

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

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# 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.<sup>1,2</sup> 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.<sup>3,4</sup> 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.<sup>5,6</sup> 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.<sup>7,8</sup> 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.<sup>6,9–15</sup>

## Editorial see p 1850 Clinical Perspective on p 1902

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.<sup>16–19</sup> Thus, Nox1 is a potential therapeutic target in diabetes mellitus–related vasculopathies. Similar roles in vascular disease have been suggested for Nox2,

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Nox4 and Nox5.<sup>10,20–23</sup> 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.<sup>20,21</sup> The role of the more recently discovered isoform Nox5 in the vasculature has not been clearly determined, and this isoform is not present in the mouse or rat.<sup>6,24</sup>

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.<sup>5,6,18</sup> GKT137831 now represents the first specific, orally and chronically active Nox inhibitor with a relative specificity for Nox1 and Nox4.<sup>25,26</sup>

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*<sup>−/−</sup>]) 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 isoform.

## Methods

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

### Animal Models

Nox1<sup>27</sup> and Nox4<sup>20</sup> knockout mice were backcrossed onto *ApoE*<sup>−/−</sup> mice (ARC, WA, Australia) for 10 generations to generate double knockout animals (Transgenix, USA). Six-week-old *Nox4*<sup>−/−</sup>*ApoE*<sup>−/−</sup>, *Nox4*<sup>+/+</sup>*ApoE*<sup>−/−</sup>, *Nox1*<sup>−/−</sup>*ApoE*<sup>−/−</sup>, *Nox1*<sup>+/+</sup>*ApoE*<sup>−/−</sup> and *ApoE*<sup>−/−</sup> male mice were rendered diabetic by 5 daily IP injections of streptozotocin (Sigma-Aldrich, St Louis, MO) at a dose of 55 mg/kg.<sup>28</sup> A subgroup of diabetic and nondiabetic *ApoE*<sup>−/−</sup> mice were administered the Nox inhibitor, GKT137831 (GKT) by daily gavage at a dose of 60 mg/kg/d for 10 weeks. After 10 weeks, animals were anaesthetised by sodium pentobarbitone IP (100 mg/kg body weight; Euthatal, Sigma-Aldrich, Castle Hill, NSW, Australia) and organs were rapidly dissected.

### Nox Inhibition

GKT137831, a member of the pyrazolopyridine dione family, is a specific inhibitor of both Nox1 and Nox4.<sup>25,26</sup>

### 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.<sup>29</sup>

### 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.<sup>30</sup>

## 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.<sup>31</sup> Gene expression were analyzed quantitatively as previously described.<sup>31</sup>

## Immunohistochemistry

Paraffin sections of aorta were used to stain for nitrotyrosine (Millipore, Billerica, MA), F4/80 (Abcam, Cambridge, MA), monocyte chemoattractant protein 1 (MCP-1; BioVision, CA) and 4-Hydroxynonenal (4-HNE) (Abcam, Cambridge MA) as previously described.<sup>31</sup>

## Measurement of H<sub>2</sub>O<sub>2</sub> and Superoxide Production in Aortic Tissue

Aortic hydrogen peroxide generation was measured by Amplex red using a commercial kit (Molecular Probes, Eugene, OR).<sup>32</sup> Aortic superoxide was measured using high pressure liquid chromatography calibrated to measure DHE by a previously established method.<sup>33</sup>

## 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.<sup>34</sup> 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).<sup>32,35</sup>

## Statistical Analysis

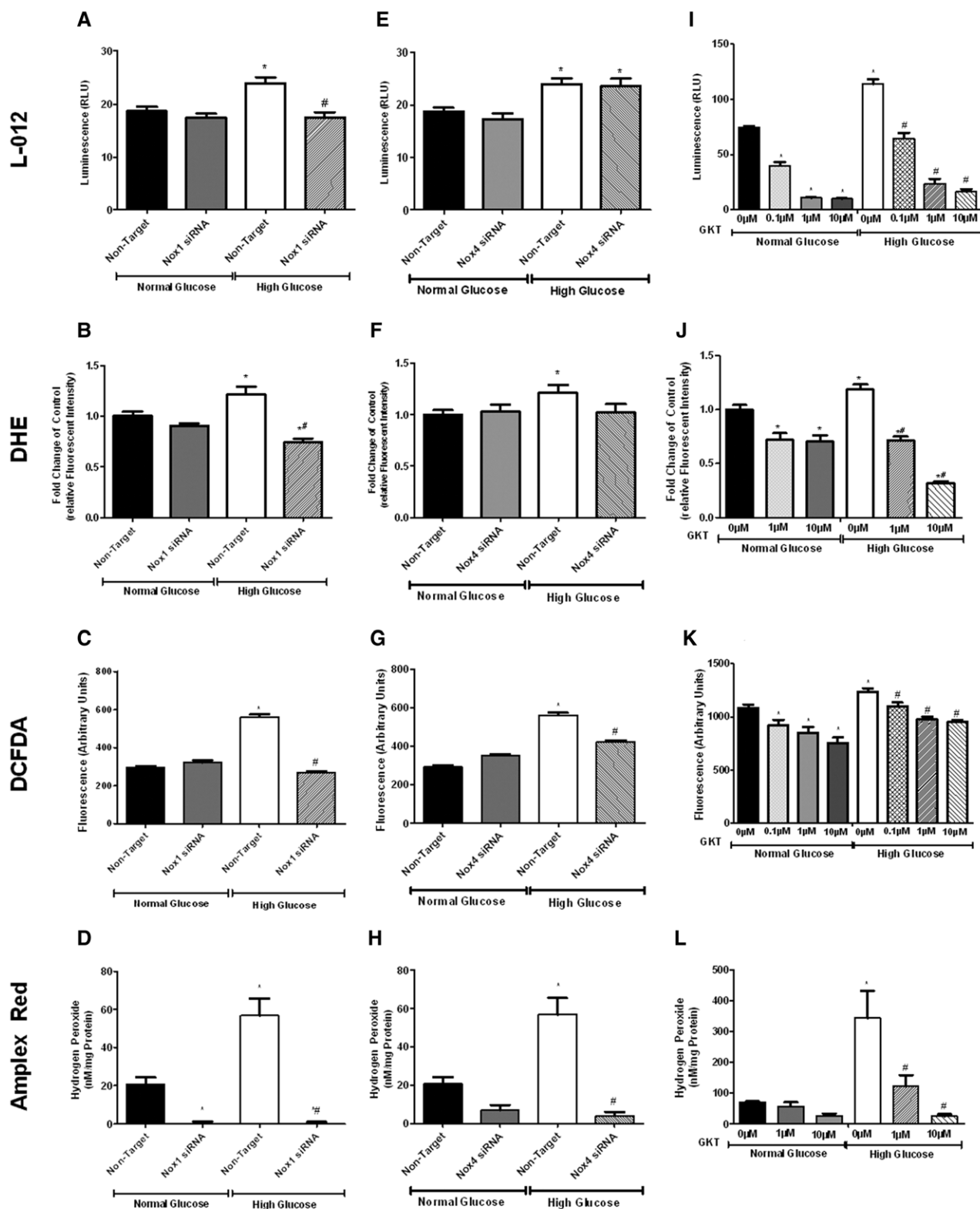
Data were analyzed for normality using the Shapiro-Wilk test before being analysed either with a 1-way ANOVA or 2-way ANOVA using SPSS Statistics version 20 (IBM) with an LSD post hoc test for multiple comparison of the means. Effects of drug dose were assessed by ANCOVA with repeated measures. *P* < 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 isoform, 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,  $F=16.6$ ,  $P<0.001$ ). 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  $\mu\text{mol/L}$ , 1  $\mu\text{mol/L}$ , or 10  $\mu\text{mol/L}$ ) 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), DHE ( $F=49.1$ ,  $P<0.01$ , Figure 1J), DCFDA ( $F=22.0$ ,  $P<0.01$ , 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 enhanced 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 caused a significant reduction in the expression of MCP-1, VCAM-1, CTGF, and fibronectin and collagen IV when compared with HAECs grown in high glucose alone (Table 2).

**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**

	Normal Glucose (5 mmol/L)		High Glucose (25 mmol/L)	
	Nontarget	Nox1 siRNA	Nontarget	Nox1 siRNA
Nox1	1.0 $\pm$ 0.30	0.1 $\pm$ 0.01*	2.5 $\pm$ 0.42*	0.1 $\pm$ 0.01*#
VCAM-1	1.0 $\pm$ 0.27	0.6 $\pm$ 0.15*	4.9 $\pm$ 0.66*	1.0 $\pm$ 0.34#
MCP-1	1.0 $\pm$ 0.27	1.1 $\pm$ 0.17	4.7 $\pm$ 1.04*	0.7 $\pm$ 0.29#
CTGF	1.0 $\pm$ 0.32	0.7 $\pm$ 0.21	1.8 $\pm$ 0.26*	0.6 $\pm$ 0.22#
Collagen IV	1.0 $\pm$ 0.38	2.4 $\pm$ 0.66	4.2 $\pm$ 0.17*	1.9 $\pm$ 0.72#
Fibronectin	1.0 $\pm$ 0.25	1.4 $\pm$ 0.28	3.1 $\pm$ 0.64*	2.2 $\pm$ 0.35#

Data are Mean $\pm$ 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 knock-out animals bred onto the *ApoE*<sup>−/−</sup> background (>10 generations) and observed atherosclerotic plaque area as major read-out. In addition, diabetic wild-type *ApoE*<sup>−/−</sup> 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,

**Table 2. RT-PCR Analysis in HAEC Cells Grown in the Presence of Normal (5 mmol/L) or High Glucose (25 mmol/L) Conditions With and Without GKT137831 (GKT) at a Dose of 1  $\mu\text{mol/L}$  or 10  $\mu\text{mol/L}$  for 24 Hours**

	Normal Glucose			High Glucose (25 mmol/L)		
	0 $\mu\text{mol/L}$ GKT	1 $\mu\text{mol/L}$ GKT	10 $\mu\text{mol/L}$ GKT	0 $\mu\text{mol/L}$ GKT	1 $\mu\text{mol/L}$ GKT	10 $\mu\text{mol/L}$ GKT
VCAM-1	1.0 $\pm$ 0.32	1.4 $\pm$ 0.17#	1.7 $\pm$ 0.24#	8.5 $\pm$ 1.17*	2.3 $\pm$ 0.37*	2.4 $\pm$ 0.47*#
MCP-1	1.0 $\pm$ 0.27	1.6 $\pm$ 0.43#	1.2 $\pm$ 0.43#	5.9 $\pm$ 0.74*	2.1 $\pm$ 0.37#	1.8 $\pm$ 0.35#
CTGF	1.0 $\pm$ 0.15	1.9 $\pm$ 0.70#	1.9 $\pm$ 0.50#	3.6 $\pm$ 0.35*	1.1 $\pm$ 0.29#	0.8 $\pm$ 0.25#
Collagen IV	1.0 $\pm$ 0.20	1.0 $\pm$ 0.24#	1.3 $\pm$ 0.26#	3.5 $\pm$ 0.38*	2.1 $\pm$ 0.37#	2.3 $\pm$ 0.61*#
Fibronectin	1.0 $\pm$ 0.33	1.0 $\pm$ 0.20	0.9 $\pm$ 0.20#	1.7 $\pm$ 0.27*	0.6 $\pm$ 0.22#	0.6 $\pm$ 0.09#

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

\* $P<0.05$  compared to Normal Glucose 0  $\mu\text{M}$  GKT, # $P<0.05$  compared to High Glucose 0  $\mu\text{M}$  GKT.

**Table 3. Metabolic and Biological Data for Control and Diabetic *Nox1<sup>+/-</sup>ApoE<sup>-/-</sup>*, *Nox1<sup>-/-</sup>ApoE<sup>-/-</sup>*, *Nox4<sup>+/-</sup>ApoE<sup>-/-</sup>* and *Nox4<sup>-/-</sup>ApoE<sup>-/-</sup>* After 10 Weeks**

	Nox1 Deletion Studies				Nox4 Deletion Studies			
	<i>Nox1<sup>+/-</sup>ApoE<sup>-/-</sup></i>		<i>Nox1<sup>-/-</sup>ApoE<sup>-/-</sup></i>		<i>Nox4<sup>+/-</sup>ApoE<sup>-/-</sup></i>		<i>Nox4<sup>-/-</sup>ApoE<sup>-/-</sup></i>	
	Control	Diabetic	Control	Diabetic	Control	Diabetic	Control	Diabetic
Body weight, g	27±1.29	21±0.51*	30±0.22	22±0.65*	29±0.30	24±0.52*†	30±0.85	24±0.75*#†
Systolic BP, mm Hg	101±3	105±2	94±3	98±4	100±3	98±3	99±6	106±4
BG, mmol/L	13.0±0.66	20.0±1.82*	10.2±0.50	20.3±1.49*	11.9±0.50	21.1±1.74*†	13.2±0.89	20.4±1.97*†
HbA1c, %	3.9±0.19	13.4±0.78*	4.5±0.31	15.3±0.69*	7.1±0.23	15.7±0.67*†	4.6±0.29	12.1±0.98*†
Cholesterol, mmol/L	9.7±0.53	15.2±1.26*	9.2±0.50	13.4±1.34*	13.0±1.11	15.0±1.79*	10.1±0.95	12.8±1.50*
Triglycerides, mmol/L	2.1±0.84	4.1±1.52	1.4±0.18	4.5±0.69*	2.3±0.94	3.0±0.67*#	2.4±0.28	3.2±0.75
HDL, mmol/L	2.9±0.93	4.4±1.59	2.3±0.31	1.7±0.32	3.4±0.35	2.7±0.42	2.3±0.38	2.3±0.37
LDL, mmol/L	7.5±0.62	11.6±1.25*	6.2±0.47	9.5±0.91*	8.4±0.97	10.9±0.43*#	6.6±0.58	9.0±1.04

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<sup>+/-</sup>ApoE<sup>-/-</sup>* Control, # compared with *Nox1<sup>+/-</sup>ApoE<sup>-/-</sup>* Diabetic and † compared with *Nox1<sup>-/-</sup>ApoE<sup>-/-</sup>* Control, \* vs *Nox4<sup>+/-</sup>ApoE<sup>-/-</sup>* Control, # vs *Nox4<sup>+/-</sup>ApoE<sup>-/-</sup>* Diabetic and † vs *Nox4<sup>-/-</sup>ApoE<sup>-/-</sup>* Control.

which were not affected by deletion of Nox1 or Nox4 (Tables 3 and 4). Similarly, GKT137831-treated diabetic *ApoE<sup>-/-</sup>* mice had similar body weights, blood pressures, and metabolic data, including total cholesterol and LDL levels compared with untreated diabetic *ApoE<sup>-/-</sup>* mice (Table 4).

#### Atherosclerotic Plaque Area

Aortic atherosclerotic plaque area was measured in the aortic arch as well as thoracic and abdominal parts 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<sup>-/-</sup>ApoE<sup>-/-</sup>* mice, both in the total aorta and even more pronounced in the aortic arch when compared to diabetic *Nox1<sup>+/-</sup>ApoE<sup>-/-</sup>* mice (Figure 2A). In accordance, treatment of diabetic *ApoE<sup>-/-</sup>* mice with GKT137831 fully prevented the development of atherosclerotic plaques within the total aorta (diabetes mellitus,  $F=6.6$ ,  $P<0.01$ ; GKT137831 treatment  $F=9.2$ ,  $P<0.01$ ; interaction of GKT137831 and diabetes

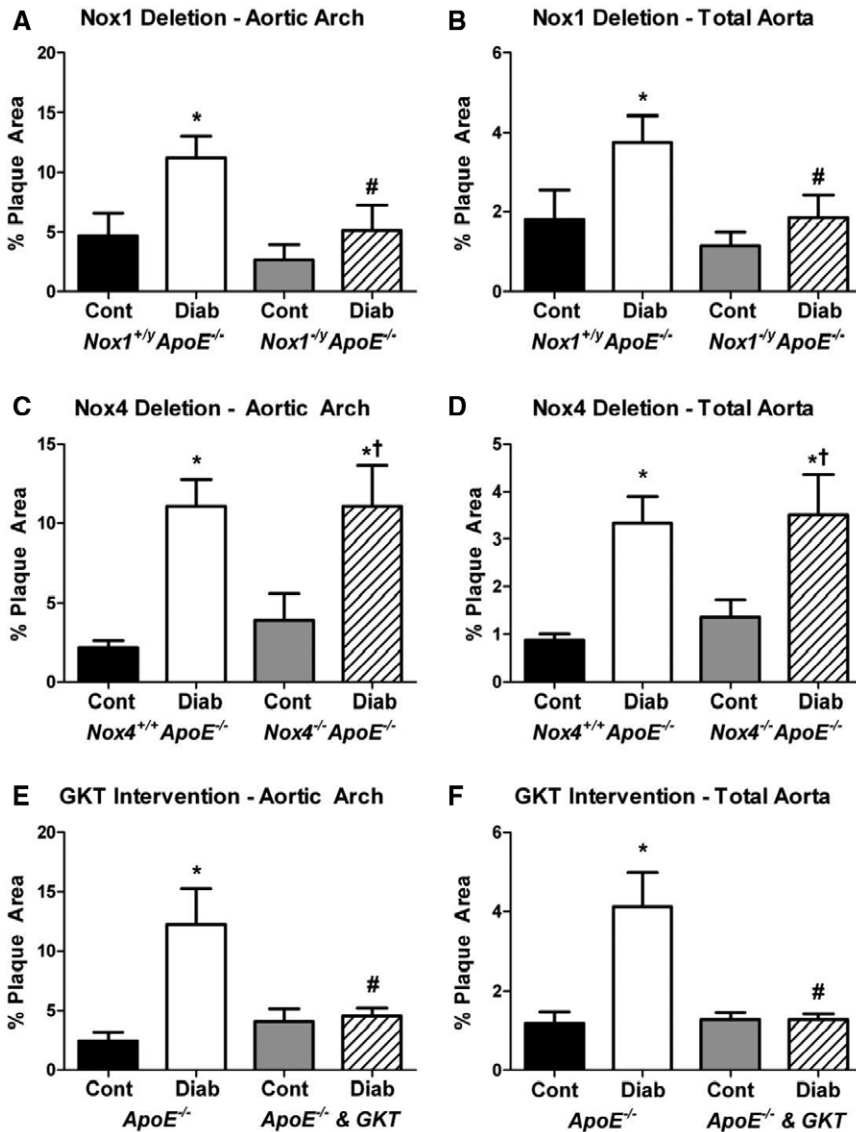
mellitus,  $F=6.7$ ,  $P<0.01$ ) and aortic arch in comparison to untreated diabetic *ApoE<sup>-/-</sup>* mice. Treatment of nondiabetic *ApoE<sup>-/-</sup>* mice did not result in any change in atherosclerotic plaque area when compared with untreated nondiabetic *ApoE<sup>-/-</sup>* mice (Figure 2E and 2F). In contrast, in diabetic *Nox4<sup>-/-</sup>ApoE<sup>-/-</sup>* 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<sup>-/-</sup>* 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 4. Metabolic and Biological Data for *ApoE<sup>-/-</sup>* Control and Diabetic Treated With or Without GKT137831 (GKT) for 10 Weeks (60 mg/kg/d)**

	<i>ApoE<sup>-/-</sup></i>		<i>ApoE<sup>-/-</sup></i> & GKT137831	
	Control	Diabetic	Control	Diabetic
Body weight, g	28±1.17	24.8±0.77*	28.2±0.42	24.8±0.40*†
Systolic BP, mm Hg	102±3	99±3	105±3	107±3
BG, mmol/L	10.4±0.94	21.8±2.11*	13.1±0.50	23.3±1.62*
HbA1c, %	4.5±0.47	11.4±0.88*	4.9±0.78	10.8±0.90*†
Cholesterol, mmol/L	6.7±0.53	12.3±1.05*	7.7±0.41	10.5±0.80*†
Triglycerides, mmol/L	0.8±0.07	1.7±0.39*	0.8±0.14	1.0±0.18#
HDL, mmol/L	1.6±0.11	2.4±0.14*	1.7±0.17	1.7±0.25#
LDL, mmol/L	4.7±0.45	9.0±0.70*	5.9±0.43	7.4±0.61*†

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<sup>-/-</sup>* Control, # compared with *ApoE<sup>-/-</sup>* Diabetic and † compared with *ApoE<sup>-/-</sup>* Control & GKT137831.



**Figure 2.** Deletion of Nox1 and treatment of *ApoE<sup>-/-</sup>* mice with GKT137831 reduced the development of atherosclerosis in diabetes mellitus. Atherosclerotic plaque area measured at 10 weeks after induction of diabetes mellitus within the aortic arch (**A**, **C**, and **E**) and total aorta (**B**, **D**, and **F**) for control (Cont) and diabetic (Diab) *Nox1<sup>+/-</sup>ApoE<sup>-/-</sup>*, *Nox1<sup>-/-</sup>ApoE<sup>-/-</sup>* mice, and control and diabetic *Nox4<sup>+/-</sup>ApoE<sup>-/-</sup>*, *Nox4<sup>-/-</sup>ApoE<sup>-/-</sup>* mice. In addition, plaque area was measured in control (Cont) and diabetic (Diab) *ApoE<sup>-/-</sup>* mice treated with or without GKT137831 (GKT) for 10 wks (60 mg/kg/d). Data are mean±SEM (n=8–10/group). Groups were analyzed separately using ANOVA with a LSD post hoc test.  $P<0.05$  \*compared with *Nox1<sup>+/-</sup>ApoE<sup>-/-</sup>* Control, #compared with *Nox1<sup>+/-</sup>ApoE<sup>-/-</sup>* or *ApoE<sup>-/-</sup>* Diabetic, \* vs *Nox4<sup>+/-</sup>ApoE<sup>-/-</sup>* Control, and † vs *Nox4<sup>+/-</sup>ApoE<sup>-/-</sup>* Diabetic and † vs *Nox4<sup>-/-</sup>ApoE<sup>-/-</sup>* control. Nox indicates NADPH oxidase.

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.

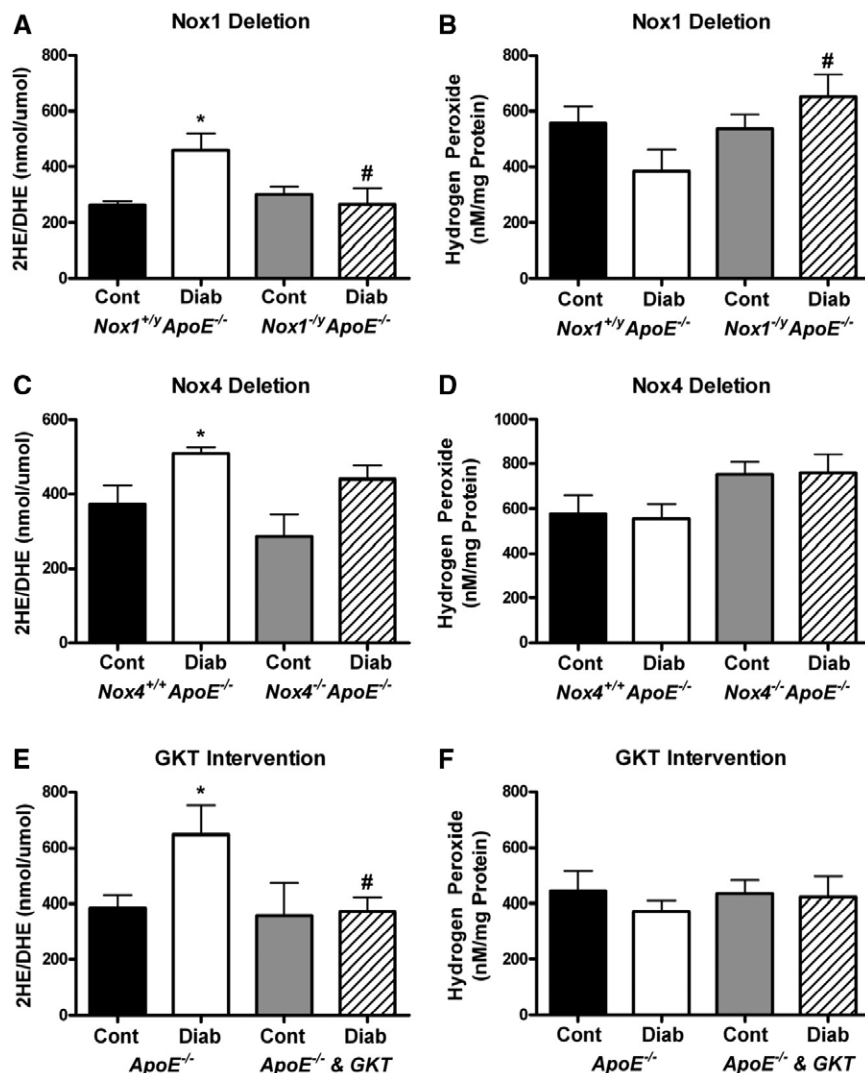
#### Aortic ROS (Superoxide and $H_2O_2$ ) Production

Only superoxide production (as assessed by DHE staining using HPLC) but not extracellular  $H_2O_2$  (as assessed using Amplex Red) was significantly increased in the diabetic *ApoE<sup>-/-</sup>* groups compared with nondiabetic *ApoE<sup>-/-</sup>* 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<sup>-/-</sup>ApoE<sup>-/-</sup>* animals when compared with control and diabetic *Nox1<sup>+/-</sup>ApoE<sup>-/-</sup>* mice (Figure 3A,  $F=6.7$ ,  $P<0.01$ ). The production of superoxide was significantly attenuated in diabetic *ApoE<sup>-/-</sup>* treated with GKT137831 compared with untreated diabetic *ApoE<sup>-/-</sup>* animals (Figure 3E). Administration of GKT137831 to both control and diabetic *ApoE<sup>-/-</sup>* mice did not have a significant effect on  $H_2O_2$  levels when compared with untreated control

and diabetic *ApoE<sup>-/-</sup>* mice (Figure 3F). Furthermore, in contrast to diabetic *Nox1<sup>-/-</sup>ApoE<sup>-/-</sup>* mice, diabetic *Nox4* deficient *ApoE<sup>-/-</sup>* mice did not show attenuation of superoxide or  $H_2O_2$  production within the aorta (Figure 3C). These data suggested that Nox1-derived superoxide but not  $H_2O_2$  is the key form of ROS triggering diabetic atherosclerosis.

#### Aortic Nitritative and Oxidative Stress

ROS can interact with either NO and nitrite to cause nitritative 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<sup>-/-</sup>ApoE<sup>-/-</sup>* mice in comparison with diabetic *Nox1<sup>+/-</sup>ApoE<sup>-/-</sup>* mice. Similarly, diabetic *ApoE<sup>-/-</sup>* mice treated with GKT137831 displayed a significant reduction in NT staining when compared with the untreated diabetic *ApoE<sup>-/-</sup>* mice (Figure 4). GKT137831 treatment also resulted in reduced



**Figure 3.** Diabetes mellitus–induced elevation in superoxide production is ameliorated by deletion of Nox1 and treatment of *ApoE<sup>-/-</sup>* with GKT137831. Whole aorta superoxide production measured by DHE/HPLC (A, C, and E) and  $H_2O_2$  (B, D, and F) released measured by Amplex red in aortas from control (Cont) and diabetic (Diab) *Nox1<sup>+/+</sup>ApoE<sup>-/-</sup>*, *Nox1<sup>-/-</sup>ApoE<sup>-/-</sup>* (A and B) in addition to control and diabetic *Nox4<sup>+/+</sup>ApoE<sup>-/-</sup>* and *Nox4<sup>-/-</sup>ApoE<sup>-/-</sup>* (C and D) mice after 10 wks. Furthermore, superoxide and hydrogen peroxide were measured in control and diabetic *ApoE<sup>-/-</sup>* mice treated with or without GKT137831 (GKT, 60 mg/kg/d) after 10 weeks (E and F). Data are mean±SEM (n=6/group). Groups were analyzed separately using ANOVA with a LSD post hoc test,  $P<0.05$  \*compared with *Nox1<sup>+/+</sup>ApoE<sup>-/-</sup>* or *ApoE<sup>-/-</sup>* Control, #compared with *Nox1<sup>+/+</sup>ApoE<sup>-/-</sup>* or *ApoE<sup>-/-</sup>* Diabetic, \* vs *Nox4<sup>+/+</sup>ApoE<sup>-/-</sup>* Control, # vs *Nox4<sup>+/+</sup>ApoE<sup>-/-</sup>* Diabetic and † vs *Nox4<sup>-/-</sup>ApoE<sup>-/-</sup>* Control. DHE indicates dihydroethidium; and HPLC, high pressure liquid chromatography.

4-HNE staining in diabetic *ApoE<sup>-/-</sup>* but not control *ApoE<sup>-/-</sup>* 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<sup>-/-</sup>* mice when compared with diabetic *Nox4<sup>+/+</sup>ApoE<sup>-/-</sup>* 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<sup>-/-</sup>ApoE<sup>-/-</sup>* mice in comparison with control and diabetic *Nox1<sup>+/+</sup>ApoE<sup>-/-</sup>* mice (Figure 6A,  $F=8.2$ ,  $P<0.01$ ). Similarly, diabetic *ApoE<sup>-/-</sup>* mice treated with GKT137831 demonstrated reduced F4/80 staining in comparison with untreated diabetic *ApoE<sup>-/-</sup>* mice (Figure 6C). Again, deletion of Nox4 did not result in a significant reduction in staining for F4/80 in diabetic *Nox4<sup>-/-</sup>ApoE<sup>-/-</sup>* mice in comparison with diabetic *Nox4<sup>+/+</sup>ApoE<sup>-/-</sup>* mice (Figure 6B).

#### Aortic Vascular Adhesion

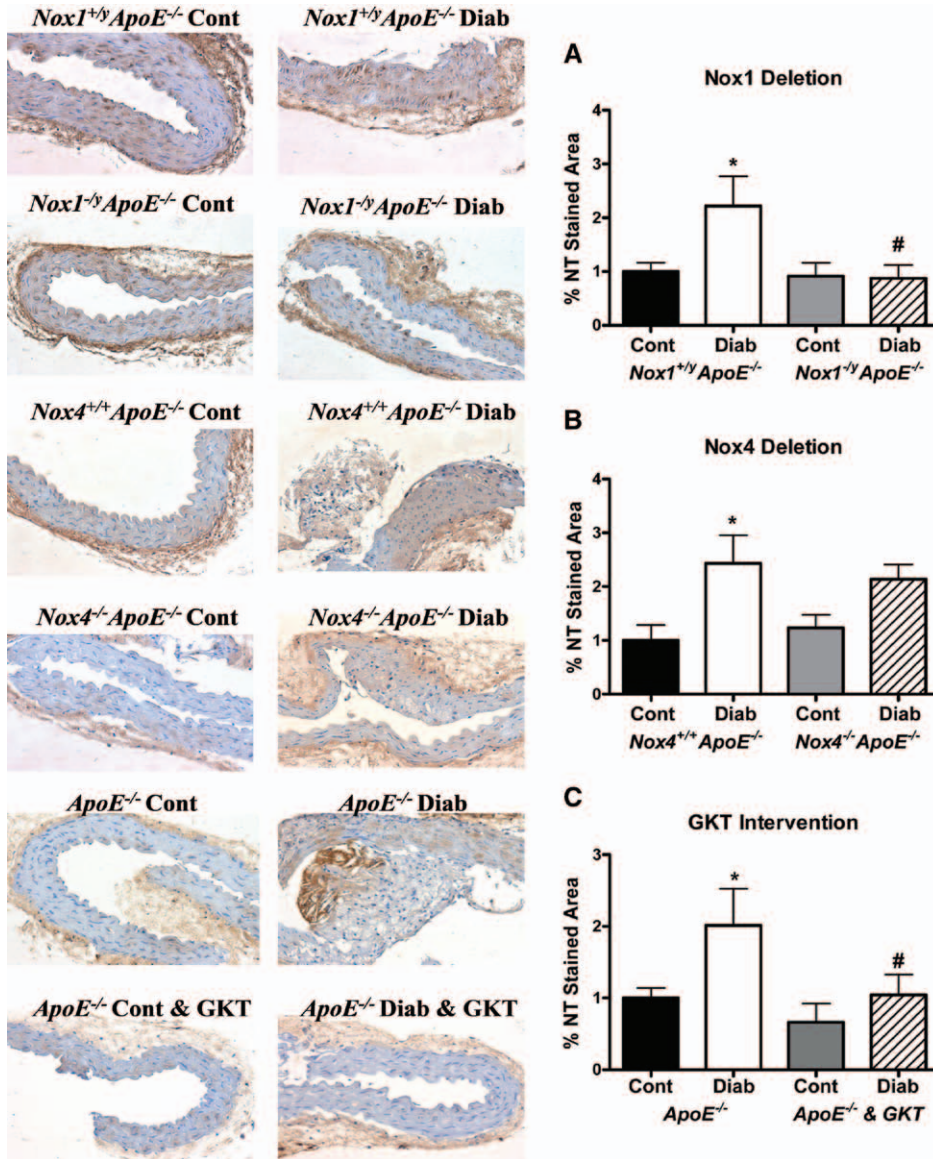
Adhesion of leukocytes to the endothelial cell layer is a key step in the initiation of atherosclerosis.<sup>36</sup> Examination of leukocyte adhesion to the aorta using vessel chamber experiments over a period of 10 minutes demonstrated a significant increase in the number of leukocytes that adhered to the aortic wall of diabetic *ApoE<sup>-/-</sup>* mice in comparison to nondiabetic *ApoE<sup>-/-</sup>* mice (Figure 7A and 7B). Deletion of Nox1 significantly reduced the number of leukocytes attached to the aortic wall compared with diabetic *ApoE<sup>-/-</sup>* mice. Similarly treatment of diabetic *ApoE<sup>-/-</sup>* mice with GKT137831 significantly reduced the number of adherent leukocytes (Figure 7A and 7B). Deletion of the Nox4 isoform did not result in attenuation of the number of leukocytes adhering to the aortic wall (Figure 7A and 7B).

#### 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





**Figure 4.** Nox1 deletion and GKT137831 treatment attenuates diabetes mellitus–induced elevation of aortic nitrotyrosine levels. Nitrotyrosine immunohistochemistry photomicrographs and quantification in the aorta of control (Cont) and diabetic (Diab) *Nox1<sup>+/-</sup>ApoE<sup>-/-</sup>*, *Nox1<sup>-/-</sup>ApoE<sup>-/-</sup>* (A) and control and diabetic *Nox4<sup>+/-</sup>ApoE<sup>-/-</sup>* and *Nox4<sup>-/-</sup>ApoE<sup>-/-</sup>* (B) mice. In addition, nitrotyrosine was measured in control (Cont) and diabetic (Diab) *ApoE<sup>-/-</sup>* mice (C) treated with or without GKT137831 (GKT) for 10 wks (60 mg/kg/d). Data are mean±SEM (n=8–10/group). Groups were analyzed separately using ANOVA with a LSD post hoc test,  $P<0.05$  \*compared with *Nox1<sup>+/-</sup>ApoE<sup>-/-</sup>* or *ApoE<sup>-/-</sup>* Control, #compared with *Nox1<sup>+/-</sup>ApoE<sup>-/-</sup>* or *ApoE<sup>-/-</sup>* Diabetic, \* vs *Nox4<sup>+/-</sup>ApoE<sup>-/-</sup>* Control, # vs *Nox4<sup>+/-</sup>ApoE<sup>-/-</sup>* Diabetic and † vs *Nox4<sup>-/-</sup>ApoE<sup>-/-</sup>* Control.

diabetes mellitus–induced increase was again attenuated in the diabetic but not control *Nox1<sup>-/-</sup>ApoE<sup>-/-</sup>* ( $F=9.9$ ,  $P<0.01$ ) and in the GKT137831-treated diabetic *ApoE<sup>-/-</sup>* mice (Figure 7C and 7E), however there was no change in MCP-1 gene expression in diabetic *Nox4<sup>-/-</sup>ApoE<sup>-/-</sup>* mice compared with *Nox4<sup>+/-</sup>ApoE<sup>-/-</sup>* 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<sup>-/-</sup>* mice with GKT137831 resulted in reduced MCP-1 staining (Figure 7H).

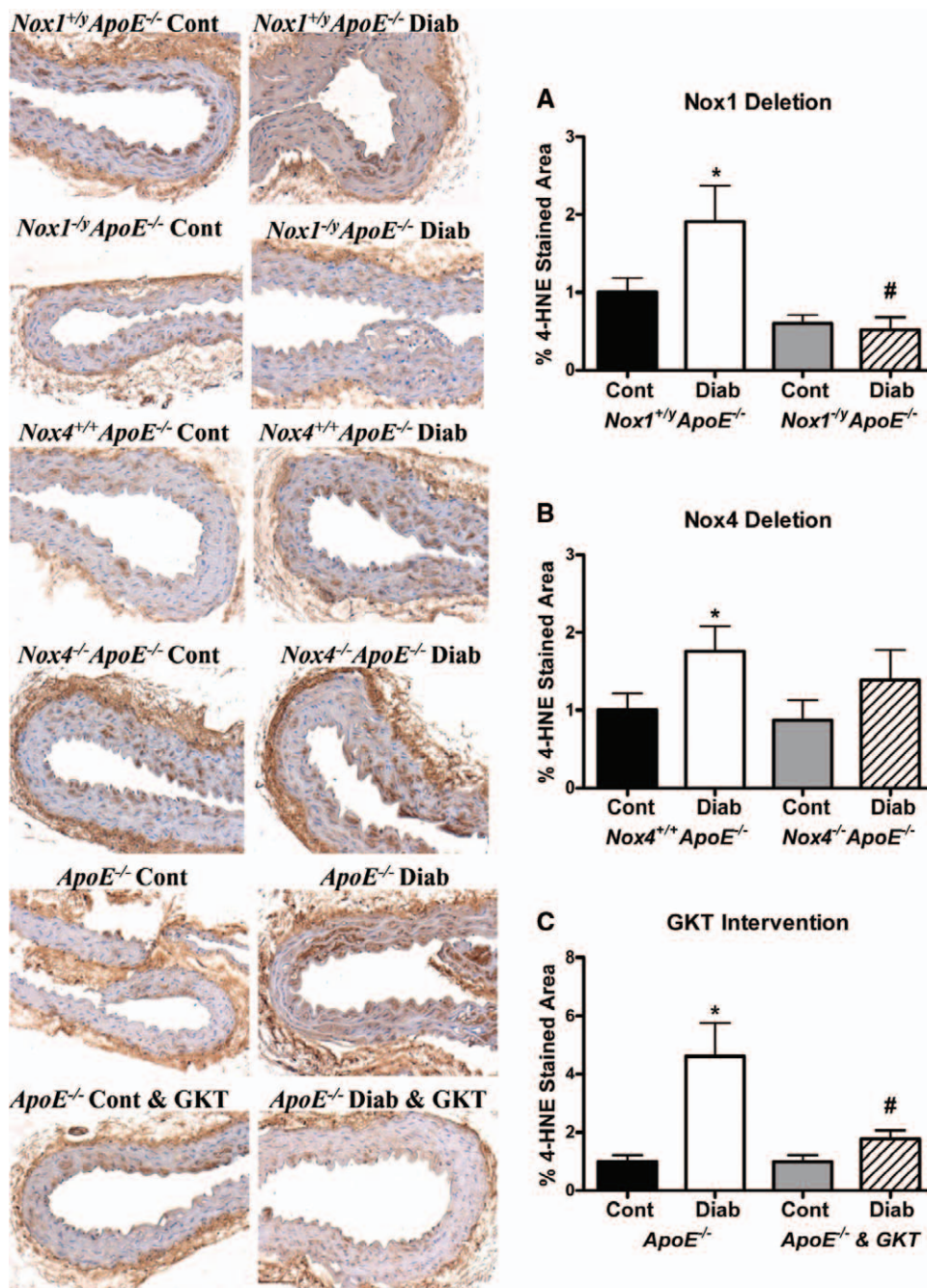
As expected, diabetes mellitus was associated with a significant increase in the gene expression of aortic VCAM-1, which was attenuated in *Nox1<sup>-/-</sup>ApoE<sup>-/-</sup>* and GKT137831 treated diabetic *ApoE<sup>-/-</sup>* mice but not in *Nox4<sup>-/-</sup>ApoE<sup>-/-</sup>* mice (Figure 8A–8C).

Fibrosis is also a potent feature of diabetes mellitus–associated atherosclerosis.<sup>37</sup> 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<sup>-/-</sup>ApoE<sup>-/-</sup>* animals, and treatment of diabetic *ApoE<sup>-/-</sup>* 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<sup>-/-</sup>ApoE<sup>-/-</sup>* 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



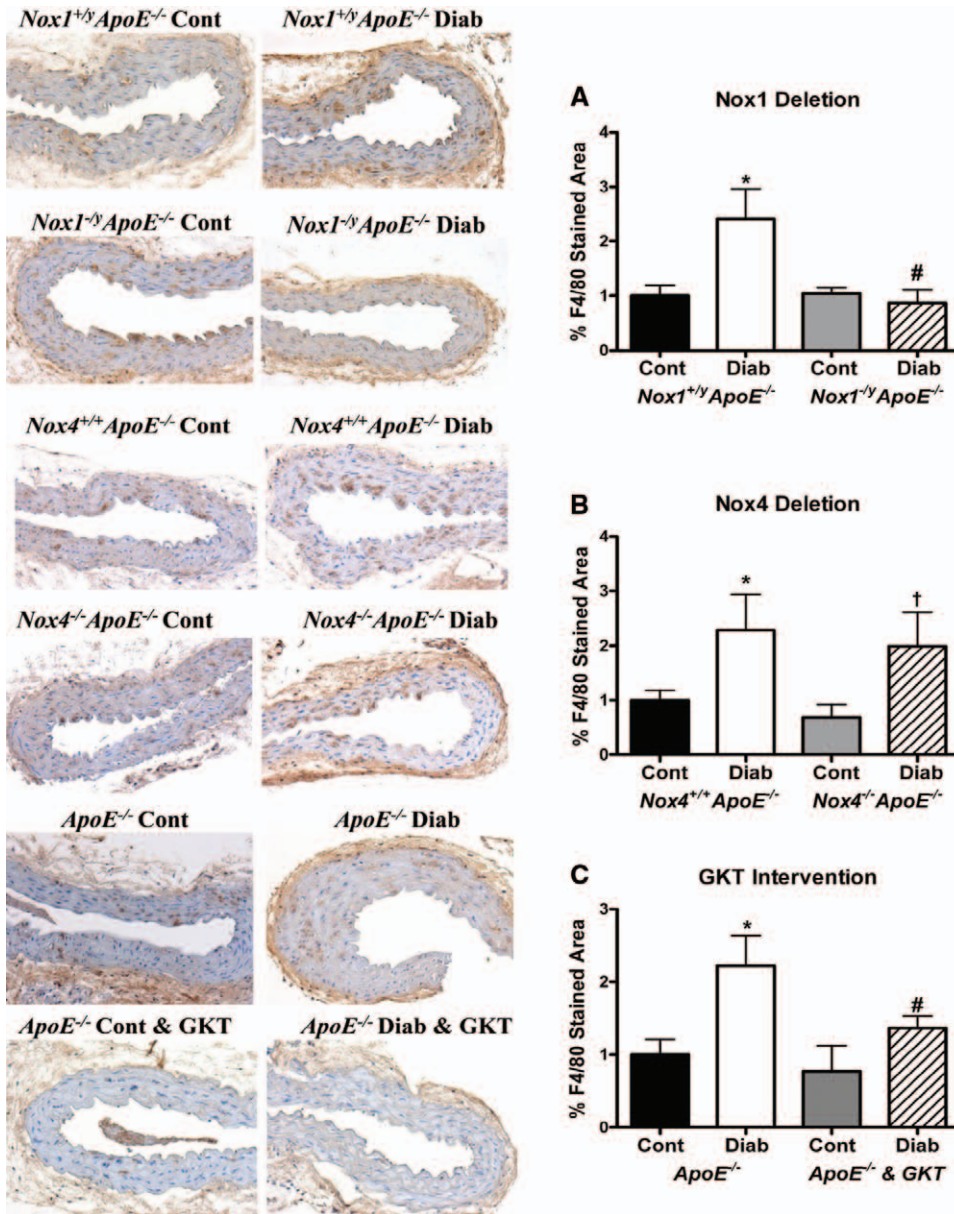
**Figure 5.** Aortic lipid peroxidation (4-Hydroxynoneal) is reduced in diabetic *Nox1<sup>-/-</sup>ApoE<sup>-/-</sup>* and diabetic GKT137831 *ApoE<sup>-/-</sup>* treated mice. 4-Hydroxynoneal immunohistochemistry photomicrographs and quantification in aortas of control (Cont) and diabetic (Diab) *Nox1<sup>+/y</sup>ApoE<sup>-/-</sup>*, *Nox1<sup>-/-</sup>ApoE<sup>-/-</sup>* (A) and control and diabetic *Nox4<sup>+/+</sup>ApoE<sup>-/-</sup>* and *Nox4<sup>-/-</sup>ApoE<sup>-/-</sup>* (B) mice. In addition, 4-Hydroxynoneal was measured in control (Cont) and diabetic (Diab) *ApoE<sup>-/-</sup>* mice (C) treated with or without GKT137831 (GKT) for 10 wks (60 mg/kg/d). Data are mean±SEM (n=8–10/group). Groups were analyzed separately using ANOVA with a LSD post hoc test,  $P<0.05$  \*compared with *Nox1<sup>+/y</sup>ApoE<sup>-/-</sup>* or *ApoE<sup>-/-</sup>* Control, #compared with *Nox1<sup>+/y</sup>ApoE<sup>-/-</sup>* or *ApoE<sup>-/-</sup>* Diabetic, \* vs *Nox4<sup>+/+</sup>ApoE<sup>-/-</sup>* Control, # vs *Nox4<sup>+/+</sup>ApoE<sup>-/-</sup>* Diabetic and † vs *Nox4<sup>-/-</sup>ApoE<sup>-/-</sup>* Control.

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.<sup>36</sup> 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<sup>-/-</sup>* 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 fibrogenesis 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 a diminished cardiovascular burden as a result of atherosclerosis.

Cardiovascular disease is the major cause for mortality and morbidity in diabetic patients.<sup>1,2</sup> 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.<sup>36,37</sup>



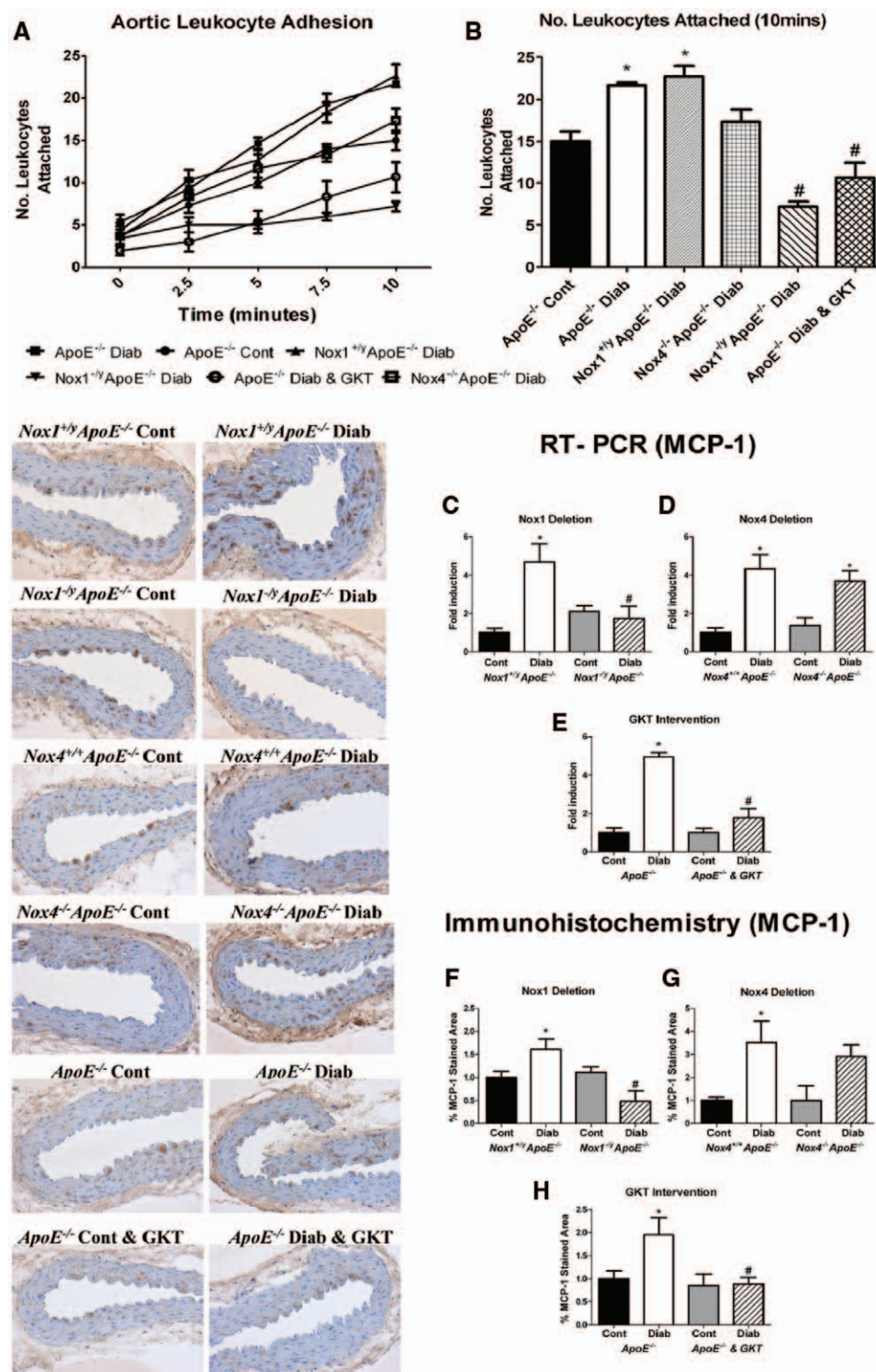


**Figure 6.** Reduced diabetes mellitus-induced aortic macrophage infiltration in diabetic *Nox1<sup>-/-</sup>ApoE<sup>-/-</sup>* and diabetic GKT137831 *ApoE<sup>-/-</sup>* treated mice. Macrophage infiltration (F4/80) as assessed by immunohistochemistry; photomicrographs and quantification in the aorta of control (Cont) and diabetic (Diab) *Nox1<sup>+/-</sup>ApoE<sup>-/-</sup>*, *Nox1<sup>-/-</sup>ApoE<sup>-/-</sup>* (A) and control and diabetic *Nox4<sup>+/-</sup>ApoE<sup>-/-</sup>* and *Nox4<sup>-/-</sup>ApoE<sup>-/-</sup>* (B) mice. Furthermore, macrophage infiltration was assessed in control (Cont) and diabetic (Diab) *ApoE<sup>-/-</sup>* mice (C) treated with or without GKT137831 (GKT) for 10 wks (60 mg/kg/d). Data are mean±SEM with n=8 to 10 per group. Groups were analyzed separately using ANOVA with a LSD post hoc test,  $P<0.05$  \*compared with *Nox1<sup>+/-</sup>ApoE<sup>-/-</sup>* or *ApoE<sup>-/-</sup>* Control, #compared with *Nox1<sup>+/-</sup>ApoE<sup>-/-</sup>* or *ApoE<sup>-/-</sup>* Diabetic, \* vs *Nox4<sup>+/-</sup>ApoE<sup>-/-</sup>* Control, # vs *Nox4<sup>+/-</sup>ApoE<sup>-/-</sup>* Diabetic and † vs *Nox4<sup>-/-</sup>ApoE<sup>-/-</sup>* Control.

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<sup>-/-</sup>* and *Nox4<sup>-/-</sup>* with *ApoE<sup>-/-</sup>* mice. Only Nox1 deletion in *ApoE<sup>-/-</sup>* mice prevented the accelerated development of atherosclerosis after 10 weeks of diabetes mellitus, whereas deletion of Nox4 in diabetic *ApoE<sup>-/-</sup>* mice did not protect mice from plaque development. This antiatherosclerotic effect of Nox1 deletion observed in diabetic *Nox1<sup>-/-</sup>ApoE<sup>-/-</sup>* 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.<sup>38–41</sup> 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,<sup>11,18,27,42</sup> 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.<sup>43</sup> Indeed, Nox1 has been



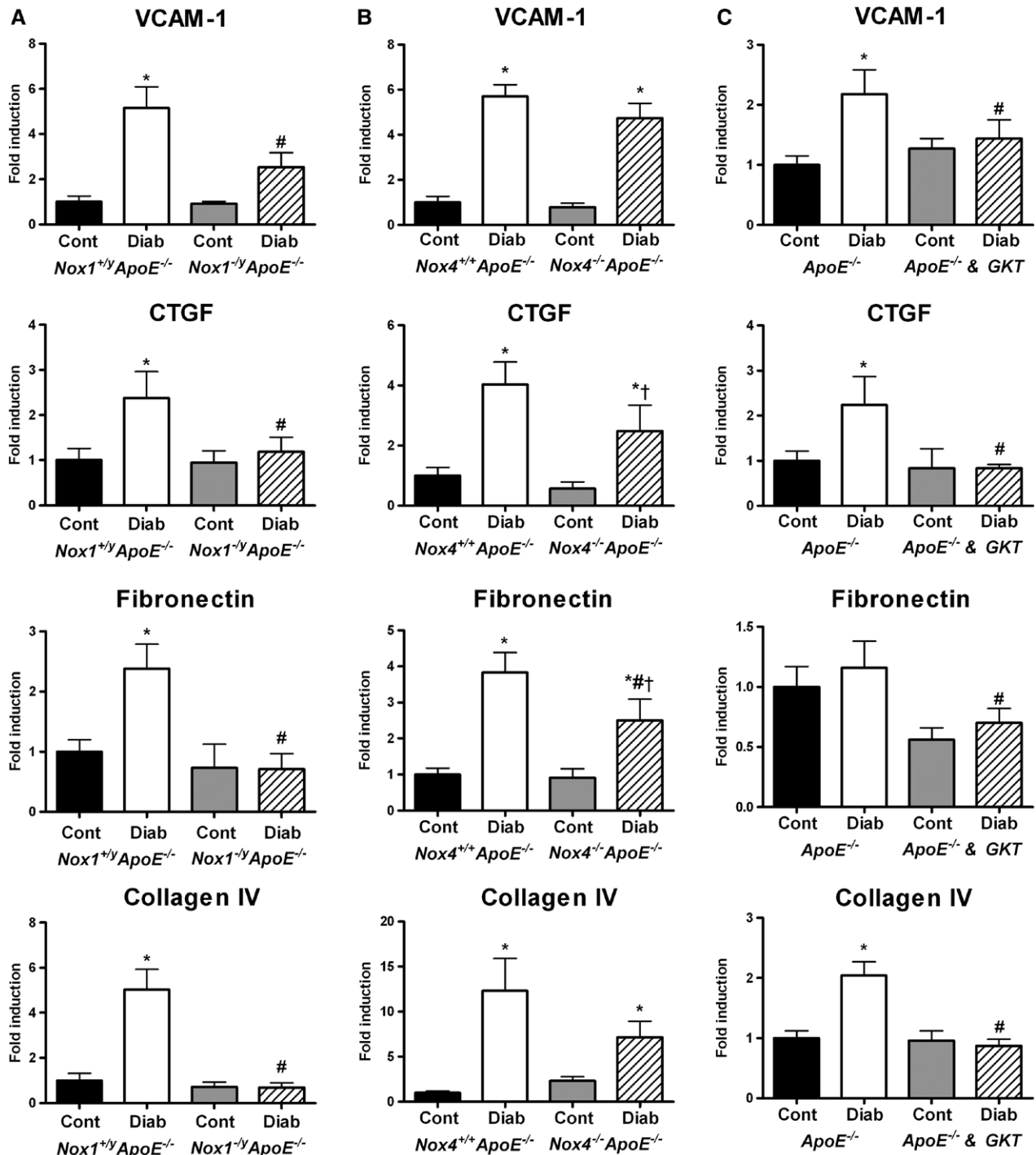
**Figure 7.** Reduced diabetes mellitus-induced aortic adhesion of leukocytes and monocyte chemoattractant protein 1 expression in  $Nox1^{-/-}ApoE^{-/-}$  and GKT137831-treated diabetic  $ApoE^{-/-}$  mice. Aortic leukocyte adhesion (A and B) in addition to monocyte chemoattractant protein 1 (MCP-1) immunohistochemistry quantification and gene expression as assessed by RT-PCR in the aortas of control (Cont) and diabetic (Diab)  $Nox1^{+/+}ApoE^{-/-}$ ,  $Nox1^{-/-}ApoE^{-/-}$  (C, F) and control and diabetic  $Nox4^{+/+}ApoE^{-/-}$  and  $Nox4^{-/-}ApoE^{-/-}$  (D, G) mice. Furthermore, MCP-1 gene and protein were measured in the aorta of control (Cont) and diabetic (Diab)  $ApoE^{-/-}$  mice (E, H) treated with or without GKT137831 (GKT) for 10 wks (60 mg/kg/d). Data are mean $\pm$ SEM (n=8–10/group). Groups were analyzed separately using ANOVA with a LSD post hoc test,  $P<0.05$ . \*compared with  $Nox1^{+/+}ApoE^{-/-}$  or  $ApoE^{-/-}$  Control, #compared with  $Nox1^{+/+}ApoE^{-/-}$  or  $ApoE^{-/-}$  Diabetic, \* vs  $Nox4^{+/+}ApoE^{-/-}$  Control, # vs  $Nox4^{+/+}ApoE^{-/-}$  Diabetic and + vs  $Nox4^{-/-}ApoE^{-/-}$  Control.

implicated in a range of processes related to vascular disease, although these studies have focused on earlier events such as endothelial dysfunction<sup>44</sup> or factors that predispose to atherosclerosis such as hypertension or increases in the potent vasoconstrictor AngII.<sup>45</sup> For example, Nox1 has been shown to be upregulated by AngII in SHR.<sup>45</sup> 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.<sup>46</sup> 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.<sup>44</sup>

The role of Nox1 in atherosclerosis has not been extensively explored. However, studies by Sheehan et al<sup>47</sup> 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





**Figure 8.** Amelioration of diabetes mellitus-induced aortic expression of proinflammatory and profibrotic markers in diabetic *Nox1<sup>-/-</sup>ApoE<sup>-/-</sup>* and GKT137831-treated diabetic *ApoE<sup>-/-</sup>* mice. RT-PCR analysis for inflammation and fibrosis markers in aortas of control (Cont) and diabetic (Diab) *Nox1<sup>+/y</sup>ApoE<sup>-/-</sup>*, *Nox1<sup>-/-</sup>ApoE<sup>-/-</sup>* (A) and control and diabetic *Nox4<sup>+/+</sup>ApoE<sup>-/-</sup>* and *Nox4<sup>-/-</sup>ApoE<sup>-/-</sup>* (B) mice. Furthermore, gene expression was analysed in aortas of control (Cont) and diabetic (Diab) *ApoE<sup>-/-</sup>* 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<sup>+/y</sup>ApoE<sup>-/-</sup>* or *ApoE<sup>-/-</sup>* Control, #compared with *Nox1<sup>+/y</sup> ApoE<sup>-/-</sup>* or *ApoE<sup>-/-</sup>* Diabetic, \* vs *Nox4<sup>+/+</sup>ApoE<sup>-/-</sup>* Control, # vs *Nox4<sup>+/+</sup>ApoE<sup>-/-</sup>* Diabetic and † vs *Nox4<sup>-/-</sup>ApoE<sup>-/-</sup>* Control.

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.<sup>37</sup> In our studies we have used the STZ-induced diabetic *ApoE<sup>-/-</sup>* mouse, a model considered to be the most appropriate model to study

advanced atherosclerosis in the context of diabetes mellitus.<sup>28</sup> 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.<sup>47</sup> Furthermore, the role of

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,<sup>25,26</sup> 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,<sup>48</sup> 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,<sup>49</sup> 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<sup>-/-</sup> 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.<sup>26</sup>

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<sup>-/-</sup> mice and in particular in Nox2<sup>-/-</sup>ApoE<sup>-/-</sup> 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.<sup>50,51</sup> 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.<sup>12,52</sup>

## 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<sup>-/-</sup> 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.

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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.

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### CLINICAL PERSPECTIVE

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.