Nox2 NADPH Oxidase Has a Critical Role in Insulin Resistance–Related Endothelial Cell Dysfunction

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Insulin resistance is characterized by excessive endothelial cell generation of potentially cytotoxic concentrations of reactive oxygen species. We examined the role of NADPH oxidase (Nox) and specifically Nox2 isoform in superoxide generation in two complementary in vivo models of human insulin resistance (endothelial specific and whole body). Using three complementary methods to measure superoxide, we demonstrated higher levels of superoxide in insulin-resistant endothelial cells, which could be pharmacologically inhibited both acutely and chronically, using the Nox inhibitor gp91ds-tat. Similarly, insulin resistance–induced impairment of endothelial-mediated vasorelaxation could also be reversed using gp91ds-tat. siRNA-mediated knockdown of Nox2, which was specifically elevated in insulin-resistant endothelial cells, significantly reduced superoxide levels. Double transgenic mice with endothelial-specific insulin resistance and deletion of Nox2 showed reduced superoxide production and improved vascular function. This study identifies Nox2 as the central molecule in insulin resistance–mediated oxidative stress and vascular dysfunction. It also establishes pharmacological inhibition of Nox2 as a novel therapeutic target in insulin resistance–related vascular disease. ***Diabetes*** **62:2130–2134, 2013**

Insulin resistance is a multisystem disorder of energy homeostasis, cell growth, and tissue repair, which has been shown to be pivotal to the initiation and progression of type 2 diabetes (1). As a result, type 2 diabetes is characterized by a portfolio of disorders including atherosclerotic coronary artery disease, stroke, and peripheral vascular disease (2). Atherosclerosis is characterized by a deleterious change in endothelial cell phenotype, a hallmark of which is excess generation of cytotoxic concentrations of reactive oxygen species (ROS) such as superoxide and failure of endogenous vascular antioxidant systems to adequately deal with this—a scenario described as oxidative stress (3).

Several studies support a role for insulin resistance in the generation of pathophysiological concentrations of ROS and the development of premature atherosclerosis (4). Our own studies in experimental models of insulin resistance at a whole-body level and specific to the endothelium demonstrated that insulin resistance per se is a substrate for increased generation of ROS and accelerated atherosclerosis (5,6). ROS are thought to promote atherosclerosis through a number of different mechanisms including but not limited to enhanced oxidation of lipoproteins, activation of proinflammatory genes, alteration of vascular smooth muscle cell phenotype, and reduction of bioavailability of the antiatherosclerotic signaling radical nitric oxide (NO).

A major source of ROS to emerge over the last decade is NADPH oxidase (Nox) (7,8). Nox was originally identified in phagocytes where it exists as a multisubunit complex consisting of a membrane-bound cytochrome b_{558} and at least four cytosolic subunits, which translocate to the membrane upon activation. It is now clear that the Noxs are a family of enzymes with each isoform being distinguished by the membrane-spanning catalytic Nox or Duox subunit that transfers electrons from NADPH to molecular oxygen, termed Nox1-Nox5 (8). We have provided evidence that increased Nox-derived ROS may be a unifying mechanism underlying insulin resistance–related oxidative stress and atherosclerosis (5,6). In the current study, we aimed to examine the therapeutic potential of inhibiting Nox and, specifically, Nox2 to reduce oxidative stress and improve endothelial-dependent vasodilatation in insulin resistance.

RESEARCH DESIGN AND METHODS

Breeding, maintenance of gene-modified mice, and metabolic testing. We used male mice with endothelial-specific mutated (dominant negative) human insulin receptor (IR) overexpression (ESMIRO) or with haploinsufficiency of IR at the whole-body level ($IR^{+/-}$). ESMIRO mice aged 3–5 months (when we have demonstrated higher ROS generation [6]) and $IR^{+/-}$ mice aged 6–8 months (when we have demonstrated significantly increased endothelial cell superoxide generation [5]) were used. All experiments were conducted under U.K. Home Office Project license no. 40/2988. Tail venous blood sampling, glucose, and insulin tolerance tests were performed as previously described (5,6).

Pulmonary endothelial cell isolation and culture. Pulmonary endothelial cells (PECs) were isolated by immunoselection with CD146 antibody–coated magnetic beads using published method and cultured in MV2 medium (Promocell), supplemented with 10% FCS, 100 units/mL penicillin, and 100 μg/mL streptomycin (9).

Pharmacological inhibition of Nox. To inhibit Nox acutely, PEC or aortic rings were exposed to gp91ds-tat (50 μmol/L for 30 min). For chronic inhibition, mice were implanted with osmotic mini-pumps (Alzet), containing gp91ds-tat or control peptide as previously reported (10). The pump delivered 10 mg/kg/day of drug for 28 days.

Transfection and PCR. PECs were transfected with small interfering RNA (siRNA) (Nox2: GGUCUUAUUUUGAAGUGUUtt) or scrambled control using electroporation (Amaxa, Cologne, Germany) and used after 60- to 72-h RNA isolation, and reverse transcription was performed as we previously described (9). PCR primer sequences are provided in Supplementary Table 1. SYBR green–based real-time PCR was performed in an ABI prism 7900HT Sequence Detection System (Applied Biosystems).

Immunoblotting. Protein extracts were resolved on 4–12% Bis-Tris gels (Invitrogen) and transferred to polyvinylidene fluoride membranes. Blots were

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probed with primary antibody (Nox2 [BD Biosciences]) or β-actin (Santa Cruz) and peroxidase-conjugated secondary antibody and developed with enhanced chemiluminescence (Millipore).

Assessment of superoxide generation

Lucigenin-enhanced chemiluminescence. Superoxide production in PECs was measured as previously described (5,6). Pellets of PECs were resuspended in PBS containing 5% FCS and 0.5% BSA, and the luminescence was measured upon addition of a nonredox cycling concentration (5 μmol/L) of lucigenin and 100 μmol/L NADPH.

Aortic vasomotor function. Vasomotor function was assessed in aortic rings as previously described (5,6). Rings mounted in an organ bath were equilibrated at a resting tension of 3 g for 45 min before experiments. Contraction and relaxation responses were measured by cumulative addition of phenylephrine (PE) (1 nmol/L to 10 μmol/L) and acetylcholine (Ach) (1 nmol/L to 10 μmol/L); insulin-mediated vasorelaxation in rings preconstricted with PE was examined as previously reported (6,9).

High-performance liquid chromatographic measurement of conversion of dihydroethidium to oxyethidium in aorta. With use of previously described methods, chromatographic separation was carried out with a NovaPak C18 column in a high-performance liquid chromatography (HPLC) system (Dionex). Dihydroethidium (DHE) was monitored by absorption at 245 nm, and 2-hydroxyethidium and ethidium were monitored by fluorescence detection (11).

Fluorescence-activated cell sorter analysis DHE fluorescence. PECs were loaded with 10 μmol/L DHE and analyzed by flow cytometry as previously described (5,6). A minimum of 5,000 events/test was analyzed in a FACScalibur flow cytometer (Becton-Dickinson).

Statistical methods. Results are expressed as means ± SEM. All results are representative of at least three independent repeats. Comparisons were made using paired or unpaired Student t tests or repeated-measures ANOVA as appropriate; where repeated t tests were performed, a Bonferroni correction was applied. $P < 0.05$ was considered statistically significant.

RESULTS

Acute inhibition of Nox reduces superoxide production in insulin-resistant endothelial cells. As previously described (6), aortic relaxation to the endothelium and NO-dependent vasodilator Ach was blunted in ESMIRO mice (Fig. 1*A*). For examination of the effect of inhibiting Nox on this response, aortic rings were exposed to gp91ds-tat or scrambled peptide prior to the study. Gp91ds-tat significantly enhanced Ach-induced relaxation compared with scrambled peptide (Fig. 1*A*). Also, gp91ds-tat significantly reduced PE-induced contraction (Supplementary Fig. 1*A*). However, gp91ds-tat did not alter insulin resistance of ESMIRO aorta as shown by no reduction in PE-induced contraction response in gp91ds-tat pretreated rings by insulin treatment (Supplementary Fig. 1*B*). Superoxide production at baseline measured using lucigenin-enhanced chemiluminescence in PECs of ESMIRO was significantly greater than in wild-type cells (Fig. 1*B*). For examination of the effect of acute inhibition of Nox on superoxide production, PECs were exposed to gp91ds-tat. Both gp91ds-tat and nonspecific antioxidant tiron significantly reduced superoxide level in ESMIRO PECs (Fig. 1*B*). Baseline superoxide measured with DHE using FACS in PECs (Fig. 1*C*) and by HPLC in aorta (Fig. 1*D*) showed similar results. Blunting of the difference in superoxide levels when the endothelium of ESMIRO aorta was denuded confirmed that endothelial cells are the primary source of excessive superoxide in aorta (Supplementary Fig. 1*C*).

As previously described (5), $IR^{+/-}$ mice also had impaired Ach-induced aortic relaxation. It was improved after gp91ds-tat treatment (Fig. 1*E*). Gp91ds-tat treatment also improved basal NO bioavailability as shown by a significantly higher N^G-monomethyl-L-arginine (L-NMMA)–mediated increase in PE-induced aortic ring contraction (Supplementary Fig. 2). Both lucigenin assay (in PECs) and

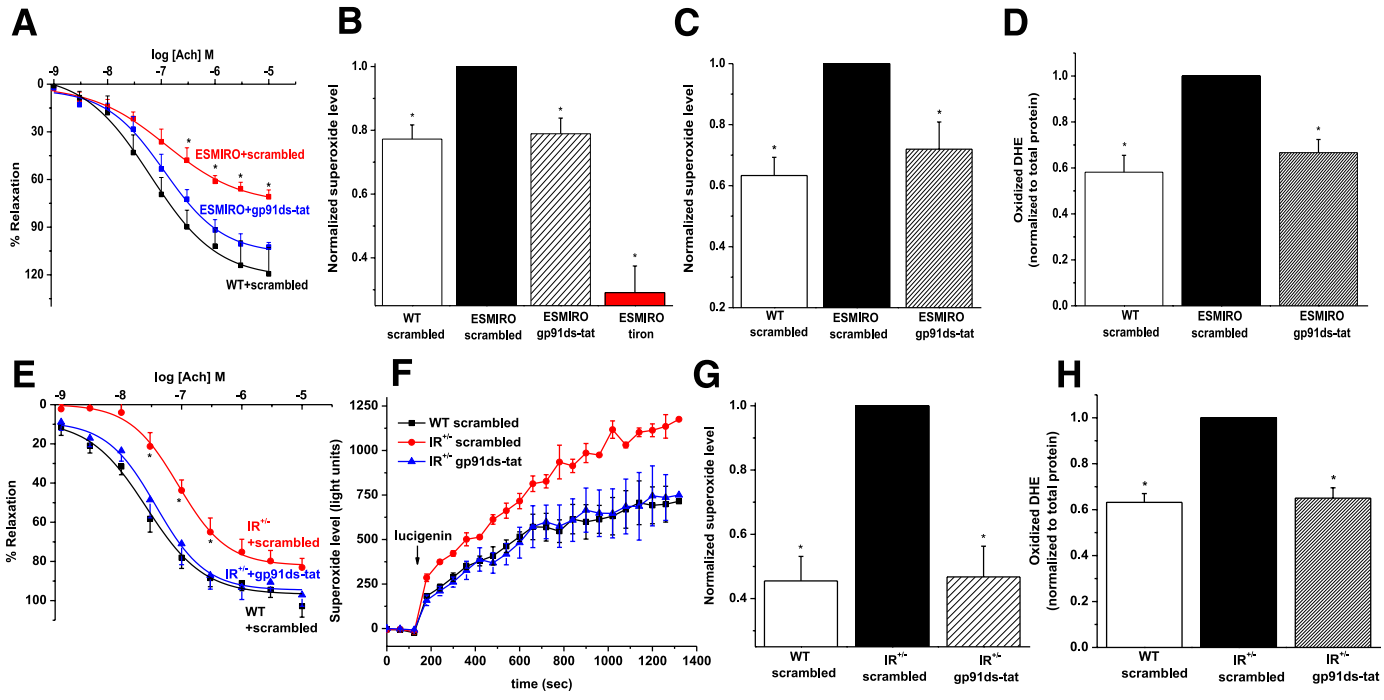

FIG. 1. Acute pharmacological inhibition of Nox in insulin resistance. *A*: Relaxation curves of aortic rings from ESMIRO mice and wild-type (WT) littermates with indicated acute treatments in response to Ach ($n = 5$ each). Normalized superoxide levels in PECs of ESMIRO and WT mice after acute treatment with gp91ds-tat or scrambled peptide measured by lucigenin-enhanced chemiluminescence assay (*B*) and FACS-based DHE analysis (*C*). *D*: Normalized oxidized DHE level in aorta of WT and ESMIRO mice after acute treatment with gp91ds-tat or scrambled peptide measured by HPLC-based DHE analysis. *E*: Relaxation curves of aortic rings from $IR^{+/-}$ and wild-type littermates with indicated acute treatments in response to Ach. Representative trace (*F*) and mean (*G*) data showing superoxide levels in PECs of WT and $IR^{+/-}$ mice measured by lucigenin-enhanced chemiluminescence after indicated acute treatments. *H*: Normalized oxidized DHE levels in aorta of WT and $IR^{+/-}$ mice after acute treatment with gp91ds-tat or scrambled peptide measured by HPLC-based DHE analysis (*$P < 0.05$).

DHE HPLC assay (in aorta) showed higher basal superoxide production in $IR^{+/-}$, which was significantly reduced upon incubation with gp91ds-tat (Fig. 1*F–H*).

For further examination of the specific isoform of Nox responsible for excessive superoxide, PECs from ESMIRO mice were examined using real-time PCR and Western blotting. The data showed significantly greater expression of *Nox2* mRNA and protein in ESMIRO (Fig. 2*A* and *B*) than in wild-type littermates. Expression of other Nox family genes was unchanged (Fig. 2*A*). While analysis of whole aorta and lung showed similar increments in Nox2 expression, spleen failed to show any difference (Supplementary Fig. 3*A*). In endothelium-denuded ESMIRO aorta, there was no difference in *Nox2* expression (Supplementary Fig. 3*B* and *C*), establishing the localization of excessive *Nox2* in aorta as the endothelium. In PECs of $IR^{+/-}$ also, *Nox2* expression was higher (Fig. 2*C*). To confirm that Nox2 is the principal source of superoxide, we used siRNA to knock down *Nox2* in PECs of ESMIRO. siRNA reduced *NOX2* expression but had no effect on *NOX4* expression (Fig. 2*D* and Supplementary Fig. 4). Lucigenin-enhanced chemiluminescence confirmed that *Nox2* siRNA significantly reduced superoxide generation in ESMIRO PECs (Fig. 2*E* and *F*).

Chronic pharmacological inhibition of Nox enhances vasomotor function in insulin resistant vessels. To investigate the therapeutic potential of longer-term inhibition of Nox, we administered gp91ds-tat or scrambled peptide to ESMIRO and $IR^{+/-}$ mice via osmotic mini-pump for 28 days. Gp91ds-tat had no effect on body mass, organ weight, glucose or insulin tolerance, or plasma insulin (Fig. 3*A–C*, *E*, and *G* and Supplementary Fig. 5). Gp91ds-tat infusion significantly enhanced Ach-induced aortic relaxation compared with scrambled peptide in ESMIRO and $IR^{+/-}$ (Fig. 3*D* and *H*). Gp91ds-tat had no effect on SNP-mediated vasorelaxation (data not shown). For examination of whether gp91ds-tat induces changes in expression of ROS-related genes, real-time PCR was performed on RNA isolated from lungs of ESMIRO mice infused with gp91ds-tat or scrambled peptide. There was a significant decrease in *SOD1* expression in gp91ds-tat treated animals (Supplementary Fig. 6).

Chronic genetic inhibition of Nox2 reduces superoxide production and enhances vasomotor function. Gp91ds-tat is thought to be specific for Nox2 but may affect other structurally similar Nox isoforms (12). Hence, to examine the effect of specifically inactivating Nox2 we generated ESMIRO/$Nox2^{y/-}$ by crossing ESMIRO mice with Nox2 holoinsufficient mice ($Nox2^{y/-}$). Nox2 was successfully deleted in ESMIRO/$Nox2^{y/-}$ as shown on both mRNA (Supplementary Fig. 7*A*) and protein (Fig. 4*A*) levels. There was no difference in behavior, grooming, body weight, organ weight, blood pressure, glucose or insulin tolerance, or plasma insulin level (Fig. 4*B–D* and Supplementary Fig. 7*B–D*).

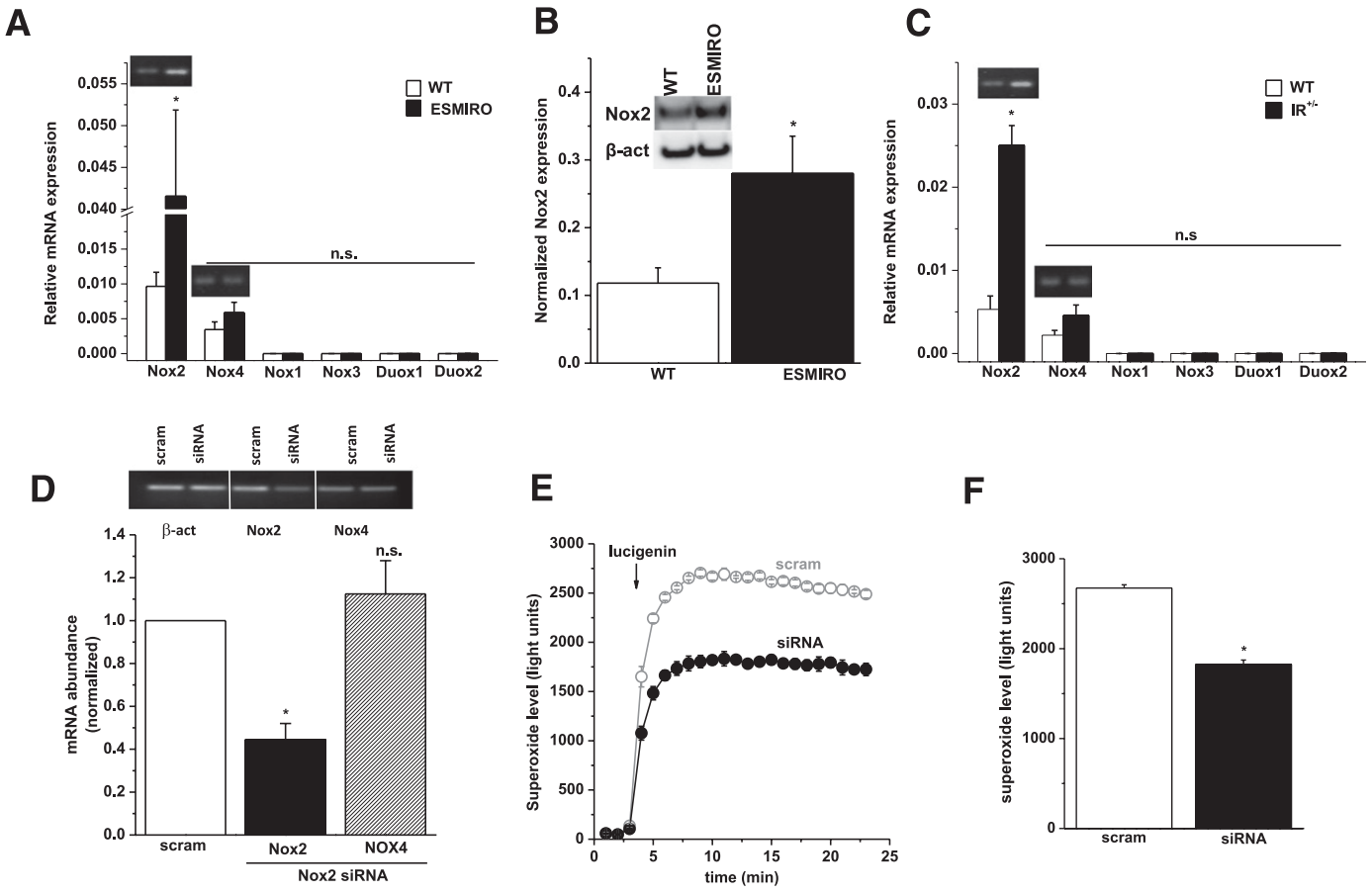

FIG. 2. Nox expression and acute knockdown of Nox2 in insulin resistance. *A*: Relative expression of different isoforms of Nox in PECs of wild-type (WT) and ESMIRO mice (insets, gel electrophoresis of *Nox2* and *Nox4* gene PCR). *B*: Normalized data showing Nox2 protein expression in PECs of WT and ESMIRO mice (n = 4; inset, an example of Nox2 blot and β-actin loading control). *C*: Relative expression of different isoforms of Nox in PECs of WT and $IR^{+/-}$ mice (inset, gel electrophoresis of *Nox2* and *Nox4* gene PCR). *D*: Relative expression of Nox2 and Nox4 in PECs of ESMIRO mice after Nox2 or scrambled siRNA treatment (insets, gel electrophoresis of β-actin, *Nox2*, and *Nox4* gene PCR). Representative trace (*E*) and mean (*F*) data show superoxide levels in PECs of ESMIRO mice after siRNA treatment (*P < 0.05).

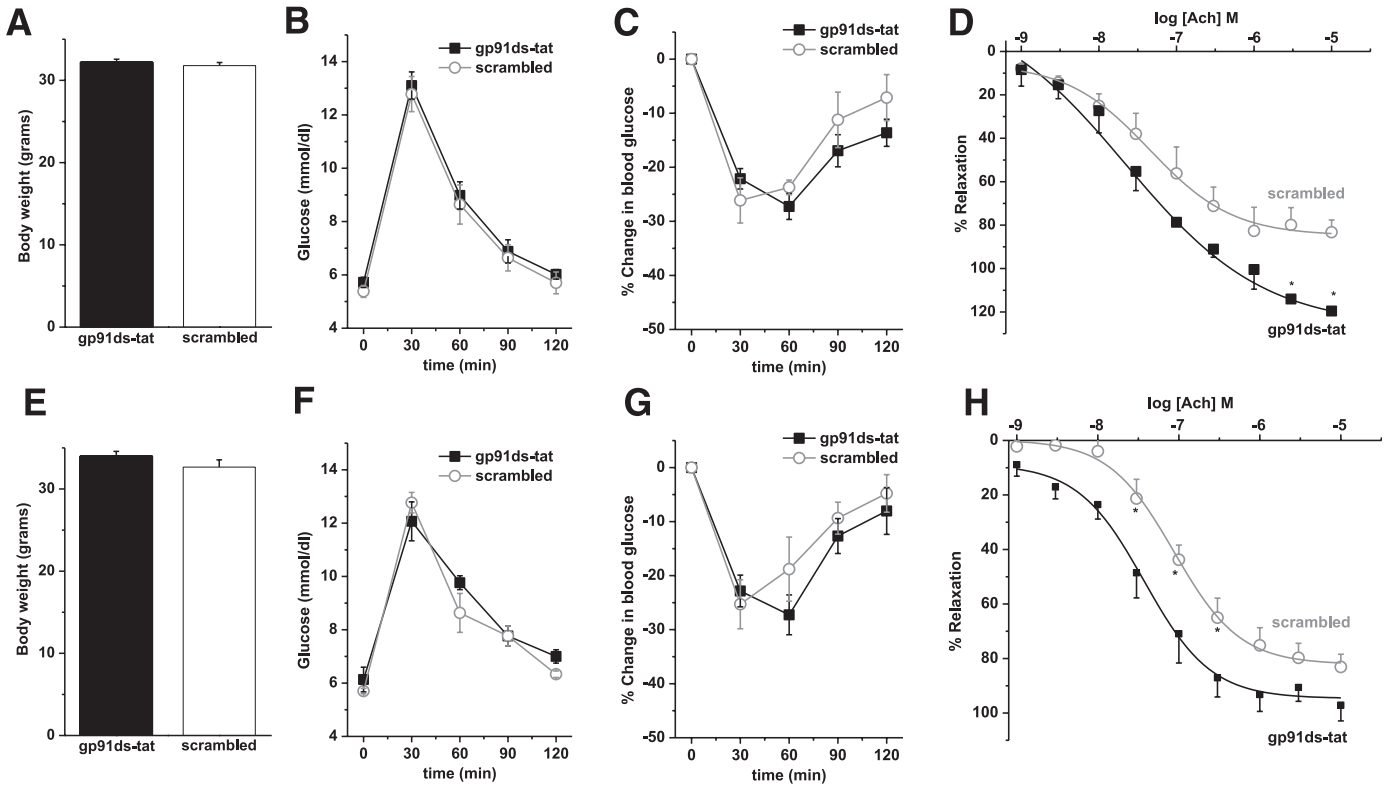

FIG. 3. Chronic pharmacological inhibition of Nox in insulin resistance. *A*: Body weights. *B*: Glucose tolerance test. *C*: Insulin tolerance test of ESMIRO mice infused with gp91ds-tat or scrambled peptide via osmotic mini-pump. *D*: Relaxation curves of aortic rings from ESMIRO mice infused with gp91ds-tat or scrambled peptide in response to Ach. *E*: Body weight. *F*: Glucose tolerance test. *G*: Insulin tolerance test of $IR^{+/-}$ mice infused with gp91ds-tat or scrambled peptide via osmotic mini-pump. *H*: Relaxation curves of aortic rings from $IR^{+/-}$ mice infused with gp91ds-tat or scrambled peptide in response to Ach (*$P < 0.05$).

Lucigenin-enhanced chemiluminescence showed that basal superoxide level was significantly reduced in ESMIRO/$Nox2^{y/-}$ PECs compared with ESMIRO (Fig. 4*E*). HPLC assay also showed a similar result in aorta of ESMIRO/$Nox2^{y/-}$ mice. Aortic rings from ESMIRO/$Nox2^{y/-}$ mice had significantly enhanced Ach-induced relaxation compared with ESMIRO (Fig. 4*G*). Real-time PCR analysis of RNA isolated from ESMIRO/$Nox2^{y/-}$ and ESMIRO lungs showed a significant decrease in *SOD1* expression in ESMIRO/$Nox2^{y/-}$ (Supplementary Fig. 8).

DISCUSSION

The principal findings of the present report are as follows: *1*) Acute and chronic inhibition of Nox using an inhibitory peptide reduces oxidative stress and restores defective vasomotor function in complementary in vivo models of insulin resistance. *2*) Acute knockdown of Nox2 reduces oxidative stress in insulin-resistant endothelial cells. *3*) Deletion of Nox2 in vivo restores aortic vasomotor function and reduces oxidative stress. *4*) Chronic inhibition of Nox or Nox2 has no effect on glucose homeostasis.

Both *Nox2* and *Nox4* are expressed in the endothelium and may contribute to ROS production. We previously demonstrated increased Nox4 mRNA expression in coronary microvascular endothelial cells from ESMIRO mice (6). Nox4 has been shown to be regulated at an mRNA level and have a unique pattern of ROS generation (13) and could therefore contribute to insulin resistance–related oxidative stress. Here, we show that specifically inhibiting Nox2 as opposed to Nox4 restored vascular function in insulin-resistant mice. This is of particular importance, as Nox4 may have favorable effects on vascular function (11) and angiogenesis (14).

This report and our previously published work now provide a unifying mechanism for the oxidative stress associated with insulin resistance. In obese mice (15) and lean mice with whole-body (5) and endothelium-specific (6) insulin resistance, we have demonstrated reduced NO bioavailability and increased superoxide generation. The principal source of superoxide was Nox. Consistent with these studies, Nox has recently emerged as a major source of superoxide in insulin-resistant humans (16,17). Despite compelling experimental evidence supporting a role for ROS in atherosclerosis, clinical trials of antioxidants have been disappointing (18). In this context, our study showing favorable effects of specifically targeting Nox2 isoform of NADPH oxidase in insulin resistance–associated endothelial cell dysfunction gains significant importance.

In conclusion, we have demonstrated that inhibiting Nox and specifically Nox2 in vivo improves endothelial cell function in mice with insulin resistance. This approach did not adversely affect glucose homeostasis. Our data establish Nox2 as an attractive target to prevent early atherosclerosis in insulin resistance.

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No potential conflicts of interest relevant to this article were reported.

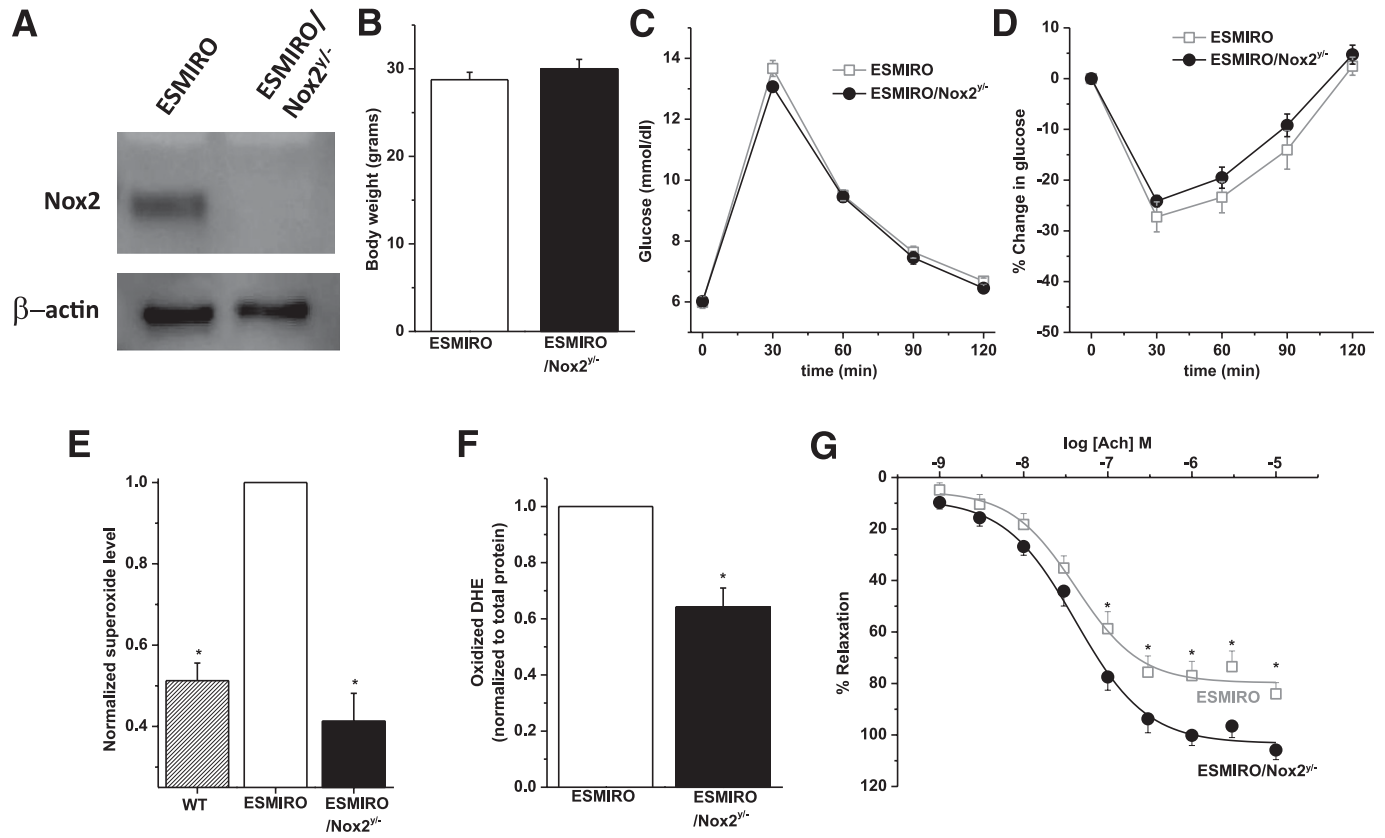

FIG. 4. Genetic deletion of Nox2 in insulin resistance. *A*: Representative Western blot of Nox2 protein in PECs of ESMIRO and ESMIRO/Nox2$^{y/-}$. Body weights (*B*) and glucose tolerance (*C*) and insulin tolerance (*D*) tests of ESMIRO and ESMIRO/Nox2$^{y/-}$ mice. *E*: Normalized superoxide level in PECs of ESMIRO and ESMIRO/Nox2$^{y/-}$ measured by lucigenin-enhanced chemiluminescence assay. *F*: Normalized oxidized DHE level in aorta of ESMIRO and ESMIRO/Nox2$^{y/-}$ mice measured by HPLC-based DHE analysis. *G*: Aortic relaxation curves of ESMIRO and ESMIRO/Nox2$^{y/-}$ mice in response to increasing concentrations of Ach (*$P < 0.05$).

P.S. performed experiments, analyzed data, and wrote the manuscript. H.V. and H.I. performed experiments and analyzed data. R.M.C. contributed to discussion and reviewed and edited the manuscript. N.Y., M.G., S.G., A.S., P.K., C.X.S., V.K.G., and J.S. performed experiments and analyzed data. D.J.B., S.B.W., K.M.C., and A.M.S. contributed to discussion and reviewed and edited the manuscript. M.T.K., A.M.S., K.M.C., and S.B.W. obtained funding and designed experiments. M.T.K. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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