA there was a significant attenuation in ROS production (Figure 1E–1H). Transfection of HAECs with either Nox1 or Nox4 did not have any effect on the expression of the remaining Nox isoforms (Table II in the online-only Data Supplement). Treatment of HAECs with the Nox inhibitor GKT137831 (0.1 μmol/L, 1 μmol/L, or 10 μmol/L) resulted in a reduced production of high glucose–induced ROS formation (Figure 1I–1L). Indeed, there was a dose-dependent effect of GKT137831 on ROS, as assessed by L-012 (F=15.5, P<0.01, Figure 1I), DCFDA (F=22.0, P<0.01, Figure 1J), DHE (F=49.1, P<0.01, Figure 1K), and Amplex Red (F=11.9, P<0.01, Figure 1L).

**Gene Expression of Proinflammatory and Profibrotic Markers**

To establish whether the role of Nox1 in HAEC ROS generation correlated with a similar role in inflammation and fibrosis, both inflammatory markers (including MCP-1 and vascular cellular adhesion molecule-1 [VCAM-1]) as well as fibrotic markers (including the connective tissue growth factor [CTGF]) and extracellular matrix proteins such as collagen IV and fibronectin were tested. Indeed, knockdown of Nox1 in HAECs by siRNA resulted in attenuation of the high glucose–induced expression of MCP-1, VCAM-1, CTGF, collagen IV, and fibronectin (Table 1). In contrast, silencing of Nox4 by siRNA did not affect the mRNA levels of proinflammatory and profibrotic markers (Table III in the online-only Data Supplement).

Treatment of HAECs under high glucose conditions with GKT137831 (0.1 μmol/L, 1 μmol/L, or 10 μmol/L) resulted in a reduced production of high glucose–induced ROS formation (Figure 1I–1L). Indeed, there was a dose-dependent effect of GKT137831 on ROS, as assessed by L-012 (F=15.5, P<0.01, Figure 1I), DCFDA (F=22.0, P<0.01, Figure 1J), DHE (F=49.1, P<0.01, Figure 1K), and Amplex Red (F=11.9, P<0.01, Figure 1L).

Table 1. RT-PCR Analysis in HAEC Cells Transfected With siRNA Specific for Nox1 and Then Grown in the Presence of Normal (5 mmol/L) or High Glucose (25 mmol/L) Conditions for 24 Hours

<table>
<thead>
<tr>
<th>Gene</th>
<th>Normal Glucose (5 mmol/L)</th>
<th>High Glucose (25 mmol/L)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Nontarget</td>
<td>Nox1 siRNA</td>
</tr>
<tr>
<td>Nox1</td>
<td>1.0±0.30</td>
<td>0.1±0.01*</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>1.0±0.27</td>
<td>0.6±0.15*</td>
</tr>
<tr>
<td>MCP-1</td>
<td>1.0±0.27</td>
<td>1.1±0.17*</td>
</tr>
<tr>
<td>CTGF</td>
<td>1.0±0.32</td>
<td>0.7±0.21</td>
</tr>
<tr>
<td>Collagen IV</td>
<td>1.0±0.38</td>
<td>2.4±0.66</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>1.0±0.25</td>
<td>1.4±0.28</td>
</tr>
</tbody>
</table>

Data are Mean±SEM (n=6/group). CTGF indicates connective tissue growth factor; HAEC, human aortic endothelial cell; MCP-1, monocyte chemoattractant protein; Nox, NADPH oxidase; and VCAM-1, vascular cell adhesion molecule.

*P<0.05 compared with Nontarget grown in Normal Glucose, #P<0.05 compared with Nontarget grown in High Glucose.

Together, our in vitro studies suggest that Nox1, but not Nox4, is a major source of vascular ROS and critical for inducing inflammation and fibrosis in high glucose conditions.

**Mouse In Vivo Studies**

To test our in vitro findings in an in vivo setting, we induced insulin-deficient diabetes mellitus using STZ in Nox1 knockout animals bred onto the ApoE−/− background (>10 generations) and observed atherosclerotic plaque area as major read-out. In addition, diabetic wild-type ApoE−/− mice were treated by gavage once a day with the Nox inhibitor GKT137831 for 10 weeks at a dose of 60 mg/kg/d after induction of diabetes mellitus.

**Metabolic Parameters**

As expected, at the end of the study all diabetic animals had lower body weights (Tables 3 and 4), elevated glucose and HbA1c levels in comparison with their nondiabetic controls. Deletion of Nox1 or Nox4 or pharmacological Nox inhibition with GKT137831 did neither have any effect on body weight, glycemic control, nor lipid levels in any diabetic mouse groups (Tables 3 and 4). Systolic blood pressure was also unchanged among all groups. Diabetic animals displayed a significant elevation in serum cholesterol, triglyceride, and LDL levels.
which were not affected by deletion of Nox1 or Nox4 (Tables 3 and 4). Similarly, GKT137831-treated diabetic ApoE−/− mice had similar body weights, blood pressures, and metabolic data, including total cholesterol and LDL levels compared with untreated diabetic ApoE−/− mice (Table 4).

### Atherosclerotic Plaque Area

Aortic atherosclerotic plaque area was measured in the aortic arch as well as thoracic and abdominal portions of the aorta. All diabetic animals showed a significant increase in total atherosclerotic plaque areas, which was most prominent in the aortic arch (Figure 2A, 2C, and 2E and Figure I in the online-only Data Supplement). Importantly, deletion of Nox1 attenuated the development of plaque formation in the aorta of diabetic Nox1+/−ApoE−/− mice, both in the total aorta and even more pronounced in the aortic arch when compared to diabetic Nox1+/−ApoE−/− mice (Figure 2A). In accordance, treatment of diabetic ApoE−/− mice with GKT137831 fully prevented the development of atherosclerotic plaques within the total aorta (diabetes mellitus, F=6.7, P<0.01) and aortic arch in comparison to untreated diabetic ApoE−/− mice. Treatment of nondiabetic ApoE−/− mice did not result in any change in atherosclerotic plaque area when compared with untreated nondiabetic ApoE−/− mice (Figure 2E and 2F). In contrast, in diabetic Nox 4−/−ApoE−/− mice, plaque area was unchanged after 10 weeks of diabetes mellitus (Figure 2C and 2D), and there was no difference in atherosclerotic plaque area within the thoracic or abdominal regions across any group (Table IV in the online-only Data Supplement). Therefore, Nox4 is unlikely to be involved in the development of atherosclerosis under diabetic conditions, at least in mice. We also induced diabetes mellitus in Nox2−/− mice using STZ. However, these mice showed increased susceptibility to Gram-negative infections with >50% mortality after 10 weeks and 100% mortality at week 20 of diabetes mellitus unless treated with antibiotics (Figure II in the online-only Data Supplement). Thus, we consider Nox2 as a rather inappropriate target to prevent or treat diabetes mellitus–associated atherosclerosis. Therefore, we did not further investigate the role of Nox2.

### Table 3. Metabolic and Biological Data for Control and Diabetic Nox1+/−ApoE−/−, Nox1−/yApoE−/− Nox4+/+ApoE−/− and Nox4−/−ApoE−/− After 10 Weeks

<table>
<thead>
<tr>
<th>Nox1 Deletion Studies</th>
<th>Nox4 Deletion Studies</th>
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<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>27±1.29</td>
</tr>
<tr>
<td>Systolic BP, mm Hg</td>
<td>101±3</td>
</tr>
<tr>
<td>BG, mmol/L</td>
<td>13.0±0.66</td>
</tr>
<tr>
<td>HbA1c, %</td>
<td>3.9±0.19</td>
</tr>
<tr>
<td>Cholesterol, mmol/L</td>
<td>9.7±0.53</td>
</tr>
<tr>
<td>Triglycerides, mmol/L</td>
<td>2.1±0.84</td>
</tr>
<tr>
<td>HDL, mmol/L</td>
<td>2.9±0.93</td>
</tr>
<tr>
<td>LDL, mmol/L</td>
<td>7.5±0.62</td>
</tr>
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</table>

Groups were analysed separately, data are mean±SEM (n=10–15/group). BG indicates blood glucose; BP, blood pressure; HbA1c, hemoglobin A1c; HDL, high-density lipoprotein; LDL, low-density lipoprotein; Nox, and NADPH oxidase.

P<0.05 * compared with Nox1+/−ApoE−/− Control, # compared with Nox1+/−ApoE−/− Diabetic and † compared with Nox4+/+ApoE−/− Control, * vs Nox4+/−ApoE−/− Control, † vs Nox4−/−ApoE−/− Control.

### Table 4. Metabolic and Biological Data for ApoE−/− Control and Diabetic Treated With or Without GKT137831 (GKT) for 10 Weeks (60 mg/kg/d)

<table>
<thead>
<tr>
<th></th>
<th>ApoE−/−</th>
<th>ApoE−/− &amp; GKT137831</th>
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<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Diabetic</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>28±1.17</td>
<td>24.8±0.77*</td>
</tr>
<tr>
<td>Systolic BP, mm Hg</td>
<td>102±3</td>
<td>99±3</td>
</tr>
<tr>
<td>BG, mmol/L</td>
<td>10.4±0.94</td>
<td>21.8±2.11*</td>
</tr>
<tr>
<td>HbA1c, %</td>
<td>4.5±0.47</td>
<td>11.4±0.88*</td>
</tr>
<tr>
<td>Cholesterol, mmol/L</td>
<td>6.7±0.53</td>
<td>12.3±1.05*</td>
</tr>
<tr>
<td>Triglycerides, mmol/L</td>
<td>0.8±0.07</td>
<td>1.7±0.39*</td>
</tr>
<tr>
<td>HDL, mmol/L</td>
<td>1.6±0.11</td>
<td>2.4±0.14*</td>
</tr>
<tr>
<td>LDL, mmol/L</td>
<td>4.7±0.45</td>
<td>9.0±0.70*</td>
</tr>
</tbody>
</table>

Data are mean±SEM (n=10–15/group). BG indicates blood glucose; BP, blood pressure; HbA1c, hemoglobin A1c; HDL, high-density lipoprotein; and LDL, low-density lipoprotein.

P<0.05 * compared with ApoE−/− Control, # compared with ApoE−/− Diabetic and † compared with ApoE−/− Control & GKT137831.
These results supported the in vitro findings in HAECs and translated to a mouse experimental in vivo and pharmacological proof-of-principle setting by suggesting that Nox1, but not Nox4, is a major player in diabetes mellitus–induced vascular pathologies and pharmacologically targetable.

**Aortic ROS (Superoxide and H$_2$O$_2$) Production**

Only superoxide production (as assessed by DHE staining using HPLC) but not extracellular H$_2$O$_2$ (as assessed using Amplex Red) was significantly increased in the diabetic ApoE−/− groups compared with nondiabetic ApoE−/− mice (Figure 3A–3F). Further supporting a role of Nox1, we observed a significant reduction in superoxide production in the aortas from diabetic but not control Nox1+/−ApoE−/− animals when compared with control and diabetic Nox1+/−ApoE−/− mice (Figure 3A, F=6.7, P<0.01). The production of superoxide was significantly attenuated in diabetic ApoE−/− treated with GKT137831 compared with untreated diabetic ApoE−/− animals (Figure 3E). Administration of GKT137831 to both control and diabetic ApoE−/− mice did not have a significant effect on H$_2$O$_2$ levels when compared with untreated control and diabetic ApoE−/− mice (Figure 3F). Furthermore, in contrast to diabetic Nox1+/−ApoE−/− mice, diabetic Nox4 deficient ApoE−/− mice did not show attenuation of superoxide or H$_2$O$_2$ production within the aorta (Figure 3C). These data suggested that Nox1-derived superoxide but not H$_2$O$_2$ is the key form of ROS triggering diabetic atherosclerosis.

**Aortic Nitrative and Oxidative Stress**

ROS can interact with either NO and nitrite to cause nitrative stress or lipids to cause peroxidation and oxidative stress, which can be measured by nitrotyrosine (NT) staining and 4-HNE, respectively. In the aortic wall, both NT and 4-HNE were increased in all diabetic animals when compared with vessels from nondiabetic control mice (Figures 4 and 5). Again, deletion of Nox1 resulted in a significant attenuation in NT and 4-HNE staining in the aorta of diabetic Nox1−/−ApoE−/− mice in comparison with diabetic Nox1+/−ApoE−/− mice. Similarly, diabetic ApoE−/− mice treated with GKT137831 displayed a significant reduction in NT staining when compared with the untreated diabetic ApoE−/− mice (Figure 4). GKT137831 treatment also resulted in reduced...
4-HNE staining in diabetic ApoE−/− but not control ApoE−/− mice (Figure 5, F=11.3, P<0.01).

In line with our previous results, deletion of Nox4 neither altered NT nor 4-HNE staining in aortas of diabetic ApoE−/− mice when compared with diabetic Nox1+/yApoE−/− mice (Figures 4 and 5).

**Macrophage Infiltration**

Macrophage infiltration, a hallmark of atherosclerosis, was assessed by F4/80 immunohistochemistry within whole aortas. It was significantly increased in all diabetic mice compared with aortic vessels from nondiabetic control mice (Figure 6). Deletion of Nox1 resulted in a significant reduction in F4/80 staining in the aorta of diabetic but not control Nox1+/yApoE−/− mice in comparison with control and diabetic Nox1+/yApoE−/− mice (Figure 6A, F=8.2, P<0.01). Similarly, diabetic ApoE−/− mice treated with GKT137831 demonstrated reduced F4/80 staining in comparison with untreated diabetic ApoE−/− mice (Figure 6C). Again, deletion of Nox4 did not result in a significant reduction in staining for F4/80 in diabetic Nox4+/−ApoE−/− mice in comparison with diabetic Nox4+/−ApoE−/− mice (Figure 6B).

**Aortic Vascular Adhesion**

Adhesion of leukocytes to the endothelial cell layer is a key step in the initiation of atherosclerosis.36 Examination of leukocyte adhesion to the aorta using vessel chamber experiments over a period of 10 minutes demonstrated a significant increase in the number of leukocytes that adhered to the aortic wall of diabetic ApoE−/− mice in comparison to nondiabetic ApoE−/− mice (Figure 7A and 7B). Deletion of Nox1 significantly reduced the number of adherent leukocytes (Figure 7A and 7B). Deletion of Nox4 isoform did not result in attenuation of the number of leukocytes adhering to the aortic wall.

**Vascular Adhesion Markers and Inflammation**

Because inflammation and adhesion of monocytes to the vascular wall are critical steps in the initiation and development of diabetic atherosclerosis, we examined vascular expression of MCP-1 and VCAM-1. There was a significant increase in the gene expression of MCP-1 in the aorta of diabetic animals (Figure 7C–7E). This
diabetes mellitus–induced increase was again attenuated in the diabetic but not control Nox1−/−ApoE−/− (F=9.9, P<0.01) and in the GKT137831-treated diabetic ApoE−/− mice (Figure 7C and 7E), however there was no change in MCP-1 gene expression in diabetic Nox4−/−ApoE−/− mice compared with Nox4+/+ApoE−/− diabetic mice (Figure 7D). Immunohistochemistry confirmed the diabetes mellitus–induced upregulation of MCP-1 protein expression in the aortas of diabetic mice, which was significantly attenuated by deletion of Nox1 (F=14.1, P<0.01) but not Nox4 (Figure 7F–7H). Similarly, treatment of diabetic ApoE−/− mice with GKT137831 resulted in reduced MCP-1 staining (Figure 7H).

As expected, diabetes mellitus was associated with a significant increase in the gene expression of aortic VCAM-1, which was attenuated in Nox1+/+ApoE−/− and GKT137831 treated diabetic ApoE−/− mice but not in Nox4−/−ApoE−/− mice (Figure 8A–8C). Fibrosis is also a potent feature of diabetes mellitus–associated atherosclerosis. Accordingly, the profibrotic growth factor, CTGF, as well as the matrix proteins, fibronectin and collagen IV, were significantly upregulated at the mRNA level in the aortas of all diabetic mice (Figure 8A–8C). Deletion of Nox1 in diabetic Nox1−/−ApoE−/− animals, and treatment of diabetic ApoE−/− diabetic animals with GKT137831 resulted in significantly attenuated gene expression of fibronectin and CTGF (Figure 8A and 8C). However, in accordance with our other findings, deletion of Nox4 in diabetic Nox4−/−ApoE−/− animals did not result in reduced expression of CTGF and fibronectin (Figure 8B). With respect to collagen IV mRNA levels, Nox1 deletion and GKT137831 reduced gene expression in the diabetic but not control mice (Nox1 deletion, F=17.0, P<0.01; GKT137831 treatment, F=11.2, P<0.01) but there was no effect of Nox4 gene deletion (Figure 8A–8C).

**Discussion**

This series of experiments provides strong evidence of a major pathophysiological role for Nox1, but not Nox4, in advanced atherosclerosis in diabetes mellitus. By using both genetic and pharmacological approaches, we have demonstrated that
in the setting of diabetes mellitus, Nox1 mediates oxidative stress, inflammation, and fibrosis and determines plaque size. Our studies provide novel mechanistic data demonstrating that Nox1 inhibition attenuates diabetes mellitus–induced adhesion of inflammatory cells to the vascular wall, a key initiating step in the development of atherosclerosis. In this study 2 major vascular isoforms of the enzyme NADPH oxidase, Nox1 and Nox4, were assessed using Nox isoform KO on the atherosclerosis-prone ApoE−/− background in the absence and presence of the proatherosclerotic stimulus, insulin-deficient diabetes mellitus. In vitro, using human endothelial cells, silencing of Nox1 using an siRNA approach attenuated high glucose–induced ROS production and expression of proinflammatory cytokines and profibrotic growth factors. Furthermore, the novel Nox inhibitor GKT137831 attenuated superoxide production, inflammation, and vascular adhesion as well as fibrosis in vitro and in vivo. This pharmacological strategy provides a potentially new clinical approach to reduce oxidative stress in the diabetic setting ultimately leading to diminished cardiovascular burden as a result of atherosclerosis.

Cardiovascular disease is the major cause for mortality and morbidity in diabetic patients. It has been shown that atherosclerosis in diabetes mellitus exhibits characteristic features including a more pronounced inflammatory phenotype characterized by enhanced vascular macrophage infiltration, upregulation of proinflammatory cytokines, and adhesion molecules, leading to accelerated adhesion of leucocytes to the vascular wall.
To directly compare the role of Nox1 and Nox4 in a model of advanced atherosclerosis in the context of diabetes mellitus, we generated double knockout mice by crossing Nox1−/− and Nox4−/− with ApoE−/− mice. Only Nox1 deletion in ApoE−/− mice prevented the accelerated development of atherosclerosis after 10 weeks of diabetes mellitus, whereas deletion of Nox4 in diabetic ApoE−/− mice did not protect mice from plaque development. This antiatherosclerotic effect of Nox1 deletion observed in diabetic Nox1−/yApoE−/− mice was associated with reduced generation of ROS. Activation of inflammatory and fibrotic markers are critical processes in the development of diabetes mellitus–related atherosclerosis.38–41 Consistent with a link between Nox1 and the atherosclerotic process, Nox1 deletion was associated with attenuation of diabetes mellitus–induced vascular macrophage infiltration, expression of adhesion molecules and chemokines as well as fibrogenesis. To complement these in vivo findings demonstrating a role for Nox1 in diabetes mellitus–associated atherosclerosis and in the regulation of diabetes mellitus–induced upregulation of adhesion molecules, we assessed ex vivo dynamic adhesion which was enhanced in the diabetic milieu. Indeed, Nox1 but not Nox4 deletion led to attenuation of diabetes mellitus–associated enhanced vascular adhesion. Therefore, our studies suggest that Nox1 is the most important Nox isoform and source of ROS for the development of diabetes mellitus–accelerated atherosclerosis and as such a promising therapeutic target.

Within the vasculature, Nox1 expression is low under normal conditions but is increased under pathological conditions such as hypertension,11,18,27,42 implicating Nox1 as a potential drug target for vasculoprotection. Recent studies have suggested, albeit not investigated in the diabetic context, that Nox1 plays a role in vascular smooth muscle cell migration, proliferation, and extracellular matrix formation and has been implicated in neointima formation.43 Indeed, Nox1 has been
implicated in a range of processes related to vascular disease, although these studies have focused on earlier events such as endothelial dysfunction or factors that predispose to atherosclerosis such as hypertension or increases in the potent vasoconstrictor AngII. For example, Nox1 has been shown to be upregulated by AngII in SHR. In the balloon injury model in rats a recent report has described a role for Nox1 involving the direct binding of this enzyme to the AT1 receptor.

In STZ-diabetes mellitus, activation of Nox1 but not Nox4 or Nox2 has been implicated in eNOS uncoupling and endothelial dysfunction, although these studies were performed in a nonatherosclerosis prone mouse model. The role of Nox1 in atherosclerosis has not been extensively explored. However, studies by Sheehan et al have suggested that Nox1 plays a role in a different model of atherosclerosis using high-fat feeding and in neointima formation. There are several differences between our studies and the findings by Sheehan et al. First, all our studies were performed in a
hyperglycemic milieu where atherosclerosis is considered to be accelerated as a result of interactions between metabolic and hemodynamic factors, including upregulation of the local vascular renin–angiotensin system in the vasculature. In our studies we have used the STZ-induced diabetic ApoE−/− mouse, a model considered to be the most appropriate model to study advanced atherosclerosis in the context of diabetes mellitus.

In contrast to our model, which explored a more advanced clinically relevant model of atherosclerosis, the study by Sheehan et al demonstrated rather modest plaque development with much less pronounced effects of Nox1 deficiency on atherosclerosis. Furthermore, the role of

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**Figure 8.** Amelioration of diabetes mellitus–induced aortic expression of proinflammatory and profibrotic markers in diabetic Nox1+/yApoE−/− and GKT137831-treated diabetic ApoE−/− mice. RT-PCR analysis for inflammation and fibrosis markers in aortas of control (Cont) and diabetic (Diab) Nox1+/yApoE−/−, Nox1+/yApoE−/− (A) and control and diabetic Nox4+/ApoE−/− and Nox4−/−ApoE−/− (B) mice. Furthermore, gene expression was analysed in aortas of control (Cont) and diabetic (Diab) ApoE−/− mice (C) treated with or without GKT137831 (GKT) for 10 wks (60 mg/kg/d). Data are mean±SEM (n=6–8/group). Groups were analyzed separately using ANOVA with a LSD post hoc test, \( P<0.05 \) *compared with Nox1+/yApoE−/− or ApoE−/− Control, *compared with Nox1+/yApoE−/− or ApoE−/− Diabetic, * vs Nox4+/ApoE−/− Control, * vs Nox4+/ApoE−/− Diabetic and * vs Nox4−/−ApoE−/− Control.
other Nox isoforms such as Nox4 in atherosclerosis was not considered in that study.

To enhance the potential clinical translation of the findings, we included a complementary pharmacological approach by using the Nox inhibitor GKT137831, which inhibits both Nox1 and Nox4. Treatment with GKT137831 mimicked the antiatherosclerotic effect of Nox1 deletion. This included inhibition of ROS generation and attenuation of diabetes mellitus–induced increased adhesion of leucocytes to the vascular wall resulting in a reduction in vascular macrophage infiltration, inflammation, and fibrosis ultimately leading to attenuation of plaque formation. Importantly, this agent did not lead to increased susceptibility to infection. GKT137831 also inhibits Nox4, and previously it has been claimed that Nox4 silencing in microvascular complications, specifically diabetic nephropathy, is protective, albeit using potentially less specific antisense techniques. Although Nox4 has been reported to have vascular effects such as actions on angiogenesis in hindlimb ischemia, albeit in a normoglycemic setting, no effects on atherosclerosis per se have been previously defined with respect to this particular Nox isoform. In this study, no benefit on atherosclerosis was observed in Nox4−/− mice, nor did any in vitro or ex vivo studies demonstrate any improvement in vascular adhesion or reduction in proinflammatory molecules. Thus, our studies do not support a vasculoprotective role for Nox4 in diabetes mellitus.

GKT137831 does not appear to have significantly inhibited Nox2 at the dose used in this study. Indeed, previous detailed studies on the effects of GKT137831 on Nox2 and on relevant proteins implicated in neutrophil defense have been reported, and indeed GKT137831 was shown to not affect Nox2-dependent events such as innate microbial killing.

To complement the in vivo studies, a series of in vitro experiments in human endothelial cells were performed, which were consistent with the in vivo findings, demonstrating a central role for Nox1 in ROS generation and regulation of a range of proinflammatory and profibrotic molecules. Similar benefits of GKT137831 in suppressing endothelial ROS production in a dose-dependent manner and altering expression of key molecules such as MCP-1 and VCAM-1 were also observed. A modest effect of the siRNA to Nox1 on Nox4 expression was observed, although the significance of these findings are unclear.

Nox2 is also upregulated in hyperglycemic states, and thus a pathological role for this isoform should be considered. However, STZ-induced diabetes mellitus in Nox2−/− mice and in particular in Nox2−/−ApoE−/− double KO mice was associated with increased susceptibility to infections resulting in almost 100% mortality. This is reminiscent of humans with mutations in Nox2 and reduced Nox2 activity who develop chronic granulomatous disease (CGD) and are susceptible to infections. This lethality of Nox2 deletion in diabetes mellitus–associated atherosclerosis indicates that Nox2 is likely to be an inappropriate target in the context of diabetes mellitus. In humans, a role for another Nox isoform, Nox5, which is not present in rodents has been suggested in the vascular system and warrants further investigation in man.

Conclusion
This study has demonstrated a major role for Nox1 derived ROS in diabetes mellitus–associated atherosclerosis. Specifically, genetic deletion of Nox1 in diabetic ApoE−/− mice was associated with reduced diabetes mellitus–associated atherosclerotic plaque development, and this may be attributable at least in part to reduced adhesion of inflammatory cells to the vascular wall, a key initiating step in atherosclerosis development. The Nox inhibitor GKT137831 mimicked those effects both in vivo and in vitro. Thus, our studies indicate a previously not reported major pathophysiological role for Nox1 in atherosclerosis particularly in a diabetic setting. Thus, Nox1 inhibition represents a novel therapeutic approach in the prevention and treatment of diabetes mellitus–associated vascular disorders, such as atherosclerosis.

Acknowledgments
We thank Dr Ying He (Ottawa Hospital Research Institute, Ottawa, Canada), Dr Natalie Lumsden, Ann-Marrie Jefferys, Edward Grixiti, Maryann Arnstein, Kylie Gilbert (Baker IDI Heart & Diabetes Institute, Melbourne, Australia) for their technical expertise, and Prof Karl-Heinz Krause (Department of Pathology and Immunology, Center Medical University, University of Geneva, Geneva, Switzerland) for providing Nox1+/−/P2 generation.

Sources of Funding
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Disclosures
C.S. and F.H. are paid employees and own shares in Genkyotex SA, Geneva, Switzerland. The other authors report no conflicts.

References


Diabetic patients have increased risk of cardiovascular complications, including myocardial infarction, stroke, and peripheral vascular disease. The underlying mechanisms responsible for this acceleration of cardiovascular disease in diabetes mellitus remain unknown. Antioxidants have in general been disappointing for a range of reasons, including dosing problems, local availability, and pro-oxidant effects. It is now recognized that NADPH oxidase (Nox)–derived reactive oxygen species may be an important mediator of vascular disease, particularly in diabetes mellitus. In this series of experiments we demonstrate for the first time a key role for Nox1 in the development of diabetes mellitus–accelerated atherosclerosis. The genetic deletion of Nox1 but not Nox4 was associated with reduced adhesion of inflammatory cells to the vascular wall, as well as leading to less vascular macrophage infiltration and fibrosis. Importantly, from a clinical translational point of view, these results were replicated using a pharmacological Nox inhibitor, GKT137381, which is already in clinical development. Furthermore, the in vivo data were complemented by in vitro studies using siRNA silencing to knockdown selectively Nox1 or Nox 4, and we were able to replicate the effects on reactive oxygen species formation, inflammation, and fibrosis as observed in vivo. These studies provide the first definitive evidence that Nox1 is a critical target for advanced atherosclerosis, particularly in the diabetic context, and that this isoform is pharmacologically targetable, thereby increasing the clinical translational potential of these findings.


NADPH Oxidase 1 Plays a Key Role in Diabetes Mellitus—Accelerated Atherosclerosis

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Gray SP; Nox1 plays a Key Role in Diabetes Accelerated Atherosclerosis

Supplemental Material

**Animal Models:** All animals were housed at the Precinct Animal Centre of the Baker IDI Heart & Diabetes Institute, with animal experiments conducted in accordance to the principles devised by the Alfred Medical Research & Education Precinct (AMREP) Animal Ethics Committee under guidelines laid down by the National Health and Medical Research Council of Australia. The animals had unrestricted access to water and feed and were maintained on a 12 hour light and dark cycle in a pathogen free environment on standard mouse chow (Specialty Feeds, WA, Australia).

Nox1\(^1\) and Nox4\(^2\) knockout mice were backcrossed onto ApoE\(^{-/-}\) mice (ARC, WA, Australia) for 10 generations to generate double knockout animals (Transgynix, USA). Six-week-old Nox4\(^{-/-}\)ApoE\(^{-/-}\), Nox4\(^{+/+}\)ApoE\(^{-/-}\), Nox1\(^{-/-}\)ApoE\(^{-/-}\), Nox1\(^{+/+}\)ApoE\(^{-/-}\) and ApoE\(^{-/-}\) male mice were rendered diabetic by five daily i.p. injections of streptozotocin (Sigma-Aldrich, St Louis, MO, USA) at a dose of 55mg/kg in citrate buffer\(^3\). Only animals with a blood glucose level >15 mmol/l 1 week after the induction of diabetes were included in the study. A subgroup of diabetic and non-diabetic ApoE\(^{-/-}\) mice were administered the Nox inhibitor, GKT137831 (GKT) by daily gavage at a dose of 60mg/kg/day for 10 weeks. Ten weeks post induction of diabetes systolic BP was assessed by non-invasive tail cuff system in conscious mice\(^4\). After 10 weeks, the animals were anaesthetised by an i.p. injection of sodium pentobarbitone (100mg/kg body weight; Euthatal, Sigma-Aldrich, Castle Hill, NSW, Australia). Glycated hemoglobin was measured in erythrocyte lysates by HPLC (Bio-Rad, USA). Total glucose, cholesterol and triglyceride concentrations were measured in plasma with a standard commercial enzymatic assay (Beckman Coulter Diagnostics, Gladsvile, NSW, Australia). Organs were rapidly dissected and weights obtained before being either snap frozen in liquid nitrogen or fixed in buffered formalin (10% v/v).
Nox Inhibition: GKT137831, a member of the pyrazolopyridine dione family is an inhibitor of both Nox1 and Nox4 isoforms (Nox1 Ki = 110nM and Nox4 Ki = 140nM) when analysed in cell free assays for ROS production using membrane preparations heterologously over-expressing specific Nox enzyme isoforms and has specificity for Nox4 and Nox1 enzymes as shown in an extensive in vitro off-target pharmacological profiling on 170 different proteins including ROS producing and redox-sensitive enzymes. GKT137831 is 15-fold less potent on Nox2 (Ki = 1750nM) and does not inhibit of highly specific Nox2-driven innate immune responses, including the neutrophil burst at concentrations up to 100µM, bacterial killing in vitro or in vivo (when used at a concentration of up to 20µM or administered at 100mg/kg orally, respectively). At concentrations of up to 10µM, GKT137831 does not demonstrate any affinity to xanthine oxidase or uncoupled NOS, and does not scavenge superoxide.

Atherosclerotic Plaque Area Quantification: Assessment of plaque area was undertaken using en face analysis as previously described, after staining with Sudan IV-Herxheimer’s solution (BDH, Poole UK). Digitised photographs of opened aortas were obtained using a dissecting microscope (Olympus SZX9; Olympus Optical, Tokyo, Japan) and a digital camera (Axiocam colour camera; Carl Zeiss, North Ryde, NSW, Australia). Plaque area was calculated as the proportion of aortic intimal surface area occupied by red-stained plaque (Abode Photoshop v 6.0.1; Adobe Systems, Chatswood, NSW, Australia).

Ex Vivo Vessel Chamber Studies for Adhesion of Human Leukocytes: Isolated aortas were removed from ApoE−/− Control and Diabetic, Nox1−/−ApoE−/− Diabetic, Nox4−/−ApoE−/− Diabetic and ApoE−/− Diabetic GKT137831 treated animals and mounted in a vessel chamber primed with Krebs buffer. Vessels were maintained at physiological pH by infusion of carbogen gas through the Krebs buffer at 37°C. Anticoagulated human whole blood from multiple healthy non-medicated volunteers was labelled with DiC18 (1:1000) and perfused through the aortas at 0.12 ml/min using a syringe pump (Harvard Apparatus). Images of vessel wall-cell interactions were observed using an Olympus BX51...
fluorescence microscope fitted with a x10 LWD lens (Olympus, Tokyo, Japan) and analysed suing a Hamamatsu camera, coupled to Image ProPlus software. Vessels were pre-treated TNFα (10ng/ml 4 hours at 37°C). The numbers of adhered leukocytes were counted over a 10 minute time frame in a blind manner⁹.

**Quantitative RT-PCR:** Total RNA was extracted after homogenising whole aorta (Polytron PT-MR2100; Kinematica, Littau/Lucerne, Switzerland) in TRIZol reagent (Invitrogen Australia, Mt Waverly, Vic, Australia). Contaminating DNA was removed after treatment with DNA-free DNase according to the manufacturer’s specifications (Ambion, Austin, TX, USA). Finally, DNA-free RNA was reverse transcribed into cDNA using the Superscript First Strand Synthesis System (Life Technologies BRL, Grand Island, NY, USA). Expression of genes encoding members of the NADPH oxidase family, Nox1, Nox2 and Nox4, as well as inflammatory markers, MCP-1, VCAM-1, fibrotic markers, CTGF, Collagen IV, Fibronectin, were analysed by quantitative RT-PCR using Taqman® system (ABI Prism 7500; Perkin-Elmer, Foster City, CA, USA). Primer and probe sequences have been published previously¹⁰,¹¹. Fluorescence for each cycle was analysed quantitatively and gene expression normalised relative to the expression of the housekeeping gene for 18S rRNA (18S ribosomal RNA Taqman Control Reagent kit), which was multiplexed together with the gene of interest. Probes and primers were designed using a Primer Express program and were purchased from Applied Biosystems (ABI, Foster City, CA, USA). Results were expressed relative to non-diabetic ApoE⁻/⁻ mice, which were arbitrarily assigned a value of 1.

**Immunohistochemistry:** Paraffin sections (4μm) of aorta were used to stain for nitrotyrosine (rabbit polyclonal; Millipore, Billerica, MA, USA; 1:100), F4/80 (rat monoclonal; Abcam, Cambridge, MA, USA; 1:25), MCP-1 (rabbit polyclonal; BioVision, CA, USA; 1:100) and 4-HNE (rabbit polyclonal; Abcam, Cambridge MA, USA; 1:125) as previously described. In brief, sections were dewaxed, hydrated and quenched in 3% H₂O₂ in TRIS-buffered saline (pH 7.6) to inhibit endogenous peroxidase activity. Sections were incubated with Protein Blocking Agent (Thermo Electron,
Pittsburgh, PA, USA) for 60 minutes at room temperature, then in primary antibody overnight at 4°C in a humidified chamber. Sections were incubated in the appropriate biotinylated secondary antibody (all from Vector Laboratories), followed by horseradish peroxidase-conjugated streptavidin (Vectastain Elite ABC Staining Kit; Vector Laboratories). Staining was also amplified further by Dako Catalysed Signal Amplification Kit, according to instructions (Dako Cytomation). All signals were visualised with 3,3’-diamino-benzidine terahydrochloride/H₂O₂ (DAB; Sigma-Aldrich, St Louis, MO, USA). Finally, sections were counterstained with Mayer’s haematoxylin, dehydrated and coverslipped. All sections were examined under light microscopy (Olympus BX-50; Olympus Optical) and digitised with a high-resolution camera. All digital quantification (Image-Pro Plus; v6.0) and assessments were performed in a blinded manner. Results were expressed relative to non-diabetic ApoE⁻/⁻ mice, which were arbitrarily assigned a value of 1.

*Measurement of H₂O₂ and Superoxide Production in Aortic Tissue:* Hydrogen peroxide generation was measured in 10-15mm segments of aortae by Amplex red using a commercial kit following manufacturer’s instructions (Molecular Probes, Eugene, OR, USA). In short, cytosolic extractions were prepared as previously described¹². Twenty microliters of cytosolic isolate, standards and blank were assayed in duplicates in black 96-well plates after addition of pre-warmed (37°C) working solution containing 100µM Amplex red reagent and 0.2U/ml horseradish peroxidase. Fluorescence intensity was measured on a fluorescence microplate reader after 30 minutes at excitation of 544nm and emission of 590nm at 37°C. Superoxide was measured in 10-15mm segments of aorta removed from all mice using a previously established method ¹³. In brief, tissue samples were incubated with 500µl of PBS/100 mM DTPA containing 30mM Dihydrorhodidium (DHE; Molecular Probes, Eugene, OR USA) for 30 minutes. Tissue samples were snap frozen, homogenised and resuspended in 500µl acetonitrile and sonicate and vacuumed dried. Dried samples were resuspended in 120µl of 20% Methanol, 79.9% Water and 0.1% Trifluoroacetic acid. Samples were then injected into an HPLC, calibrated for measurement of DHE¹³.
Cell Culture Experiments: Human aortic endothelial cells (HAECs) were obtained from Clonetics (Lonza, USA) and grown into 75cm² culture flasks (250,000 per flask with humidified incubator, 5% CO₂) at 37°C in the presence of EGM-2 Bulletkit supplemented with endothelial growth media. HAECs were used between passages six and eleven. EGM-2 containing 25mmol/l glucose (Sigma Chemical, St Louis, MO, USA) was used as a high glucose condition in all the experiments, while regular EGM-2, which contains 5.6mmol/l glucose, was used as the normal glucose condition. 25mM mannitol was used as an osmotic control. In all experiments, HAECs were used at 80% to 90% confluence. On the day of the experiment, cells were washed with PBS, trypsinized and re-suspended in fresh EGM-2. 30,000 cells per ml were then plated into 6-well plates and incubated for two days, at which time point media were removed and fresh media supplemented with GKT137831 at either 0µM, 1µM or 10µM and incubated for an additional 24 hours. The cells were then harvested for isolation of mRNA and gene expression analyses (RT-PCR), which were carried out as outlined above.

siRNA knockdown of Nox1 and Nox4 in vitro: The knockdown of Nox1 and Nox4 was performed using HAECs by MISSION siRNA expressing lentivirus vectors (Sigma) as described previously. The sequence for Nox1 knockdown is TRC0000046083, 5’GCCTATATGATCTGCCTACAT3’ and for Nox4 TRC0000046092, 5’GAGCCTCAGCATCTGTTCTTA3’ in addition to a non-target control 5’CAACAAGATGAAGAGCACAA3’. siRNA lentivirus particles were produced in 293FT cells by co-transfection with ViraPower Lentiviral Packaging Mix (Invitrogen, Molecular Probes, USA). The cells transduced with the MISSION Non-target shRNA control vector particles (Sigma-Aldrich, St Louis, MO, USA) were used as controls. HAECs were seeded at 1 x10⁶ cells/dish in a 100mm dish and infected by the lentivirus particles in the presence of 8µg/ml polybrene for 2 days, followed by selection in puromycin (1µg/ml; Sigma-Aldrich, St Louis, MO, USA) for 5 days. The knockdown efficiency in the cells was verified by qRT-PCR and was greater than 90% for Nox1 and 90% for Nox4.
**Measurement of ROS in vitro:** ROS was measured in HAEC cells (+/- glucose) using the Amplex red commercial kit following manufacturer’s instructions (Molecular Probes, Eugene, OR, USA). In short, HAEC cells were grown in 75cm² flasks and were pre-treated with GKT (0µM, 1µM, 10µM) for 1 hour at which point cells were harvested for whole cell were prepared as previously described.22 Twenty microliters of whole cell preparations, standards and blank were assayed in triplicates in black 96-well plates after addition of pre-warmed (37°C) working solution containing 100µM Amplex red reagent and 0.2U/ml horseradish peroxidase. Fluorescence intensity was measured on a fluorescence microplate reader after 30 minutes at excitation of 544nm and emission of 590nm at 37°C. Furthermore, HAEC cells (+/- glucose) were washed with PBS, trypsinized and re-suspended before 200µl of cells added to each well of a black 96-well microplate, and placed into incubator at 37°C for 24 hours. On the day of the experiment cells were washed with PBS (with Ca/Mg ions) and 100µl of DCFDA (Invitrogen Molecular Probes, USA) at a concentration of 10µM was added to each well and left for 40mins at 37°C. Each well was washed with PBS (with Ca/Mg ions) twice, and 100µl of PBS was added and immediately read with VICTOR microplate reader (485/535nm) every 5 minutes for 60 minutes. Additionally, DHE signal was measured in cells with fluorescent dye Dihydroethidium (DHE, Molecular probes, Eugene, OR, USA) using flow cytometry. HAECS were grown in 25cm² flasks and were pre-treated with GKT137831 (0µM, 1µM or 10µM) for 1 hour at which point cells were washed with PBS, trypsinized and re-suspended in 2ml of PBS and spun at 1800rpm for 5 minutes at room temperature. PBS was removed and cell pellet was re-suspended in 1ml of PBS and stained with DHE fluorescent dye (2µg/ml) for 30 minutes at 37°C. Cells were spun down (1800rpm for 5 minutes) and PBS removed, remaining cell pellet was re-suspended in 300µl of PBS. Fluorescence intensities were monitored and recorded using a FACSan Cell Sorter (BD Bioscience). A minimum of 10,000 events/sample was collected, with data analyzed using CellQuest Pro Software (BD Bioscience)15,16. Lastly, HAECs were washed with PBS, trypsinized and re-suspended in 20ml of fresh EGM-2 medium, with 200µl of cells added to each well of black 96-well microplate and placed into the incubator at 37°C for 24 hours. After 24 hours each well was washed with Krebs-HEPES warmed to 37°C. After washing, each well had 100µl of Krebs-HEPES supplemented with L-
012 (Wako Chemicals, Virginia, USA) at a concentration of 100µM in the dark and incubated at 37°C for 15 minutes. After incubation, plates were read in a luminometer (Berthold Technologies, Germany) and read every 5 minutes for 60 minutes at 37°C.
Supplementary Table 1:
RT-PCR analysis for the Nox1, Nox2, Nox4 and Nox5 isoforms of the NADPH oxidase family in human AEC grown under normal (5mM) glucose (NG) or high (25mM) glucose (HG) conditions. Data are Mean±SEM with 6 replicates per group. Data were analysed using a One-Way ANOVA with a LSD post-hoc test, *P<0.05 v normal glucose (NG) control.

<table>
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<tr>
<th></th>
<th>Normal Glucose (5mM)</th>
<th>High Glucose (25mM)</th>
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<tr>
<td>Nox1</td>
<td>1.0±0.30</td>
<td>2.5±0.42*</td>
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<td>Nox2</td>
<td>1.0±0.26</td>
<td>5.4±1.28*</td>
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<td>Nox4</td>
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<tr>
<td>Nox5</td>
<td>1.0±0.19</td>
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Supplementary Table 2:
RT-PCR analysis for Nox1, Nox2 and Nox4 expression in HAEC cells transfected with siRNA specific for Nox1 or Nox4. Data are Mean±SEM, with 6 replicates per group. Data were analysed using unpaired, two-tailed T-Test, *P<0.05 v Non-Target.

<table>
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<tr>
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<th>Nox4 siRNA</th>
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Supplementary Table 3:
RT-PCR analysis in HAEC cells transfected with siRNA specific for Nox4 and then grown in the presence of normal (5mM) or high glucose (25mM) conditions for 24 hours. Data are Mean±SEM, with 6 replicates per group. Data were analysed using a One-Way ANOVA with a LSD post-hoc test, *P<0.05 v Non-Target grown in Normal Glucose, #P<0.05 v Non-Target grown in High Glucose.

<table>
<thead>
<tr>
<th></th>
<th>Normal Glucose (5mM)</th>
<th>High Glucose (25mM)</th>
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<td>1.4±0.24</td>
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**Supplementary Table 4.**

Atherosclerotic plaque area within the thoracic and abdominal regions of the aorta from control and diabetic *Nox1*+/−*ApoE*−/−, *Nox1*−/−*ApoE*−/−, *Nox4*+/+*ApoE*−/−, *Nox4*−/−*ApoE*−/− and *ApoE*−/− control and diabetic treated with or without GKT137831 (GKT) for 10wks. Data are Mean±SEM with N=10-15 per group and were analysed separately using One-Way ANOVA with a LSD post-hoc test, there was no statistical significance.

<table>
<thead>
<tr>
<th></th>
<th><em>Nox1</em>+/−<em>ApoE</em>−/−</th>
<th><em>Nox1</em>−/−<em>ApoE</em>−/−</th>
<th><em>Nox4</em>+/+<em>ApoE</em>−/−</th>
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<td>Diabetic</td>
<td>Control</td>
<td>Diabetic</td>
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<tr>
<td><strong>Thoracic Aorta</strong></td>
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<td>1.3±0.31</td>
<td>0.4±0.14</td>
<td>0.6±0.29</td>
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<td><strong>Abdominal Aorta</strong></td>
<td>0.9±0.86</td>
<td>2.2±0.52</td>
<td>1.8±0.42</td>
<td>1.7±0.44</td>
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<th><em>ApoE</em>−/−</th>
<th><em>ApoE</em>−/− &amp; GKT</th>
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<td><strong>Thoracic Aorta</strong></td>
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<td>1.5±0.40</td>
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Supplementary Figure 1:

*En Face* aortic images showing aortic arch and thoracic for *ApoE<sup>−/−</sup>* Control, *ApoE<sup>−/−</sup>* Diabetic, *Nox1<sup>−/−</sup>* *ApoE<sup>−/−</sup>* Diabetic, *Nox4<sup>−/−</sup>* *ApoE<sup>−/−</sup>* Diabetic and *ApoE<sup>−/−</sup>* Diabetic treated with GKT after 10 weeks. Arrows demonstrate atherosclerotic plaques.
Supplementary Figure 2:

Survival curves for non-diabetic (C) and diabetic Nox2\textsuperscript{-/-} animals (D) over 20 weeks of diabetes. The induction of diabetes by STZ resulted in reduced survival of Nox2\textsuperscript{-/-} mice, with mortality rates > 50% after 12 weeks of diabetes and 100% by 20 weeks.
Supplementary References


