Reactive Oxygen Species Can Provide Atheroprotection via NOX4-Dependent Inhibition of Inflammation and Vascular Remodeling

Reactive Oxygen Species Can Provide Atheroprotection via NOX4-Dependent Inhibition of Inflammation and Vascular Remodeling

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Objective—Oxidative stress is considered a hallmark of atherosclerosis. In particular, the superoxide-generating type 1 NADPH oxidase (NOX1) has been shown to be induced and play a pivotal role in early phases of mouse models of atherosclerosis and in the context of diabetes mellitus. Here, we investigated the role of the most abundant type 4 isoform (NOX4) in human and mouse advanced atherosclerosis.

Approach and Results—Plaques of patients with cardiovascular events or established diabetes mellitus showed a surprising reduction in expression of the most abundant but hydrogen peroxide (H_2O_2)-generating type 4 isoform (Nox4), whereas Nox1 mRNA was elevated, when compared with respective controls. As these data suggested that NOX4-derived reactive oxygen species may convey a surprisingly protective effect during plaque progression, we examined a mouse model of accelerated and advanced diabetic atherosclerosis, the streptozotocin-treated *ApoE*$^{-/-}$ mouse, with (*NOX4*$^{-/-}$) and without genetic deletion of Nox4. Similar to the human data, advanced versus early plaques of wild-type mice showed reduced Nox4 mRNA expression. Consistent with a rather protective role of NOX4-derived reactive oxygen species, *NOX4*$^{-/-}$ mice showed increased atherosclerosis when compared with wild-type mice. Deleting NOX4 was associated with reduced H_2O_2 forming activity and attenuation of the proinflammatory markers, monocyte chemotratic protein-1, interleukin-1β, and tumor necrosis factor-α, as well as vascular macrophage accumulation. Furthermore, there was a greater accumulation of fibrillar collagen fibres within the vascular wall and plaque in diabetic *Nox4*$^{-/-}$*ApoE*$^{-/-}$ mice, indicative of plaque remodeling. These data could be replicated in human aortic endothelial cells in vitro, where Nox4 overexpression increased H_2O_2 and reduced the expression of pro-oxidants and profibrotic markers. Interestingly, Nox4 levels inversely correlated with Nox2 gene and protein levels. Although NOX2 is not constitutively active unlike NOX4 and forms rather superoxide, this opens up the possibility that at least some effects of NOX4 deletion are mediated by NOX2 activation.

Conclusions—Thus, the appearance of reactive oxygen species in atherosclerosis is apparently not always a nondesirable oxidative stress, but can also have protective effects. Both in humans and in mouse, the H_2O_2-forming NOX4, unlike the superoxide-forming NOX1, can act as a negative modulator of inflammation and remodeling and convey atheroprotection. These results have implications on how to judge reactive oxygen species formation in cardiovascular disease and need to be considered in the development of NOX inhibitory drugs. **(*Arterioscler Thromb Vasc Biol*. 2016;36:295-307. DOI: 10.1161/ATVBAHA.115.307012.)**

Key Words: atherosclerosis ■ diabetes mellitus ■ endothelial cells ■ NADPH oxidase ■ reactive oxygen species

Oxidative stress and the associated endothelial dysfunction are considered a key feature of many vascular disease states, including atherosclerosis and diabetic vasculopathies.[1] The vascular isoforms of the NADPH oxidase (NOX) family of proteins have been shown to be important mediators of this.[2] NOX1 and NOX2 have been shown to be deleterious to vascular disease development, particularly in the context of atherosclerosis.[3,4] By contrast, the role of another NOX isoform, NOX4 in vascular injury remains controversial. Both, protective and deleterious roles for NOX4 have been demonstrated in vascular disease development.[5–8] Studies in transgenic mice have demonstrated that NOX4 plays a role in vasodilatation and provides

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Nonstandard Abbreviations and Acronyms	
ApoE$^{-/-}$	apolipoprotein E
CTGF	connective tissue growth factor
H_2O_2	hydrogen peroxide
HAECs	human arterial endothelial cells
IL-1β	interleukin-1 β
MCP-1	monocyte chemotratic protein 1
NO	nitric oxide
NOX	NADPH Oxidase
ROS	reactive oxygen species
TGFβ	transforming growth factor β
TNFα	tumor necrosis factor-α

protection against hypoxia-induced vascular damage through a promotion of angiogenesis, suggesting that NOX4 may play a vasculoprotective role.[9,10] However, studies in a stroke model demonstrated a deleterious role for NOX4 because *Nox4*$^{-/-}$ animals showed reduced stroke area in association with attenuated oxidative stress, reduced blood–brain barrier leakage and neuronal apoptosis.[11] Furthermore, in the heart, using the same model of cardiac hypertrophy and cardiomyocyte injury, contrasting effects of NOX4 have been reported, which were either deleterious or protective.[12–15]

Accelerated plaque formation is observed in the context of diabetes mellitus, with atherosclerotic lesions often being more inflammatory and fibrotic. Furthermore, diabetic plaques undergo extensive vascular remodeling leading to plaque expansion and remodeling.[16,17] The superoxide-generating type 1 NADPH oxidase (NOX1) has been shown to be induced and play a pivotal role in early phases of mouse models of atherosclerosis and in the context of diabetes mellitus. Here, the effect of long-term NOX4 and NOX1 deletion were investigated in the context of diabetes mellitus.

Surprisingly, we observed a reduced Nox4 gene expression in plaques of patients with cardiovascular events as well as in diabetic patients. We therefore examined the relationship of Nox4 versus Nox1 expression with plaque development in an established murine model of accelerated plaque formation, the streptozotocin-induced diabetic *ApoE*$^{-/-}$ mouse.[18] The data are of interest not only with respect to the qualitative role of reactive oxygen species (ROS) in atherosclerosis but also for the possible safety profile of NOX1/4 inhibiting drugs currently in clinical development for cardiovascular and other indications.

Materials and Methods

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

Human Atherosclerotic Plaque Real Time-Polymerase Chain Reaction

Total RNA was extracted from diabetic and nondiabetic carotid endarterectomy specimens obtained from surgery (BioBank, Baker IDI Heart and Diabetes Institute and Department of Surgery, Alfred Hospital, Melbourne) homogenization in TRIzol reagent (Invitrogen Australia, Mt Waverly, Victoria, Australia). DNA-free RNA was reverse transcribed to generate cDNA using the Superscript First Strand Synthesis System (Life Technologies BRL, Grand Island, NY) as previously described.[3] Total RNA was also extracted from carotid endarterectomy specimens obtained from surgery (Department of Surgery, Maasland Hospital Sittard, Sittard, The Netherlands).[19] Samples were individually hybridized to Illumina Human Sentrix-8 V2.0 BeadChip (Illumina Inc, San Diego, CA).[20,21] The adjusted *P* values presented in the results are corrected according to the Benjamini and Hochberg False Detection Rate method.

Animal Model

All animal experiments conducted in accordance to the principals and guidelines devised by the Alfred Medical Research and Education Precinct Animal Ethics Committee under the guidelines laid down by the National Health and Medical Research Council of Australia. Six-week-old male mice were rendered diabetic by streptozotocin (Sigma-Aldich, St. Louis, MO) as previously described.[3,11,22] After 20 weeks, the animals were anesthetized by intraperitoneal injection of sodium pentobarbitone (100 mg/kg body weight; Euthatal, Sigma-Aldrich, Castle Hill, NSW, Australia).

Atherosclerotic Plaque Area Quantification

Assessment of plaque area was undertaken using en face analysis as previously described.[23]

Cell Culture

Human arterial endothelial cells (HAECs) were obtained from Clonetics (Lonza) and grown in endothelial growth media-2 at normal (5 mmol/L) glucose. Cells used for real-time polymerase chain reaction (RT-PCR) were plated in media supplemented in either normal (5 mmol/L) or high (25 mmol/L) glucose for 48 hours before being harvested for RNA or protein isolation. Cells used for ROS analysis were plated in 96 well plates in either normal or high glucose for 24 hours before being used for ROS analysis.

Knockdown and Overexpression of Nox4 In Vitro

The knockdown of Nox4 was performed in HAECs using MISSION shRNA expressing lentivirus vectors as described previously.[24] The knockdown efficiency in the cells was verified by RT-PCR, and it was >90% for Nox4. Stable overexpression of Nox4 was performed in HAECs using as previously described[25] and overexpression was confirmed by RT-PCR.

Knockdown of Nox2 In Vitro

The knockdown of Nox2 was performed in HAECs using Lipofectamine RNAiMAX reagent with either scramble siRNA or siRNA targeted to Nox2 (NM_000397.3). Knockdown efficiency was verified by RT-PCR, and it was >50% for Nox2.

Measurement of ROS In Vitro

ROS production in HAEC (±glucose) was measured by Amplex Red (Molecular Probes, Eugene, OR), L-012 (Wako Chemicals, Richmond, VA), and Nitrotyrosine ELISA (Abcam, Cambridge, AM).[26,27]

Quantitative RT-PCR

Total aortic RNA was extracted after homogenization (Polytron PT-MR2100; Kinematica, Littau/Lucerne, Switzerland) of whole aorta in TRIzol reagent (Invitrogen Australia). DNA-free RNA was reverse transcribed to generate cDNA using the Superscript First Strand Synthesis System (life Technologies BRL, Grand Island, NY) as previously described.[3]

Histological Stains, Immunohistochemistry, and immunofluorescence

Paraffin sections (4 μmol/L) of aorta were used for histological stains (Masson Tirchome and Picro-Sirius Red) as previously described.[28,29] Immunohistochemistry was also performed on paraffin sections of aorta for nitrotyrosine (rabbit polyclonal; Millipore, Billercia, MA;

1:100) and macrophages (F4/80 rat monoclonal; Abcam; 1:100) as previously described.[3,30,31] HAEC cells grown on cover slips with and without glucose for 48 hours were stained with Nox2 (rabbit polyclonal, Abcam; 1:100) followed by an Alexa 488-conjugated secondary antibody (Abcam; 1:500) and imaged using a confocal microscope.

Activity Assays

Aortic superoxide generation was evaluated in frozen aortic sections with the oxidative fluorescent dye dihydroethidium as described previously.[32] Hydrogen peroxide (H_2O_2) generation was measured in 10 to 15 mm segments of aortas by Amplex Red using a commercial kit following manufacturer's instructions (Molecular Probes, Eugene, OR).[3,27] Nitrotyrosine concentrations were measured in whole aortic homogenates using a commercial kit following manufacturer's instructions (Abcam). Rac-1 activity was measured in cells grown under normal and high glucose conditions using a commercial kit following manufacturer's instructions (Cytoskeleton, Denver, CO).

Biomarkers

To measure circulating levels of monocyte chemotratic protein 1 (MCP-1), plasma samples were obtained from all experimental mice 1 week before experimental end and quickly frozen at −20°C. Plasma samples were sent to Aushion BioSystems (Aushion BioSystems, Billerica, MA) for analysis of MCP-1 concentrations using the Circa Immunoassay. Aortic MCP-1, tumor necrosis factor-α (TNFα), and interleukin (IL)-1β were measured in whole protein preparations as described previously[33] and measured using commercial kit's following manufacturer's instructions (R&D Systems, Minneapolis, MN).

Western Blot

Western blots of total aortic protein were performed as previously described targeting t-SMAD3 (rabbit monoclonal; OriGene, Rockville, MD; 1:500) and p-SMAD3 (rabbit monoclonal; Abcam; 1:250).[34,35]

Statistical Analysis

Data were analyzed for normality using the Shipiro–Wilk test before being analyzed with either a *t* Test performed or a 1-way ANOVA or a 2-way ANOVA using SPSS Statistics version 20 (IBM) with a least significant difference post hoc test for multiple comparisons of the means, $P<0.05$ was considered statistically significant. All results are mean±SEM, unless otherwise specified.

Results

Nox4 Gene Expression Is Reduced in Plaques of Patients With Cardiovascular Events and in Diabetic Subjects Compared to Nondiabetic Subjects

Gene expression data using human carotid plaque material demonstrated reduced Nox4 expression in plaques removed from subjects with cardiovascular events compared with subjects without cardiovascular events (Figure 1A). In a separate cohort of nondiabetic and diabetic subjects, we similarly found a 65% reduction in Nox4 gene expression in plaques of diabetic subjects versus nondiabetic subjects (Figure 1B).

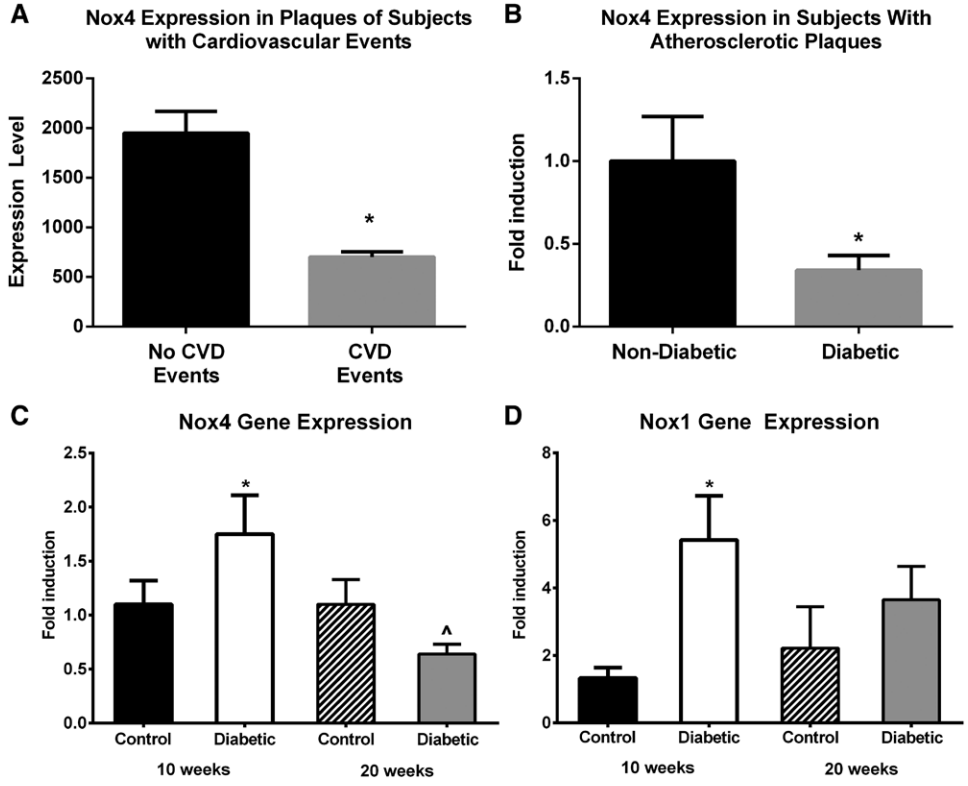

Figure 1. Gene expression data in plaques from human subjects with cardiovascular events compared with those without cardiovascular events demonstrated reduced gene expression of Nox4 (**A**). In another cohort, diabetic subjects with cardiovascular disease demonstrated a significant reduction in the expression of Nox4 (65%) compared with nondiabetic subjects with cardiovascular disease (**B**). Nox1 and Nox4 isoform expression in diabetic and nondiabetic *ApoE*$^{-/-}$ mice after 10 weeks of diabetes mellitus and 20 weeks of diabetes mellitus (**C** and **D**). After 10 weeks of diabetes mellitus, Nox1 and Nox4 are significantly elevated in expression compared with control *ApoE*$^{-/-}$ mice, in turn, after an additional 10 weeks of diabetes mellitus, totaling 20 weeks, Nox1 isoform expression is still significantly elevated while Nox4 isoform expression is significantly downregulated in comparison with *ApoE*$^{-/-}$ control mice. Data are Mean±SEM (n=8–10 per group), *$P<0.05$ compared with nondiabetic subjects with a cardiovascular event, *$P<0.05$ compared with *ApoE*$^{-/-}$ control mice, ^$P<0.05$ compared to *ApoE*$^{-/-}$ diabetic mice at 10 weeks.

The subject characteristics are summarized in Table II in the online-only Data Supplement.

Nox4 Expression Is Reduced in Advanced Mouse Atherosclerotic Plaques

We next measured Nox4 gene expression in aortic samples from diabetic and nondiabetic *ApoE*$^{-/-}$ mice, a mouse model of accelerated atherosclerosis. Gene expression of the Nox isoforms Nox1 and Nox4 were examined at 10 and 20 weeks after diabetes mellitus induction. After 10 weeks of diabetes mellitus that is characterized by early plaque development and fatty streaks, gene expression of both Nox4 and Nox1 was significantly elevated in aortas of diabetic *ApoE*$^{-/-}$ mice compared with control *ApoE*$^{-/-}$ mice (Figure 1C and 1D). After an additional 10 weeks of diabetes mellitus, at which time point more advanced plaques have developed, we observed a significant time-dependent decline in Nox4 expression in plaques (P<0.05, F=5.24), which was dependent on the diabetic status of the mouse (time and diabetes mellitus interaction, P<0.05, F=7.44, Figure 1C). In contrast, although there was a modest decline in Nox1 gene expression, this was still significantly upregulated compared with *ApoE*$^{-/-}$ control mice (Figure 1D).

Deletion of Nox4 in Diabetes Mellitus Increases the Development of Atherosclerosis

On the basis of the surprising finding that Nox4 gene expression was reduced in more advanced vascular disease associated with plaque vulnerability, we embarked on delineating the role of NOX4 in a long-term model (20 week) of atherosclerosis in the setting of diabetes mellitus, which is characterized by an increased incidence of large and advanced fibrotic atherosclerotic lesions.

We used *Nox4*$^{-/-}$*ApoE*$^{-/-}$ mice and their respective wild-type (WT) counterparts, which were rendered diabetic using streptozotocin and followed for 20 weeks of diabetes mellitus. As a comparison, we concurrently studied *Nox1*$^{-/y}$*ApoE*$^{-/-}$ mice. Our previous findings in a short-term (10 week) study had shown that NOX1 plays a detrimental role in atherosclerosis development.[3]

As anticipated induction of diabetes mellitus was associated with reduced body weight (Table I in the online-only Data Supplement). Diabetic NOX4–deficient animals were slightly heavier in comparison with diabetic Nox4–expressing mice. Deletion of NOX1 did not have an effect on body weight. Deletion of either NOX4 or NOX1 did not alter plasma glucose levels. Similarly, there was no change in blood pressure across all groups. Induction of diabetes mellitus increased circulating cholesterol and triglycerides, which was unaffected by deletion of NOX4; however, deletion of NOX1 modestly increased the concentration of circulating cholesterol and triglycerides in comparison with *Nox1*$^{+/y}$*ApoE*$^{-/-}$ diabetic mice (Table I in the online-only Data Supplement).

After 20 weeks of diabetes mellitus, all WT diabetic mice demonstrated a significant increase in atherosclerotic plaque development in comparison with control WT mice (Figure 2A–2D). Deletion of the NOX4 isoform in diabetes mellitus significantly further increased atherosclerotic plaque

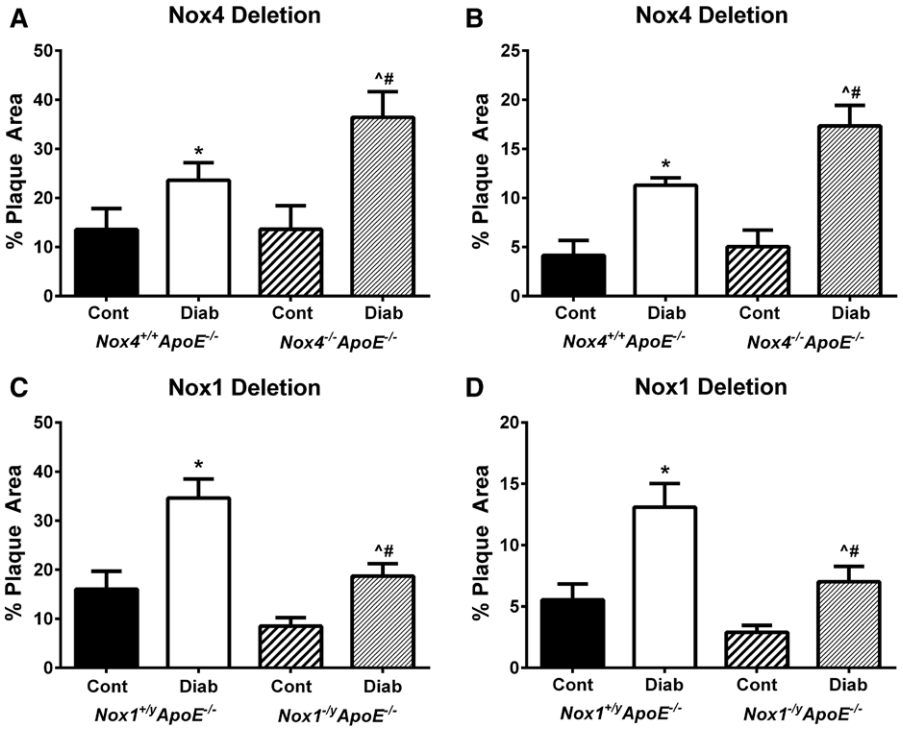

Figure 2. Atherosclerotic plaque areas in total aorta (**A** and **C**) and aortic arch (**B** and **D**) are significantly increased in diabetic *Nox4*$^{-/-}$*ApoE*$^{-/-}$ (**A** and **B**) and significantly decreased in *Nox1*$^{-/y}$*ApoE*$^{-/-}$ (**C** and **D**) when compared with their wild-type diabetic counterparts after 20 weeks of diabetes mellitus. Data are mean±SEM (n=8–10 per group), *P<0.05 compared with respective wild-type control that being *Nox4*$^{+/+}$*ApoE*$^{-/-}$ or *Nox1*$^{+/y}$*ApoE*$^{-/-}$ mice. #P<0.05 compared with wild-type diabetic, that being *Nox4*$^{+/+}$*ApoE*$^{-/-}$ or *Nox1*$^{+/y}$*ApoE*$^{-/-}$ mice. ^P<0.05 compared with knockout control, that being *Nox4*$^{-/-}$*ApoE*$^{-/-}$ or *Nox1*$^{-/y}$*ApoE*$^{-/-}$ mice.

area compared with *Nox4*$^{+/+}$*ApoE*$^{-/-}$ diabetic mice, with the greatest increase in plaque area observed in the aortic arch (Figure 2A and 2B). In contrast, deletion of the NOX1 isoform attenuated the development of atherosclerosis after 20 weeks of diabetes mellitus (Figure 2C and 2D; Figure I in the online-only Data Supplement).

Reduced H_2O_2 Formation in the Aorta of *Nox4*$^{-/-}$*ApoE*$^{-/-}$ Mice

Measurement of the main ROS generated by NOX4, H_2O_2, demonstrated a reduction in the aorta of Nox4$^{-/-}$ (Figure 3G) but not Nox1$^{-/y}$ mice (Figure 3F). Dihydrothethidium immunofluorescence (for total oxidative capacity) and nitrotyrosine (for superoxide/nitric oxide [NO] or H_2O_2/nitrite/MPO interactions) demonstrated sustained elevations in diabetic *Nox4*$^{-/-}$*ApoE*$^{-/-}$ mice (Figure 3G and 3H). Analysis of the gene expression demonstrated increased mRNA levels of Nox1, Nox2, and its assembly subunit p47phox in WT diabetic mice (Table). This was confirmed by immunohistochemical analysis in atherosclerotic plaques of *Nox4*$^{-/-}$*ApoE*$^{-/-}$, which demonstrated increased staining for NOX2 in foam and smooth muscle cells within the plaque when compared with *Nox4*$^{+/+}$*ApoE*$^{-/-}$ diabetic mice (Figure II in the online-only Data Supplement).

Dihydroethidium immunofluorescence demonstrated a significant elevation within the vasculature of both diabetic Nox4 and Nox1 WT mice. Deletion of Nox4 resulted in increased dihydroethidium staining, whereas deletion of the Nox1 resulted in a reduced dihydroethidium stain (Figure 3G and 3H); immunopositive tyrosine nitration in the aortic wall and by ELISA was increased in diabetic WT mice and remained elevated in *Nox4*$^{-/-}$ (Figure 3A and 3B) but was lowered in *Nox1*$^{-/y}$*ApoE*$^{-/-}$ diabetic mice (Figure 3B–3D). Thus, in this setting the nitrotyrosine biomarker may indicate interaction of NOX1-derived superoxide with NO to yield peroxynitrite, a potent nitrating species. However, we cannot exclude that upregulated NOX2/p47phox levels also contributed to this.

Nox4 Deficiency Increases Expression of Proinflammatory Markers and Aortic Macrophage Accumulation

With the identification of increased expression of Nox2, p47phox and sustained increased activity of dihydroethidium and nitrotyrosine accumulation within the aortic wall of diabetic *Nox4*$^{-/-}$*ApoE*$^{-/-}$ mice, we next examined inflammatory mechanisms that are promoted by oxidative stress.

There was a modest increase in circulating MCP-1 concentration in both diabetic Nox4 and Nox1 WT mice (Figure 4A and 4D). Deletion of the NOX4 isoform in diabetes mellitus resulted in a significant further elevation in MCP-1 concentrations (Figure 4A), whereas deletion of Nox1 did not alter circulating MCP-1 concentrations (Figure 4D) consistent with the diabetic *Nox4*$^{-/-}$*ApoE*$^{-/-}$ mice being in a heightened state of inflammation. Further examination of MCP-1 accumulation within the aortic wall showed increased MCP-1 protein expression in both the diabetic Nox4 and the Nox1 WT mice (Figure 4B and 4E). Deletion of the NOX4 isoform in diabetes

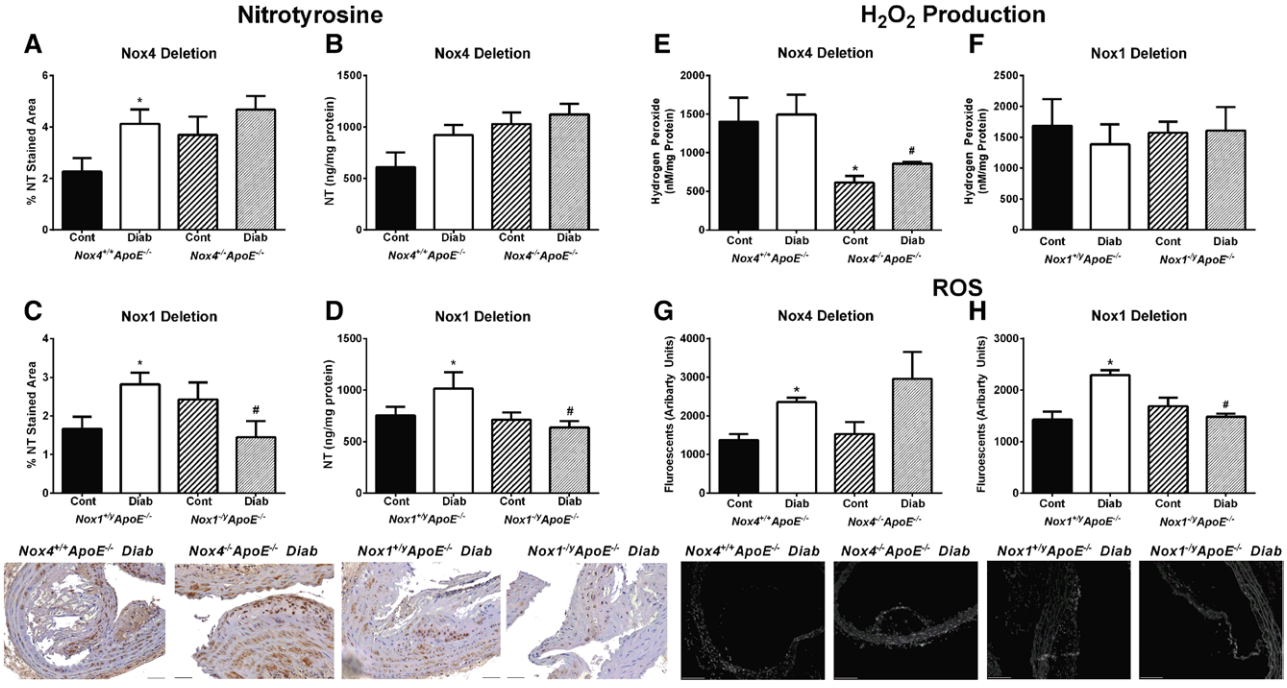

Figure 3. Oxidative stress parameters including reactive oxygen species (ROS) and nitrotyrosine accumulation within the aortic wall and protein concentration in aortic homogenates identified sustained increase in *Nox4*$^{-/-}$*ApoE*$^{-/-}$ diabetic mice compared with wild-type diabetic mice (**A** and **B**), in conjunction with reduction in H_2O_2 production in both control and diabetic Nox4-deleted mice (**E**). In contrast, deletion of Nox1 in diabetes mellitus was associated with a significant reduction in ROS and nitrotyrosine accumulation compared with wild-type diabetic mice (**C**, **D**, and **H**) with no change in hydrogen peroxide production (**F**). ROS measurements by dihydroethidium showed significant elevations in wild-type diabetic mice, which was mainlined in Nox4-deleted diabetic mice (**G**), but significantly attenuated in Nox1-deleted diabetic mice (**H**). Data are mean±SEM (n=8 per group), **P*<0.05 compared with respective wild-type control that being *Nox4*$^{+/+}$*ApoE*$^{-/-}$ or *Nox1*$^{+/y}$*ApoE*$^{-/-}$ mice. #*P*<0.05 compared with wild-type diabetic, that being *Nox4*$^{+/+}$*ApoE*$^{-/-}$ or *Nox1*$^{+/y}$*ApoE*$^{-/-}$ mice. Scale bar, 50 μm.

Table. RT-PCR Analysis for the Gene Expression of Nox Isoforms (Nox1, Nox2, Nox4, and p47phox) in Addition to Antioxidant Enzyme Gpx1, in Diabetic and Control *Nox4*$^{-/-}$*ApoE*$^{-/-}$ and *Nox1*$^{-/y}$*ApoE*$^{-/-}$ as Well as Their Respective Wild-Types

	Nox4$^{+/+}$*ApoE*$^{-/-}$		*Nox4*$^{-/-}$*ApoE*$^{-/-}$		*Nox1*$^{+/y}$*ApoE*$^{-/-}$		*Nox1*$^{-/y}$*ApoE*$^{-/-}$	
	Control	Diabetic	Control	Diabetic	Control	Diabetic	Control	Diabetic
Nox1	1.00±0.24	2.17±0.38*	0.74±0.24†	1.03±0.23†	1.00±0.31	3.07±0.41*	0.01±0.01*†	0.01±0.018*†
Nox2	1.00±0.24	1.18±0.32	1.31±0.36	2.53±0.27*†‡	1.00±0.25	3.60±1.04*	0.96±0.67	3.18±0.59*‡
Nox4	1.00±0.21	1.24±0.30	0.01±0.01*†	0.01±0.01*†	1.00±0.16	1.10±0.35	0.39±0.14*	0.91±0.18
p47phox	1.00±0.20	1.68±0.40*	1.19±0.36	2.90±0.41*†‡	1.00±0.30	3.87±1.01*	0.45±0.34	3.73±0.75*‡
Gpx1	1.00±0.11	1.69±0.41*	0.99±0.26	1.15±0.10	1.00±0.16	2.02±0.47*	0.54±0.16	1.66±0.34‡
Nrf-2	1.00±0.12	1.28±0.24	1.03±0.32	0.95±0.10	1.00±0.16	2.29±0.48*	0.46±0.10	1.67±0.33
HO-1	1.00±0.22	1.09±0.22	0.73±0.31	1.57±0.22‡	1.00±0.39	3.21±0.89*	0.33±0.14	4.20±1.17‡

Data are mean±SEM (n=6 per group).

*P<0.05 compared with respective wild-type control that being *Nox4*$^{+/+}$*ApoE*$^{-/-}$ or *Nox1*$^{+/y}$*ApoE*$^{-/-}$ mice.

†P<0.05 compared with wild-type diabetic, that being *Nox4*$^{+/+}$*ApoE*$^{-/-}$ or *Nox1*$^{+/y}$*ApoE*$^{-/-}$ mice.

‡P<0.05 compared with knockout control, that being *Nox4*$^{-/-}$*ApoE*$^{-/-}$ or *Nox1*$^{-/y}$*ApoE*$^{-/-}$ mice.

mellitus significantly further increased the aortic MCP-1 concentration (Figure 4B). However, deletion of the NOX1 isoform significantly attenuated the diabetes mellitus–induced increase in MCP-1 concentration (Figure 4E). MCP-1 accumulation would be expected to increase adherence of immune cells to the vascular wall, particularly macrophages. Increased macrophage accumulation within the aortic wall was observed in both diabetic Nox4 and Nox1 WT mice (Figure 4C and 4F). Deletion of the NOX4 isoform significantly further increased macrophage accumulation within the aortic wall (Figure 4C), whereas deletion of NOX1 significantly reduced macrophage accumulation (Figure 4F; Figure III in the online-only Data Supplement). We further examined proinflammatory factors involved in atherosclerosis, with a specific focus on those that have an upstream profibrotic role, including TNFα and IL-1β.[16] The diabetes mellitus–induced increase in aortic IL-1β gene expression was significantly attenuated in Nox1-deficient mice but was further increased in Nox4-deficient mice (Figure IVA and IVC in the online-only Data Supplement). Diabetic *Nox1*$^{+/y}$*ApoE*$^{-/-}$ and *Nox4*$^{+/+}$*ApoE*$^{-/-}$ mice demonstrated a significant elevation in TNFα levels, which was significantly further increased in diabetic *Nox4*$^{-/-}$*ApoE*$^{-/-}$ mice, but reduced in *Nox1*$^{-/y}$*ApoE*$^{-/-}$mice (Figure IVB and IVD in the online-only Data Supplement).

Nox4 Deficiency Increases Fibrillar Collagen Deposition Within Atherosclerotic Plaques in Association With Enhanced Transforming Growth Factor β-SMAD Signaling

Fibrosis is a critical step in the progression of atherosclerosis, through the establishment of plaque expansion and remodeling.[16] Diabetic *Nox4*$^{+/+}$*ApoE*$^{-/-}$ mice showed significant elevations in the gene expression of the fibrillar collagens I (Figure 5A) and III (Figure 5B), but not collagen IVα3 (Figure 5C) in the aortic wall. Deletion of the NOX4 isoform in diabetes mellitus resulted in a significant further increase in the expression of collagens I and III (Figure 5A and 5B), with a significant downregulation in the expression of collagen IVa3 (Figure 5C). In contrast, in diabetic Nox1 WT mice there was increased expression of collagens I (Figure 5E) and IVα3 (Figure 5G), but not collagen III (Figure 5F). Deletion of Nox1 in diabetes mellitus attenuated collagen I expression, with no change in the expression of collagens III and IVα3 (Figure 5E–5G). These results indicate that there is increased de novo formation and deposition of the newly formed fibrillar collagens, collagens I and III in diabetic Nox4-deleted mice, whereas the basement membrane collagen IV was reduced. Increased extracellular matrix accumulation in the plaques of diabetic *Nox4*$^{+/+}$*ApoE*$^{-/-}$ and *Nox1*$^{+/y}$*ApoE*$^{-/-}$ mice was also confirmed by qualitative Masson trichrome staining (Figure 5I). This was not attenuated in Nox4-deficient *ApoE*$^{-/-}$ mice, but it was reduced in the Nox1-deficient *ApoE*$^{-/-}$ mice. Using Picro-Sirius Red to identify qualitative individual collagen fibers as different colors when visualized under polarized light,[28] we demonstrated that diabetic Nox4–deficient mice showed increased collagen deposition within the plaques, which were predominantly lighter and newly formed fibrillar fibers including collagens I and III, and reduced accumulation of older blue- and green-stained fibers, representing collagen IV (Figure 5I, white arrows) in both, the plaque center and the fibrous cap. In contrast, Nox1-deficient mice had less accumulation of yellow- and red-stained fibers compared with *Nox1*$^{+/y}$*ApoE*$^{-/-}$ diabetic mice and a greater accumulation of green- and blue-stained fibers, consistent with a reduction in the fibrillar collagens, collagens I and III, but an increase in collagen IV fibers representing basement membrane collagens (Figure 5I, white arrows).

The profibrotic growth factor transforming growth factor β (TGFβ) has been shown to be involved in extracellular matrix accumulation within atherosclerotic plaques and has been shown to be regulated by Nox4.[36–38] In this study, we observed increased TGFβ gene expression in the aorta of diabetic Nox1 and Nox4 WT mice (Figure 5D and 5H). Deletion of Nox4 in diabetes mellitus resulted in a further significant elevation in TGFβ expression (Figure 5D), whereas Nox1 deletion did not change TGFβ expression (Figure 5H). Phosphorylated SMAD3 (p-SMAD3) and total SMAD3 (t-SMAD3) in the aorta were measured by Western

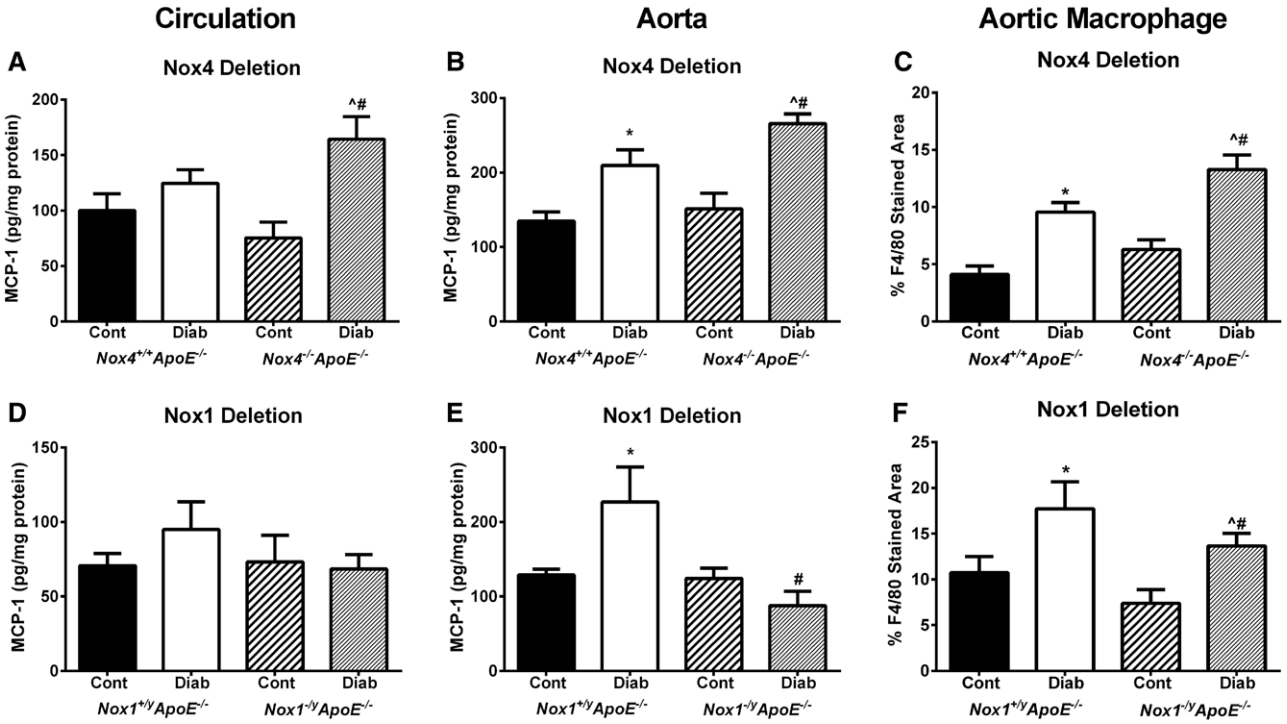

Figure 4. Deletion of Nox4 in the presence of diabetes mellitus significantly increased the concentration of the chemotactic marker monocyte chemotratic protein 1 (MCP-1) in both circulation and aortic homogenates (**A** and **B**), with consistent increases in macrophage accumulation (**C**). Significant decreases in aortic MCP-1 and macrophage accumulation were observed in *Nox1*$^{-/y}$*ApoE*$^{-/-}$ diabetic mice when compared with diabetic wild-type counterparts (**D**–**F**). Data are mean±SEM (n=8 per group), **P*<0.05 compared with respective wild-type control that being *Nox4*$^{+/+}$*ApoE*$^{-/-}$ or *Nox1*$^{+/y}$*ApoE*$^{-/-}$ mice. #*P*<0.05 compared with wild-type diabetic, that being *Nox4*$^{+/+}$*ApoE*$^{-/-}$ or *Nox1*$^{+/y}$*ApoE*$^{-/-}$ mice. ^*P*<0.05 compared with knockout control, that being *Nox4*$^{-/-}$*ApoE*$^{-/-}$ or *Nox1*$^{-/y}$*ApoE*$^{-/-}$ mice.

blot analysis, to explore altered regulation of TGFβ signaling within the aortic tissue. Deletion of the NOX4 isoform resulted in a modest increase in p-SMAD3 compared with WT diabetic mice (Figure VA in the online-only Data Supplement). In contrast, deletion of Nox1 resulted in a modest reduction in the level of p-SMAD3 when compared with WT diabetic mice (Figure VB in the online-only Data Supplement). These results suggest that increased plaque formation in the diabetic Nox4–deficient mice was associated with increased deposition of lighter younger collagen fibers, including collagens I and III in the plaque body and the fibrous cap, whereas older and heavier collagens such as collagen IV were reduced. This seems to be mediated, at least in part, through a SMAD3-TGFβ–dependent mechanism.

In Arterial Endothelial Cells Knockdown of Nox4 Results in Lower H_2O_2 Formation in Association With Enhanced Profibrotic Markers

HAECs had either Nox4 silenced in order to mimic the in vivo model of Nox4 deficiency or overexpressed to mimic an environment where there is enhanced ROS generated by Nox4. Cells were cultured under normal and high glucose conditions. Nox4 knockdown by shRNA demonstrated a 90% reduction in Nox4 mRNA levels (Figure 6A), whereas cells that were transfected to overexpress Nox4 demonstrated a 40% to 60% increase in Nox4 mRNA (Figure 6A). H_2O_2 levels were significantly lower in Nox4 knockdown cells grown under high glucose (Figure 6D), and they were significantly increased in response to Nox4 overexpression (Figure 6D). Gene expression of the profibrotic marker, connective tissue growth factor (CTGF), was significantly increased in nontarget high glucose–cultured cells, which was further elevated in Nox4-silenced cells and attenuated in high glucose–cultured cells with Nox4 overexpression (Figure 6H). These results indicated that under high glucose conditions, knockdown of Nox4 decreased endothelial H_2O_2 production in association with an increase in the profibrotic marker CTGF. Furthermore, analysis of p-SMAD3 by Western blot identified that overexpression of Nox4 under high glucose conditions resulted in a significant reduction compared with Nox4-silenced cells under high glucose conditions (Figure VI in the online-only Data Supplement). These results suggest that ROS derived from NOX4 can provide vascular protection through attenuation of proinflammatory and profibrotic mechanisms. These in vitro data replicate our in vivo findings and provide further mechanistic insight. Similar to our in vivo findings, we also found a counter-regulatory upregulation of Nox2 and p47phox when compared with cells grown under normal glucose conditions (Figure 6E–6G). In addition Rac-1 activity was increased, indicating augmented Nox2 activation in Nox4 shRNA cells grown under high glucose conditions (Figure 6I). This correlated with increased levels of nitrotyrosine staining, a possible biomarker for NOX2-derived superoxide interacting with NO. Thus, it is possible that some of the effects of NOX4 are mediated or reduced by NOX2 upregulation.

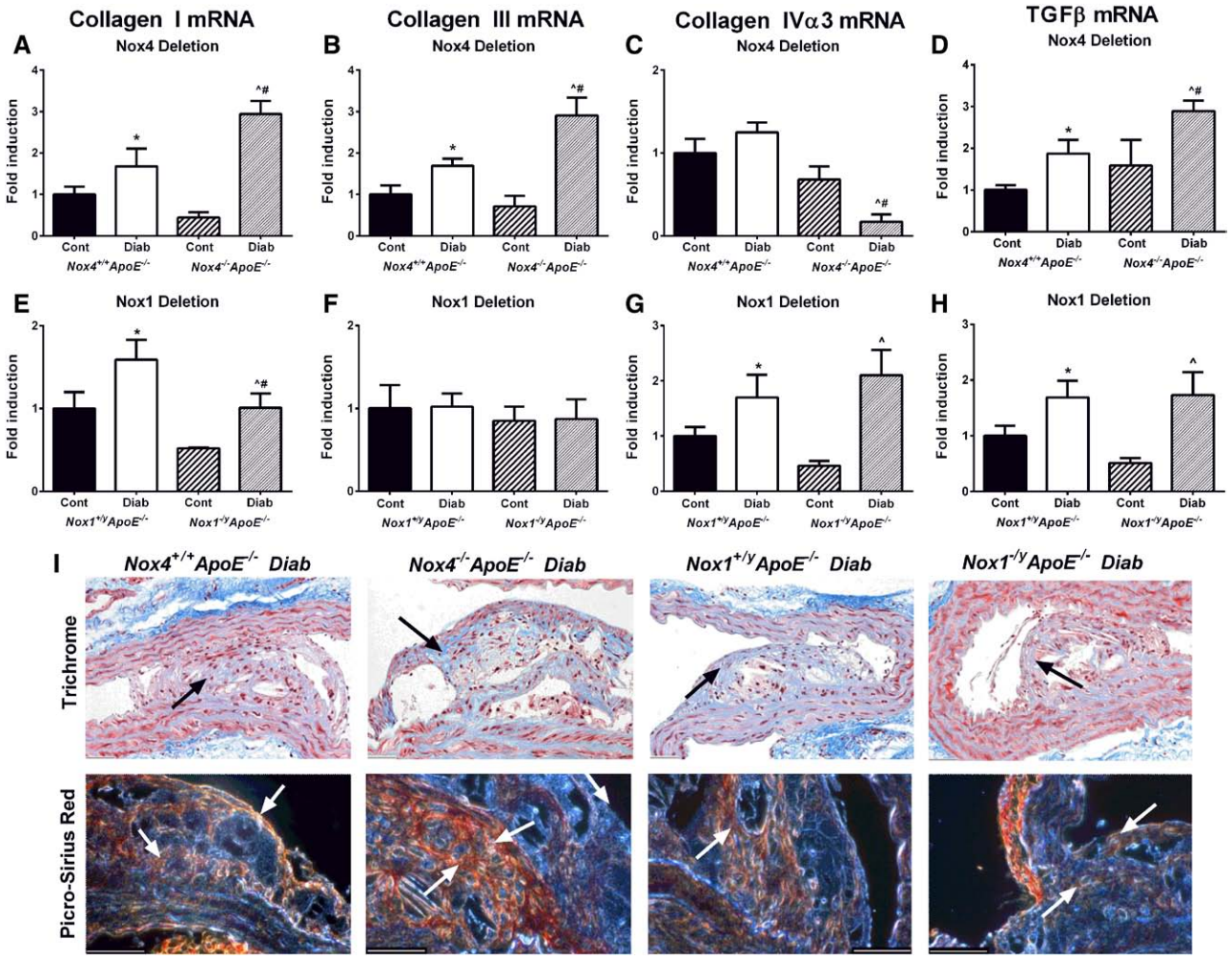

Figure 5. Gene expression analysis in *Nox4*$^{-/-}$*ApoE*$^{-/-}$ diabetic aortas identified significant increases in the expression of transforming growth factor-β (TGFβ), collagen I and III with a decrease in collagen IVα3 when compared with wild-type diabetic aortas (**A**–**D**). Complementary trichrome and Picro-Sirius Red histological stains demonstrated increase in accumulation of collagen deposition (blue stain indicated with black arrows) in Trichrome stains, which were consistent with lighter collagen fibers as indicant with orange and yellow stains in the Picro-Sirius Red stain (**I**). Deletion of Nox1 in diabetes mellitus resulted in opposite effects with reduction in collagen I gene expression and increases in collagen IVα3 gene expression with no change in TGFβ when compared with wild-type diabetic aortas (**E**–**H**). Complementary histological stains indicated reduction in collagen (evident by reduced blue accumulation) in Trichrome stains, and reduction in orange and yellow stains in the Picro-Sirius Res stain, supporting a reduction in the lighter collagen fibers (**I**). Data are mean±SEM (n=8 per group), **P*<0.05 compared with respective wild-type control that being *Nox4*$^{+/+}$*ApoE*$^{-/-}$ or *Nox1*$^{+/y}$*ApoE*$^{-/-}$ mice. ^*P*<0.05 compared with knockout control, that being *Nox4*$^{-/-}$*ApoE*$^{-/-}$ or *Nox1*$^{-/y}$*ApoE*$^{-/-}$ mice. Scale bar, 50 μm.

To test this further, Nox2 was silenced in Nox4 shRNA cells and cultured under normal and high glucose conditions using siRNA targeted to Nox2. Nox2 gene expression was significantly reduced by >50% in Nox2-silenced cells cultured under normal and high glucose conditions in both the nontarget and the Nox4 shRNA cells (Figure 6J). Examination of gene expression for MCP-1 and CTGF identified that silencing of Nox2 in Nox4 shRNA cells resulted in a significant reduction of these 2 proatherosclerotic markers when cultured under high glucose conditions (Figure 6K and 6L). These results indicate that some of the effects of NOX4 are mediated through an upregulation of NOX2 activity. To clarify this further, future studies are needed using a double knockout mouse model and, currently not available, isoform-specific inhibitors.

Discussion

Here, we show for the first time that an enzymatic source of ROS, NOX4, can protect from atherosclerosis. Oxidative stress has been postulated to be a key mediator of endothelial dysfunction and vascular disease; however, all clinical therapies addressing this by the use of antioxidants have failed by being ineffective or even causing harm.[39] It is hypothesized that this is because of the fact that ROS do not have exclusively negative effects but also serve essential signaling functions, for example, in innate immunity, hormone synthesis, and angiogenesis. The key for therapeutic exploitation of ROS will thus be to differentiate between disease-triggering and physiological sources of ROS that should rather be left untouched. What may differentiate these 2 qualities can be quantity and type of ROS being formed (eg, H_2O_2 as a

signaling molecule versus superoxide as a reactive radical) as well as type of NOX isoforms.

NOX have emerged as the only known dedicated enzymatic source of ROS, that is, their only function is to produce ROS. All other sources of ROS (mitochondria, xanthine oxidase, and endothelial NO synthase) have other primary functions and need to be altered or damaged to generate ROS. Often this initial trigger is again ROS, so-called kindling radicals, for which NOX are a likely source. Our human gene expression data suggest that Nox4 deficiency is associated with plaque vulnerability possibly enhancing the risk of cardiovascular events particularly in the context of diabetes mellitus.

This protective role of NOX4 in the vasculature can be explained by anti-inflammatory and anti-remodeling mechanisms. Interestingly, myocardial Nox4 expression is also reduced in patients with aortic valve stenosis suggesting that Nox4 deficiency maybe associated with myocardial and vascular remodeling.[40] Furthermore, deletion of Nox4 in a model of angiotensin II–induced vascular injury enhanced media hypertrophy and endothelial dysfunction[8] and in a model of cardiac hypertrophy induced cardiac hypertrophy via effects on angiogenesis and capillary density.[15] Targeted deletion of Nox4 in an angiotensin II–induced model of vascular injury resulted in a collapse of the antioxidant defense system, as reflected by changes in Nrf-2 and HO-1, contributing to enhanced NO bioavailability and hence reduced endothelial dysfunction.[8] In contrast, overexpression of Nox4 in endothelial cells resulted in an amplified response to acetylcholine- and histamine-induced vasodilatation and a reduction in blood pressure[10] and was associated with increased recovery from hypoxia and increased angiogenesis.[9]

Our studies using *Nox4*$^{-/-}$*ApoE*$^{-/-}$ mice are the first to demonstrate a protective role of Nox4 in diabetes mellitus–associated atherosclerosis. This model of streptozotocin-induced insulin-deficient diabetes melitus in *ApoE*$^{-/-}$ mice is currently, despite its possible limitations, the best available model and recommended by National Institutes of Health and Juvenile Diabetes Research Foundation to study advanced atherosclerosis in diabetes mellitus.[18] Increased plaque formation and remodeling as well as enhanced vascular inflammation in the absence of Nox4 in a model of long-term diabetes mellitus suggest a vasculoprotective role for Nox4 in atherosclerosis. These vasculoprotective effects of Nox4 include the formation of H_2O_2, downregulation of Nox2 and modulation of vascular macrophage accumulation and inflammation as well as Nox4-mediated effects on extracellular matrix remodeling. In contrast, NOX1 has deleterious effects on plaque formation in long-term atherosclerosis. Thus, our studies suggest that NOX1 and NOX4 represent an interesting example of 2 ROS sources having qualitatively opposing effects, deleterious, and protective within the same tissue and disease model. This may be related to the fact that NOX1 produces primarily superoxide and NOX4 H_2O_2 or that both isoforms are located at different subcellular compartments.

The upregulation of functional Nox2 on the gene and protein level in vitro in HAECs and in vivo in the context of Nox4 silencing may represent another potential mechanism how the lack of NOX4 leads to increased atherosclerosis. Furthermore, NOX2 is not a valid target in the context of diabetes mellitus because diabetic Nox2 knockout mice showed increased mortality and infections.[3]

Activation of inflammatory and profibrotic processes is critical in the promotion of atherosclerosis especially in diabetes mellitus.[3,32,41–45] Nox4 expression may also limit vascular inflammation and macrophage infiltration and thus lack of vascular Nox4 would presumably lead to increased activation of inflammatory pathways. In this study, we demonstrated not only increased circulating concentrations of MCP-1 but also increased aortic MCP-1 concentrations in diabetic *Nox4*$^{-/-}$*ApoE*$^{-/-}$ mice. MCP-1 is a mediator of chemotaxis of immune cells and in particular macrophages to the vascular wall. Indeed, there was increased macrophage accumulation within the aortic wall in diabetic WT mice, and this was further increased in diabetic Nox4–deficient mice. Previous studies have identified that deletion of Nox4 increases the survival of macrophages in atherosclerosis, albeit in a nondiabetic setting.[46] Our studies showing increased aortic MCP-1 protein and macrophage accumulation are consistent with the finding of enhanced macrophage recruitment and increased macrophage survival in the context of Nox4 deletion.

The later stages of atherosclerosis development are characterized by enhanced extracellular matrix deposition within plaques. Previous studies have suggested a role for Nox4 in TGFβ-mediated fibrosis with some studies suggesting a profibrotic effect of NOX4.[38] In needs to be considered that most of these studies investigated Nox4 in either kidney or pulmonary fibrosis and thus the effect of NOX4 may be site and organ specific. Our studies are the first to suggest an atheroprotective role for NOX4 in plaque remodeling in advanced atherosclerosis. We identified increased expression of newly formed fibrillar collagens such as collagens I and III in the plaque center and within the fibrous caps with a reduction in the older and heavier basement membrane and collagen IVα3 in diabetic Nox4–deficient mice when compared with diabetic WT mice.

Plaques from diabetic *Nox1*$^{-/y}$*ApoE*$^{-/-}$ mice demonstrated reduced fibrillar collagen I and III and unaltered collagen IV accumulation. Collagen types I and III are the major constitutes of total plaque protein and collagen deposition[19,47] and can be associated with smooth muscle cell transition from a contractile to a synthetic phenotype.[48] In contrast, collagen IV accumulation is associated with a more quiescent contractile phenotype of vascular smooth muscle cells. Collagens can act as an extracellular reservoir for cytokines and growth factors, including IL-1β, TGFβ, CTGF, and TNFα.[49–51]

In this study, we identified increased expression of IL-1β, TGFβ, and TNFα in the aorta of diabetic *Nox4*$^{-/-}$*ApoE*$^{-/-}$ mice. It has been demonstrated that TGFβ promotes collagen production through SMAD3 binding and increased production of CTGF.[50,52–54] In a rat model of balloon injury model, TGFβ induced overproduction of collagens, resulting in stiffer arteries.[50] Consistent with these findings, we identified increases in TGFβ expression at the gene level in the diabetic *Nox4*$^{-/-}$*ApoE*$^{-/-}$ mice, supportive of a profibrotic environment within the plaques. SMAD signaling has been suggested to play a key role in the promotion of

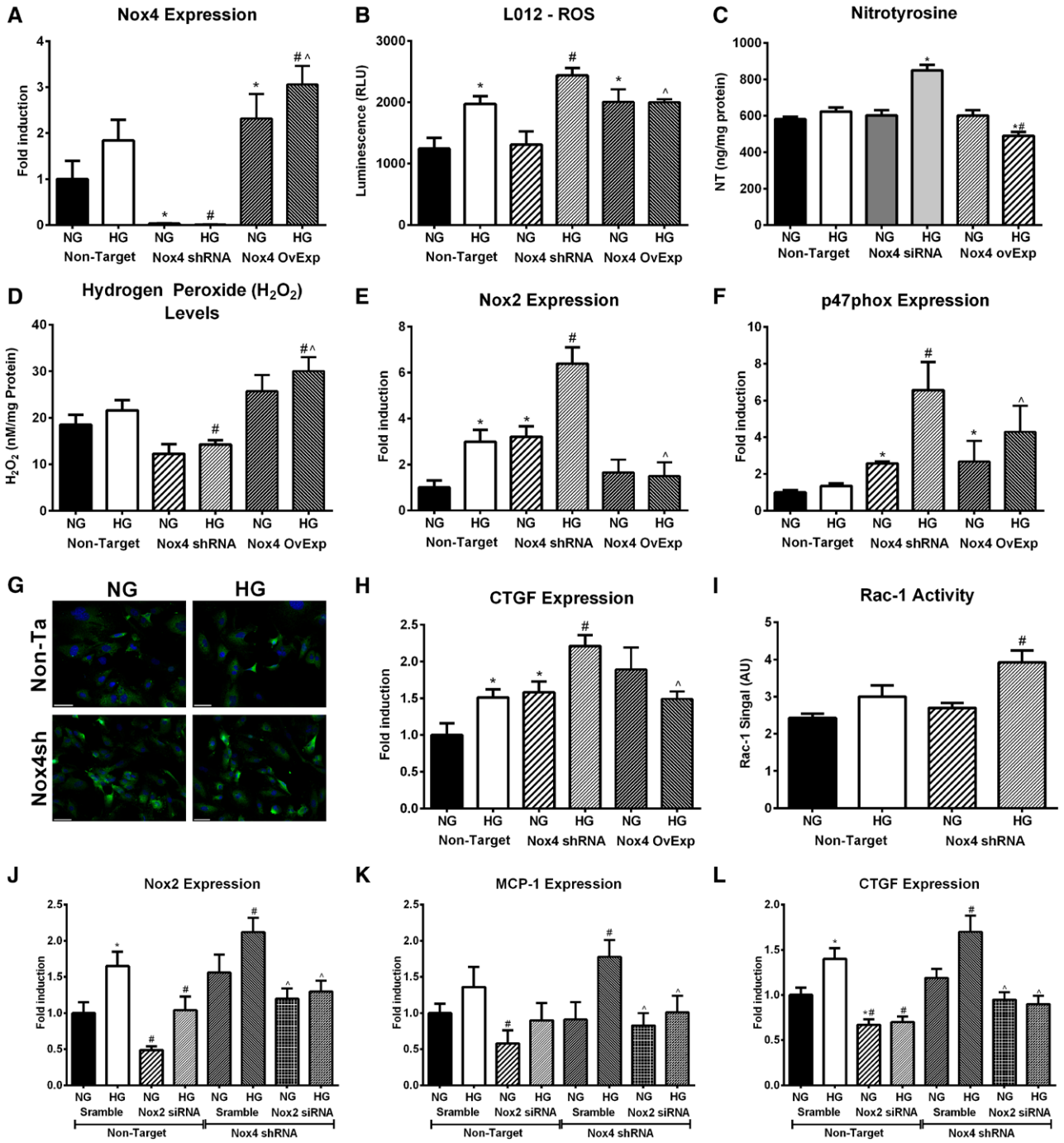

Figure 6. Human arterial endothelial cells cultured in high glucose with either Nox4 silenced (Nox4 shRNA) or overexpressed (Nox4 OvExp) demonstrated a significant increase in reactive oxygen species (ROS) in Nox4 shRNA cells, which was attenuated in Nox4 OvExp cells (**B**). Similar results were seen when examining nitrotyrosine by ELISA (**C**). Hydrogen peroxide levels were significantly reduced with Nox4 knockdown cells, which was restored with Nox4 overexpression (**D**). Examination of gene expression identified successful knockdown of Nox4 and overexpression of Nox4 compared with nontarget cells (**A**). Expression of Nox2 and p47phox was significantly upregulated in Nox4 shRNA high glucose cells, which was attenuated in Nox4 OvExp cells (**E** and **F**). Confirmatory Nox2 immunofluorescence demonstrated increased staining in Nox4 knockdown cells compared with nontarget high glucose–cultured cells (**G**). Gene expression levels of the profibrotic growth factor connective tissue growth factor (CTGF) were increased in Nox4 knockdown cells and reduced in Nox4 overexpressing cells (**H**). Rac-1 activity assay demonstrated an increase in Rac-activity in Nox4 shRNA high glucose cells compared with nontarget high glucose–cultured cells (**I**). Silencing of Nox2 in the nontarget cells demonstrated a significant reduction in Nox2 gene expression when cultured under normal and high glucose conditions. In addition, silencing of Nox2 in Nox4 shRNA cells resulted in a reduction in Nox2 gene expression under high glucose conditions when compared with Nox4 shRNA high glucose–cultured cells (**J**). Monocyte chemotratic protein 1 (MCP-1) and CTGF gene expressions were similarly reduced in cells silenced for Nox2 in Nox4 shRNA high glucose–cultured cells (**K** and **L**). Data are mean±SEM (n=6–8 per group for RT-PCR, ROS, and ELISA), **P*<0.05 compared with nontarget normal glucose (NG) cells. #*P*<0.05 compared with nontarget high glucose cells. ^*P*<0.05 compared with Nox4 siRNA high glucose cells. Scale bar, 20 μmol/L.

fibrosis within the vascular system.[36,55] Furthermore, other studies have shown that under high glucose conditions there is enhanced translocation of SMAD3 to the nucleus,[56] contributing to enhanced expression of fibrotic markers. Here, we show a modest increase in activated SMAD3 in the aorta of diabetic *Nox4*$^{-/-}$*ApoE*$^{-/-}$ mice compared with WT mice, consistent with the enhanced fibrotic environment within the atherosclerotic plaques. In vitro, we have confirmed that Nox4 deficiency in human aortic endothelial cells exposed to high glucose demonstrate reduced H_2O_2, with increased upregulation of the profibrotic growth factor, CTGF, in association with increased pSMAD3 signaling. Cosilencing of both Nox2 and Nox4 suggests that some of these proinflammatory and profibrotic changes observed with Nox4 deletion are mediated, at least in part, via Nox2 activation. NOX2 activation in endothelial cells is associated with increased expression of MCP-1 leading to further vascular immune-cell recruitment. Furthermore, these changes are associated with increased expression of profibrotic growth factors and increased SMAD3 signaling, leading to plaque remodeling. These changes observed with Nox4 deletion were rescued by Nox4 overexpression. These studies demonstrate an important atheroprotective role for Nox4 in endothelial cells. However, we cannot exclude a role for NOX4 in cells other than endothelial cells of the vasculature, which needs further investigation.

In conclusion, this is the first study to demonstrate an atheroprotective role of a specific form of ROS, H_2O_2 and its enzymatic source, Nox4, in advanced atherosclerosis development modulating inflammation and plaque remodeling. Furthermore, these results suggest that isoform-specific inhibitors are advisable when aiming at exploiting NOX for chronic therapeutic intervention. As such, this is an important example for redefining the roles of ROS in physiology and disease.

Note Added in Proof

While this manuscript was under revision, a paper by Schürmann et al[57] was published suggesting a vasculoprotective effect by Nox4.

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Disclosures

None.

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Significance

We here show that the role of reactive oxygen species in atherosclerosis is apparently qualitatively not uniform. Both, in the humans and in the mouse the H_2O_2-forming NADPH oxidase 4 is protective, possibly by limiting inflammation and vascular remodeling. In contrast, the superoxide-forming NADPH oxidase 1 is proatherosclerotic. These findings may have important translational implications for reactive oxygen species/oxidative stress as a disease biomarker and the development of isoform-specific NADPH oxidase inhibitors currently in evaluation for chronic therapeutic applications.