Effect of Endothelium-Specific Insulin Resistance on Endothelial Function In Vivo

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OBJECTIVE—Insulin resistance is an independent risk factor for the development of cardiovascular atherosclerosis. A key step in the development of atherosclerosis is endothelial dysfunction, manifest by a reduction in bioactivity of nitric oxide (NO). Insulin resistance is associated with endothelial dysfunction; however, the mechanistic relationship between these abnormalities and the role of impaired endothelial insulin signaling versus global insulin resistance remains unclear.

RESEARCH DESIGN AND METHODS—To examine the effects of insulin resistance specific to the endothelium, we generated a transgenic mouse with endothelium-targeted overexpression of a dominant-negative mutant human insulin receptor (ESMIRO). This receptor has a mutation (Ala-Thr^{1134}) in its tyrosine kinase domain that disrupts insulin signaling. Humans with the Thr^{1134} mutation are insulin resistant. We performed metabolic and vascular characterization of this model.

RESULTS—ESMIRO mice had preserved glucose homeostasis and were normotensive. They had significant endothelial dysfunction as evidenced by blunted aortic vasorelaxant responses to acetylcholine (ACh) and calcium ionophore. Furthermore, the vascular action of insulin was lost in ESMIRO mice, and insulin-induced endothelial NO synthase (eNOS) phosphorylation was blunted. Despite this phenotype, ESMIRO mice demonstrate similar levels of eNOS mRNA and protein expression to wild type. ACh-induced relaxation was normalized by the superoxide dismutase mimetic, Mn(III)tetrakis(1-methyl-4-pyridyl) porphyrin pentachloride. Endothelial cells of ESMIRO mice showed increased superoxide generation and increased mRNA expression of the NADPH oxidase isoforms Nox2 and Nox4.

CONCLUSIONS—Selective endothelial insulin resistance is sufficient to induce a reduction in NO bioavailability and endothelial dysfunction that is secondary to increased generation of reactive oxygen species. This arises independent of a significant metabolic phenotype. ***Diabetes*** **57:3307–3314, 2008**

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Recent changes in human lifestyle have led to a striking increase in the incidence of obesity and type 2 diabetes (1). Resistance to the effects of insulin on its traditional target tissues (muscle, liver, and adipose tissue) is a central pathogenic feature of these disorders (2). Insulin resistance at a whole-body level is an independent risk factor for the development of atherosclerosis (3–8). A key pathogenic step in atherogenesis is the development of endothelial cell dysfunction, manifest by a reduction in bioavailability of the antiatherosclerotic signaling molecule nitric oxide (NO) (9). Consonant with this, longitudinal studies have shown that impaired NO-dependent vasodilatation is a predictor of future cardiac events and the development of coronary artery atherosclerosis (10). Insulin resistance is associated with endothelial dysfunction (11), and this may account at least in part for its ultimately deleterious consequences. A number of studies have suggested a reciprocal relationship between insulin sensitivity and endothelial cell function (12).

The mechanistic relationship between insulin resistance and endothelial dysfunction remains unclear. Studies of vascular function in models of insulin resistance are complicated by the complex phenotype of type 2 diabetes, which includes numerous factors that may influence endothelial function, e.g., hyperinsulinemia, hyperglycemia, hypertension, and hyperlipidemia. Therefore, if the relationship between insulin resistance and endothelial dysfunction in vivo is causative, the basis for this is difficult to establish. Nevertheless, there is evidence to suggest that the direct effects of insulin on the endothelium or disrupted endothelial insulin signaling may impact on endothelial function. Insulin stimulates endothelial cell production of NO (13), and, therefore, insulin resistance at the level of the endothelium might be expected to be associated with reduced insulin-stimulated NO. Recently, Kahn and colleagues (14) generated a mouse with endothelium-targeted deficiency of the insulin receptor to study the impact of insulin resistance specific to the endothelium on metabolic homeostasis. Endothelial cell function and NO bioavailability were not addressed in this study.

In the current study, we report the effects of selective disruption of insulin signaling in the endothelium in vivo, achieved by endothelium-targeted overexpression of a mutant human insulin receptor under control of the tie-2 promoter (endothelium-specific mutant insulin receptor overexpressing [ESMIRO] mice). This mutant receptor has an alanine residue replaced by Thr^{1134}, resulting in markedly impaired insulin signaling and severe insulin resistance in heterozygous human subjects (15). Thr^{1134} receptors display normal ligand binding but are devoid of detectable insulin-stimulated tyrosine kinase activity and

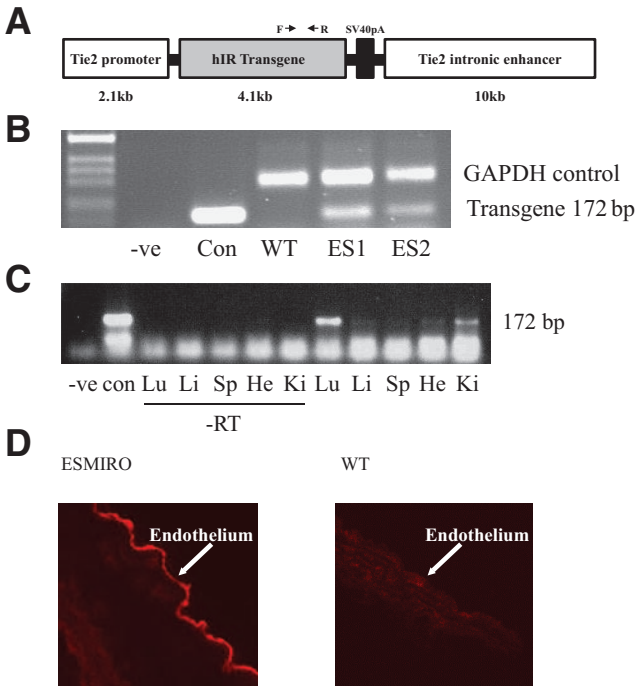

FIG. 1. *A*: Schematic of the Tie2/mutant human insulin receptor transgene. Forward (F) and reverse (R) oligonucleotide primers specific for the human insulin receptor are represented. *B*: PCR of genomic DNA from tail lysates using the primers demonstrates incorporation of the transgene into genomic DNA in both lines of ESMIRO mice. *C*: Transgene expression in different organs from ESMIRO mice. ESMIRO mice demonstrated increased transgene expression in lung (Lu) and kidney (Ki) compared with liver (Li), spleen (SP), and heart (He). −ve, negative control; con, positive control (construct); RT-ve, reverse transcriptase negative. *D*: Immunohistochemistry of sections of thoracic aorta from ESMIRO and wild-type (WT) mice. Staining with an antibody specific for the human insulin receptor demonstrated transgene protein expression in ESMIRO but not wild-type endothelium (magnification ×400). The transgene colocalized in the endothelium with von Willebrand factor (data not shown). BP, base pair. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. (Please see http://dx.doi.org/10.2337/db07-1111 for a high-quality digital representation of this figure.)

fail to mediate several of insulin's biological effects, including activation of the insulin signaling pathway (15,16). Transgenic mice overexpressing this mutant receptor in skeletal muscle have blunting of insulin-mediated insulin receptor substrate (IRS)-1 and phosphatidylinositol 3-kinase (PI 3-kinase) phosphorylation (17). Although genetic defects involving the insulin receptor are rare, impaired insulin-mediated receptor kinase activity and impaired activation of the insulin signaling pathway are characteristic of obese and insulin-resistant type 2 diabetic humans (18). Here, we report the effects of overexpression of this receptor in the endothelium in vivo on metabolic and blood pressure homeostasis and on endothelial function in conduit vessels.

RESEARCH DESIGN AND METHODS

Endothelial cell–specific transgene overexpression was achieved using the murine Tie2 promoter and intronic enhancer as previously described (19). A Thr^{1134} mutation was introduced into cDNA encoding the human insulin receptor by site-directed mutagenesis (15). The plasmid pHHNS was a gift from Keith Channon (University of Oxford, Oxford, U.K.). After excision of the *LacZ* sequence with *Sbf*I and *Mlu*I (19), the Thr^{1134} mutant human insulin receptor cDNA was cloned into pHHNS (Fig. 1*A*). Plasmid fidelity and orientation were confirmed using standard DNA sequencing and PCR analysis.

The transgene *Tie2-hIR* was excised with *Sal*I, purified, and microinjected into fertilized eggs from superovulated C57BL/6 × CBA mice. Potential founders were screened by genotyping of genomic DNA from tail lysates using primers specific for the human insulin receptor (forward, 5′-GGT GGC AGC TTT CCC CAA CAC T; reverse, 5′-AGC CTT GGC TTC AGG CAT GGT C-3′).

Animals were backcrossed eight times onto a C57BL/6J background. All studies were comparisons of male ESMIRO mice aged 8–10 weeks and age- and sex-matched wild-type littermates. Morphological assessment was performed in 8- to 10-week-old male mice (20). Two lines of ESMIRO mice were generated, and experiments were performed in both lines to exclude a positional effect of transgene integration into the murine genome.

Semiquantitative and real-time RT-PCR. Total RNA was extracted from a variety of tissues using an RNeasy Mini kit (Qiagen). Equal quantities of RNA were reverse transcribed using Superscript II RT (Invitrogen) and random decamer oligonucleotides. To assess transgene expression, PCR was performed using primers specific for the human insulin receptor (forward, 5′-GGT GGC AGC TTT CCC CAA CAC T; reverse, 5′-AGC CTT GGC TTC AGG CAT GGT C-3′).

Real-time PCR analysis of endothelial NO synthase (eNOS), NOX2, NOX4, and β-actin expression was performed (eNOS forward, GGGAAAGCTGCAGGTATTTGAT, and reverse, CACTGTGATGGCTGAACGAAGA; Nox2 forward, ACTCCTTGGGTCAGCACTGG, and reverse, GTTCCTGTCCAGTTGTCTTCG; Nox4 forward, TGAACTACAGTGAAGATTTCCTTGAAC, and reverse, GACACCCGTCAGACCAGGAAT; and β-actin forward, CGTGAAAAGATGACCCAGATCA, and reverse, TGGTACGACCAGAGGCATACAG) (20). The standard curve method was used, and results are normalized to β-actin expression.

Immunohistochemistry. Thoracic aorta was excised and mounted vertically in OCT embedding matrix (CellPath, Powys, U.K.) before freezing in cold isopentane. Eight-micrometer transverse sections were fixed in paraformaldehyde (4% in PBS, 30 min). A human-specific anti-insulin receptor antibody (1 in 200, 30 min; E5844; Spring Bioscience, Fremont, CA) was used, which does not cross-react with the murine insulin receptor. A Vector M.O.M. immunodetection kit (Vector Laboratories, Burlingame, CA) reduced background staining of endogenous mouse immunoglobulins. Antibody staining was visualized using a secondary antibody conjugated to fluorophores (Alexa Fluor Dyes; Molecular Probes). Aortic sections were also probed with anti–von Willebrand factor antibody (Sigma-Aldrich, Gillingham, U.K.).

eNOS and phospho-eNOS (Ser^{1177}) protein expression. Fasted mice were anesthetized. An injection of either human recombinant insulin (5 units; Actrapid; NovoNordisk, Bagsvaerd, Denmark) or saline vehicle (0.9% NaCl) was administered via the inferior vena cava. After 5 min, the thoracic aorta was excised. Immunoblotting was performed on total aortic tissue homogenates using mouse monoclonal antibodies against total murine eNOS and phospho-eNOS (Ser^{1177}) or a rabbit polyclonal antibody against β-actin (20).

Metabolic assessment. Intraperitoneal glucose and insulin tolerance tests were performed in conscious fasted mice. Plasma insulin was measured by enzyme-linked immunoassay using mouse insulin standards. Fasting triglycerides and fasting free fatty acids were measured by colorimetry (20,21). Homeostasis model assessment of insulin resistance (HOMA-IR) was calculated from fasting insulin and glucose measurements.

Blood pressure. Systolic blood pressure was measured using tail cuff plethysmography (20,21).

Aortic vascular function. Vascular function was studied in aortic rings mounted in an organ bath as described previously (20,21). Cumulative dose responses to phenylepherine (1 nmol/l to 10 μmol/l) were measured. Phenylepherine constriction responses were reassessed after incubation with insulin (100 mU/ml; 2 h; Actrapid) (20). Relaxation responses to acetylcholine (ACh; 1 nmol/l to 10 μmol/l), A23187 (1 nmol/l to 1 μmol/l), and sodium nitroprusside (SNP; 0.1 nmol/l to 1 μmol/l) were then measured. ACh relaxation experiments were repeated after incubation with the SOD mimetic Mn(III)tetrakis(1-methyl-4-pyridyl) porphyrin pentachloride (MnTMPyP; 10 μmol/l; 30 min) (22).

Coronary microvascular endothelial cell isolation. Six mouse hearts were used for each preparation of coronary microvascular endothelial cells (CMECs) as described previously (23,24).

Vascular superoxide production. Three different methods were used to detect superoxide. First, in situ reactive oxygen species (ROS) generation by CMECs was assessed using dihydroethidium (DHE) fluorescence (2 μmol/l; 5 min) as previously reported (24,25). CMECs were grown to 70% confluence before exposure to DHE. Peak fluorescence intensity per cell was quantified microscopically using a computerized image analysis system (Improvision) from at least 30 cells per group; $n = 6$ mice per CMEC isolation.

Second, 5 μmol/l lucigenin-enhanced chemiluminescence was performed on homogenates of CMECs or aorta to assess NADPH-dependent superoxide production (22,23). In some experiments, one of the following agents was preincubated for 10 min: 20 mmol/l tiron, 10 μmol/l diphenyleneiodonium (DPI), or 100 μmol/l NG-nitro-L-arginine methyl ester (L-NAME).

Finally, functional evidence for the generation of superoxide was obtained by assessing the effect of an SOD mimetic (MnTMPyP) on aortic relaxation responses to ACh in the ESMIRO model (24).

TABLE 1
Metabolic characteristics and systolic blood pressure of ESMIRO mice compared with their wild-type littermates

	Wild type	ESMIRO	*P* value
Fasting blood sugar (mmol/l)	6.7 ± 0.4	6.5 ± 0.2	0.68
Postglucose blood sugar (mmol/l)	16.2 ± 1.0	14.4 ± 0.7	0.17
Random blood glucose (mmol/l)	11.1 ± 1.1	13.3 ± 1.1	0.19
Fasting insulin (ng/ml)	0.30 ± 0.03	0.34 ± 0.03	0.27
Postglucose insulin (ng/ml)	0.72 ± 0.06	0.78 ± 0.10	0.61
HOMA-IR	2.4 ± 0.6	2.1 ± 0.2	0.63
Fasted triglycerides (mmol/l)	0.98 ± 0.14	0.8 ± 0.08	0.28
Fasted free fatty acids (mmol/l)	1.0 ± 0.2	0.92 ± 0.1	0.49
Systolic blood pressure (mmHg)	131 ± 4	129 ± 4	0.68

Data are means ± SE ($n = 8$). No significant differences were noted between the two groups.

Statistics. Data are expressed as means ± SE. In vascular studies, concentration-response relationships were compared using two-way repeated-measures ANOVA. Half-maximal effective concentration (EC_{50}) and maximal response (Emax) were compared using a Student's *t* test. One-way ANOVA was used to analyze chemiluminescence data (Newman-Keuls multiple comparison test for post hoc analysis). Other variables were compared using Student's *t* test. $P < 0.05$ taken as statistically significant.

RESULTS

Endothelium-specific expression of Thr^{1134} insulin receptor. Endothelial cell–specific expression of mutant Thr^{1134} receptors was achieved using a Tie-2 promoter/intronic enhancer construct (26). Two lines of transgenic mice were generated. Semiquantitative RT-PCR performed on mRNA isolated from a range of organs from ESMIRO mice demonstrated that transgene expression was greater in endothelial-rich tissues such as lung and to a lesser extent kidney as opposed to liver, spleen, and heart (Fig. 1*C*). Expression of mutant human insulin receptor protein was confirmed by immunohistochemistry on sections of thoracic aorta using an antibody specific for the human insulin receptor (Fig. 1*D*). Positive staining was found at the luminal endothelial surface of ESMIRO mouse aorta but not wild type. The transgene protein colocalized with endothelial cells as labeled with the endothelial marker, von Willebrand factor (data not shown).

Morphometric analysis. ESMIRO mice and their wild-type littermates appeared morphologically similar. No differences in fertility, lifespan, or behavior were observed. Whole-body weight, mean organ weights, and markers of adiposity were similar in ESMIRO and wild-type mice (data not shown).

Metabolic homeostasis. It has been suggested that endothelial cell insulin resistance might promote global metabolic insulin resistance secondary to decreased insulin-stimulated blood flow to insulin-sensitive tissues (27–29). However, metabolic characterization of the ESMIRO mice did not support such a mechanism. ESMIRO and wild-type mice demonstrated similar fasting blood glucose levels (6.5 ± 0.2 and 6.7 ± 0.4 mmol/l, $n = 8$) and fasting serum insulin levels (0.34 ± 0.03 and 0.30 ± 0.03 ng/ml, $n = 8$) (Table 1). Glucose tolerance testing demonstrated no difference between the two groups ($n = 11$, $P > 0.05$; data not shown). Insulin tolerance testing suggested a trend toward improved insulin sensitivity in ESMIRO mice ($n = 11$, $P = 0.05$ by repeated-measures ANOVA; data not shown). Serum insulin levels rose equally in response to an intraperitoneal glucose challenge (ESMIRO, 0.78 ± 0.10 and wild type, 0.72 ± 0.06 ng/ml). HOMA-IRs were similar in ESMIRO and wild-type mice (2.1 ± 0.2 and 2.4 ± 0.6, respectively; Table 1). Fasting serum triglycerides (0.80 ± 0.08 and 0.98 ± 0.14 mmol/l, respectively) and free fatty acids (0.92 ± 0.1 and 1.0 ± 0.2 mmol/l, respectively) were similar in both groups.

Blood pressure regulation. It has been previously suggested that insulin resistance at the vascular level may lead to hypertension (30). However, ESMIRO and wild-type mice had similar systolic blood pressures (Table 1; 131 ± 4 and 129 ± 4 mmHg, $n = 8$).

Effect of endothelial cell–specific mutant receptor expression on vasoconstrictor responses and the actions of insulin in conduit vessels. Isolated aortic rings of ESMIRO mice and wild-type mice had similar constrictor responses to 40 mmol/l KCl (Emax 0.68 ± 0.04 and 0.73 ± 0.06 g, ESMIRO and wild type, respectively) and phenylepherine (Fig. 2*A*; ESMIRO, Emax 0.84 ± 0.06 g, EC_{50} 227 ± 43 nmol/l; and wild-type, Emax 0.87 ± 0.05 g, EC_{50} 242 ± 26 nmol/l).

Acute exposure to insulin blunts the vasoconstrictor response to phenylepherine through an endothelial- and NO-dependent mechanism (20). Consistent with this, exposure of wild-type aorta to insulin caused a significant blunting of maximal phenylepherine-induced constriction (compare Fig. 2*A* and *B*). However, this effect was lost in ESMIRO mice (Fig. 2*B*; phenylepherine plus insulin Emax 0.83 ± 0.07 and 0.59 ± 0.06 g, ESMIRO and wild type, respectively; $P < 0.05$).

The endothelial-dependent vasodilator effect of insulin involves Akt-mediated phosphorylation of eNOS on residue Ser^{1177}, resulting in enhanced NO production (31). To investigate the effect of transgene expression on this effect of insulin on endothelial cells, we studied eNOS protein expression and Ser^{1177} eNOS phosphorylation in the thoracic aorta of ESMIRO and wild-type mice exposed to insulin in vivo. Basal levels of eNOS protein expression were similar in ESMIRO and wild-type mice (Fig. 2*C* and *D*). After administration of insulin, wild-type mice demonstrated a significant increase in the phospho-eNOS–to–eNOS ratio (1.00 vs. 1.78 ± 0.15, $P < 0.05$); this effect was absent in ESMIRO mice (0.63 ± 0.1 vs. 0.61 ± 0.1, NS). Therefore, overexpression of the mutant Thr1134 insulin receptor leads to impaired endothelial insulin signaling. A similar result was demonstrated in complementary experiments performed using lung tissue (data not shown).

Effect of transgene expression on endothelial-dependent vasodilator responses in conduit vessels. Vasorelaxation to the agonist ACh was blunted in ESMIRO mice compared with wild type (Fig. 3*A*; Emax 68 ± 6 and 102 ± 6%, ESMIRO and wild type, respectively, $P < 0.01$; EC_{50} 180 ± 73 and 114 ± 37 nmol/l, ESMIRO and wild type, respectively, NS). Similarly, vasodilator responses to the calcium ionophore A23187 were also blunted (Fig. 3*B*; Emax 51 ± 12 and 79 ± 8%, ESMIRO and wild type, respectively, $P < 0.05$; EC_{50} 119 ± 29 and 39 ± 8 nmol/l, ESMIRO and wild type, respectively, $P < 0.01$). However, responses to SNP were similar in ESMIRO and wild-type mice (Fig. 3*C*; Emax 109 ± 1 and 110 ± 2%, ESMIRO and wild type, respectively, NS; EC_{50} 23 ± 6 and 17 ± 2 nmol/l, ESMIRO and wild type, respectively, NS). These data

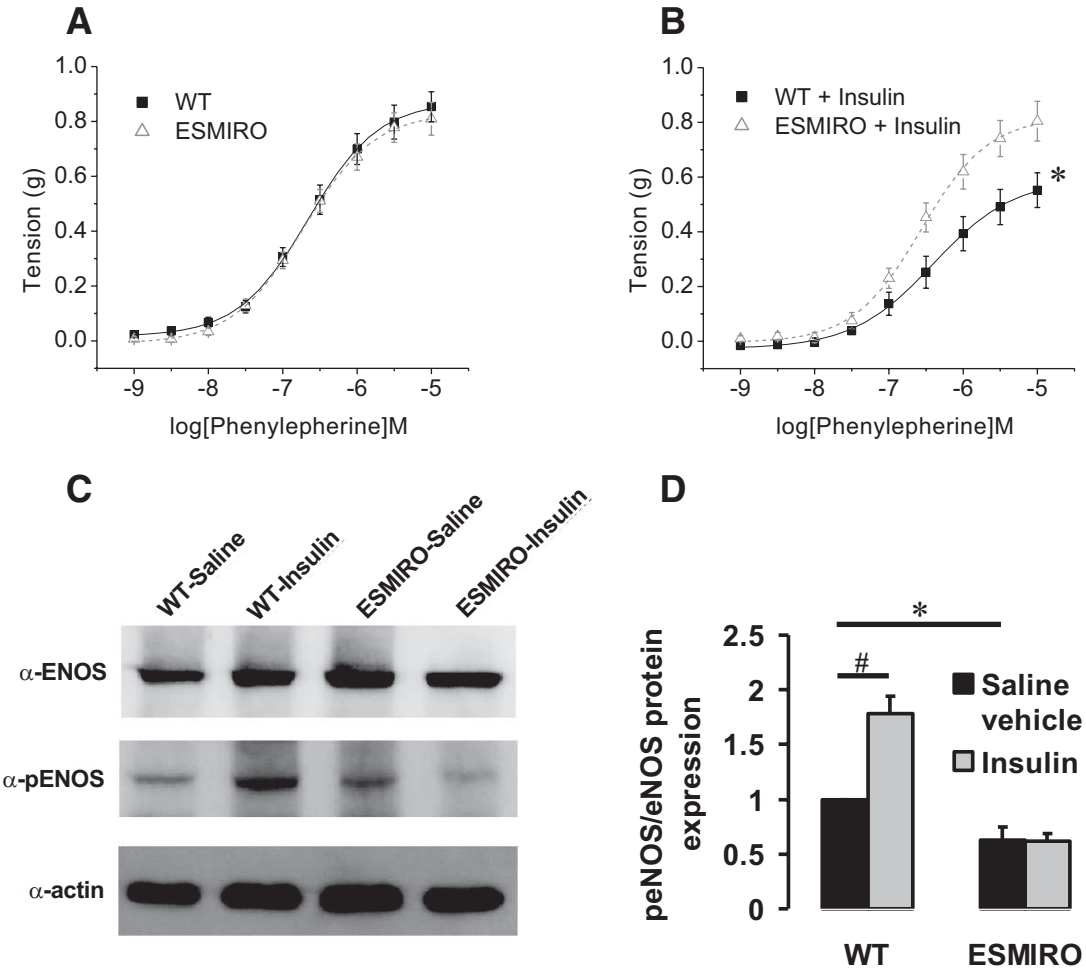

FIG. 2. The action of insulin on the aorta of ESMIRO and wild-type (WT) mice. *A*: Wild-type (■) and ESMIRO (△) mice had similar aortic vasoconstrictor responses to phenylepherine. *B*: Incubation with insulin (100 mU/ml) for 2 h significantly blunted this response in wild-type mice but had no effect in ESMIRO mice ($n = 8$). *C* and *D*: Immunoblot of eNOS and phospho-eNOS protein expression in wild-type and ESMIRO aortae ($n = 5$). There was no difference in eNOS protein expression between the two groups (corrected for β-actin). However, wild-type phospho-eNOS expression was significantly greater than that of ESMIRO mice at baseline. Furthermore, a significant increment in eNOS phosphorylation at Ser^{1177} was seen in wild-type mice after insulin injection. No such increment was seen in ESMIRO mice. *$P < 0.05$ wild type vs. ESMIRO; #$P < 0.05$ wild type vs. wild type + insulin.

demonstrate that the ESMIRO exhibits significant endothelial dysfunction.

A similar vascular phenotype was seen in a second line of ESMIRO mice (data not shown). These results exclude a positional effect after transgene integration into the murine genome.

Aortic superoxide production and its impact on endothelial function. A common mechanism of impaired endothelium-dependent vasodilatation is an increased production of ROS (32–35). We therefore assessed the effects of a SOD mimetic on ACh-induced relaxation in aortae. MnTMPyP restored vasorelaxation responses to ACh in ESMIRO aortae (Fig. 4*A*; Emax 83 ± 5 and 86 ± 7%, ESMIRO and wild type, respectively, NS), consistent with a role for increased aortic superoxide production in mediating the endothelial dysfunction seen in ESMIRO mice.

We assessed NADPH-dependent superoxide production by homogenates of thoracic aortae using lucigenin-enhanced chemiluminescence. Consistent with the functional findings in aortic vasodilatation experiments, ESMIRO aortae demonstrated increased superoxide production compared with wild type (Fig. 4*B*; 12.6.0 ± 0.5(×106) vs. 8.1 ± 0.4(×106) integrated light units, $P < 0.001$). ESMIRO and wild-type superoxide production were almost completely abolished by the antioxidant tiron ($P < 0.001$) or the flavoprotein inhibitor DPI ($P < 0.001$). The NO synthase inhibitor L-NAME had no effect on NADPH-dependent superoxide production in either group.

CMEC superoxide production. DHE fluorescence of cultured CMECs confirmed that ESMIRO mice have increased superoxide at the level of the endothelial cell compared with wild type (2,819 ± 151 and 1,439 ± 129, $P < 0.001$, $n = 6$ mice per group, Fig. 5*A*). This was confirmed by lucigenin-enhanced chemiluminescence performed on CMEC homogenates (Fig. 5*B*). As in experiments in whole aorta, NADPH-dependent superoxide production in CMECs was inhibited by tiron and DPI but not by L-NAME.

Expression of eNOS, Nox2, and Nox4 mRNA in CMECs and aorta. Consistent with the equal eNOS protein expression previously demonstrated in aorta (Fig. 2), there was no difference between groups in eNOS mRNA expression in CMECs (data not shown). However, a significant (approximately twofold) increase in mRNA expression of the NADPH oxidase isoforms Nox2 and Nox4 was seen in ESMIRO aortae (Fig. 6). Upregulation was even more pronounced in CMECs, especially that of Nox2.

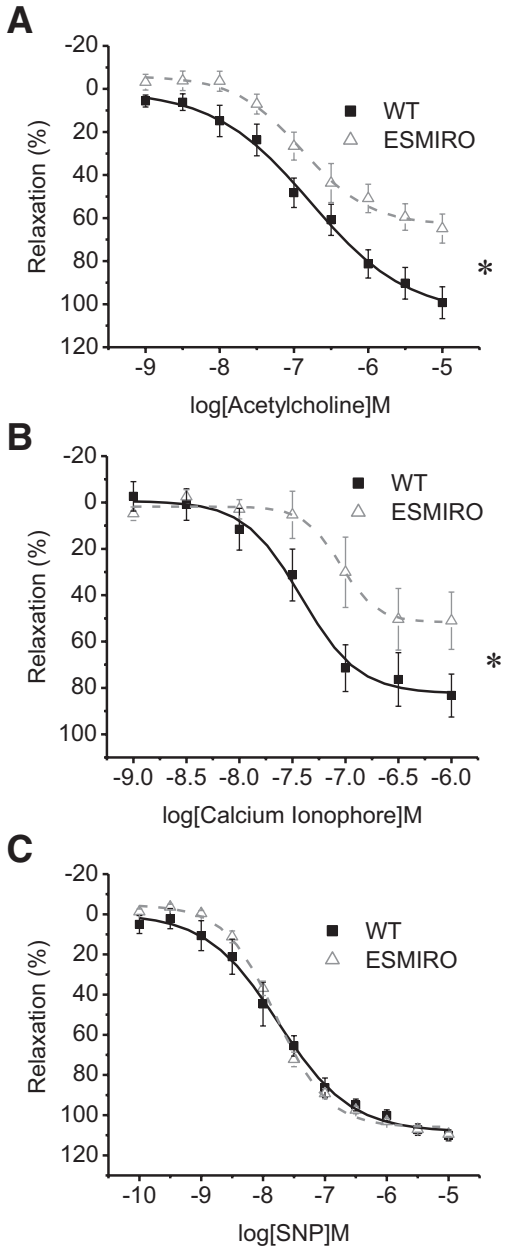

FIG. 3. Aortic relaxation responses. ESMIRO mice (△) demonstrated significantly impaired relaxation responses to ACh (*A*) and calcium ionophore A23187 (*B*) compared with wild-type (WT) littermates (■). *C*: Relaxation responses to SNP were similar in ESMIRO and wild-type mice. $n = 8$, $^*P < 0.05$.

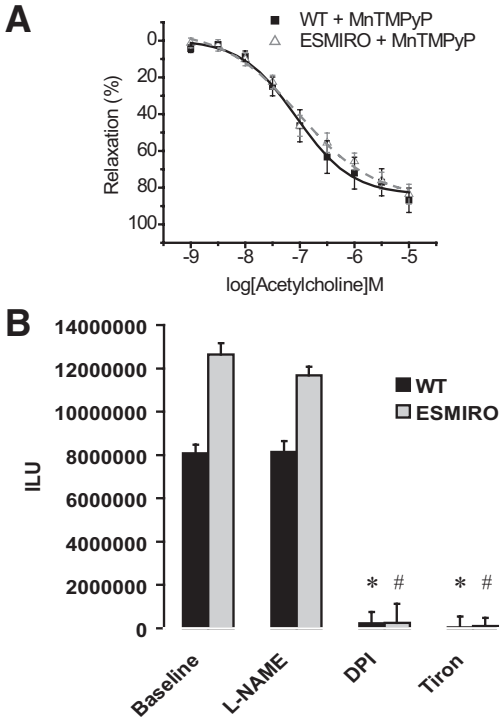

FIG. 4. Assessment of aortic superoxide production in ESMIRO and wild-type (WT) mice. ESMIRO mice demonstrate impaired relaxation responses to ACh at baseline. (see Fig. 3). *A*: Importantly, ESMIRO ACh responses (△) were normalized after incubation with the SOD mimetic, 10 μmol/l MnTMPyP, providing physiological evidence that increased superoxide production in ESMIRO aortae contributes to the impaired relaxation responses seen. *B*: Lucigenin-enhanced chemiluminescence was performed on aortic homogenates. ESMIRO aortae demonstrated increased superoxide production compared with wild type. In both groups, superoxide production was completely inhibited by the ROS scavenger tiron or the flavoprotein inhibitor DPI. There was no significant inhibition with the NOS inhibitor L-NAME. These data suggest a role for NADPH oxidase as the major source of the increased superoxide in the ESMIRO aorta. $^*P < 0.001$ with inhibitor compared with wild-type baseline; $\#P < 0.001$ with inhibitor compared with ESMIRO baseline. ILU, integrated light units.

DISCUSSION

The present study demonstrates a number of important findings that add to our understanding of the role of insulin resistance in endothelial cell homeostasis: *1*) The induction of endothelial cell–specific insulin resistance leads to markedly reduced NO bioavailability in conduit vessels without significant alteration in global insulin sensitivity. *2*) Possible mechanisms include reduced NO bioavailability secondary to increases in aortic and endothelial cell superoxide production. *3*) Endothelial cell–specific insulin resistance does not alter systolic blood pressure. *4*) Insulin action at the level of the endothelium is not a prerequisite for maintenance of normal blood glucose homeostasis.

Modulating insulin signaling with a mutant insulin receptor. To address the role of impaired insulin signaling specifically in the endothelium (as opposed to global impairment in all tissues), we generated a novel murine model of endothelial cell–specific insulin resistance by overexpressing a mutant human insulin receptor in the endothelium (15).

Insulin binding to a normal insulin receptor leads to activation of the tyrosine kinase and a rapid cascade of phosphorylation, leading to activation of multiple intracellular substrates (36). The function of these substrates is to reversibly bridge the activated insulin receptor to a variety of distal signaling molecules. As discussed above, emerging evidence supports eNOS as one of these distal signaling molecules in endothelial cells (37).

Recently, insulin receptors with a mutation where Ala^{1134} is replaced by Thr in the tyrosine kinase domain of the β-subunit have been found in humans (15). In an affected family, heterozygotes were severely insulin resistant. In in vitro transfection studies, insulin receptor processing, expression on the cell surface, and affinity of insulin binding to the mutant receptor were normal. The ability of insulin to stimulate receptor autophosphorylation and tyrosine kinase activity was, however, substantially impaired. Moreover, cells expressing Thr^{1134} insulin receptors had blunted insulin-stimulated glucose uptake

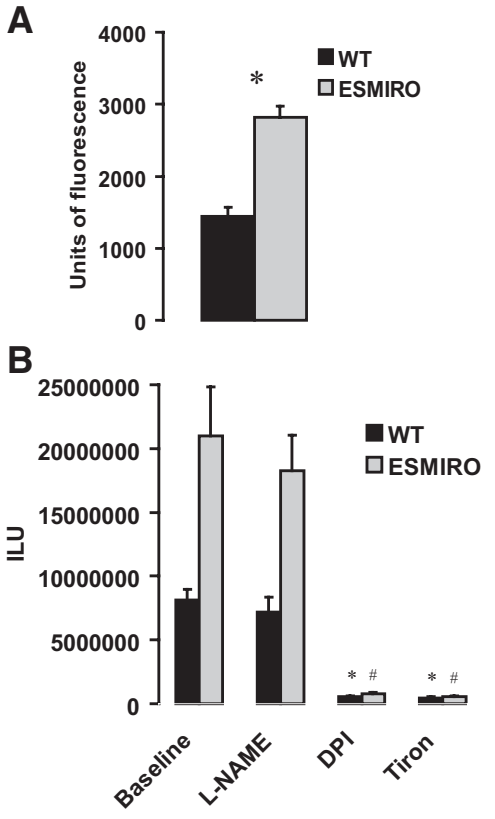

FIG. 5. Assessment of CMEC superoxide production. *A*: In situ ROS generation in CMECs was assessed through measurement of peak fluorescence of CMECs exposed to 2 μmol/l DHE (5 min). CMECs from ESMIRO mice showed significantly greater fluorescence than wild type (WT); $n = 6$ mice, $^*P < 0.05$. *B*: Using CMEC homogenates, 5 μmol/l lucigenin-enhanced chemiluminescence was repeated. Similar to the results observed in whole aortae, this suggested increased NADPH-dependent superoxide production in ESMIRO CMECs at baseline. Superoxide levels were reduced by tiron and DPI but not by L-NAME. $^*P < 0.001$ with inhibitor compared with wild-type baseline; $\#P < 0.001$ with inhibitor compared with ESMIRO baseline. ILU, integrated light units.

(16). Moller and colleagues (17) went on to produce tissue-specific transgenic mice overexpressing Thr^{1134} insulin receptors in skeletal muscle. These mice were a model of nondiabetic insulin resistance and had reduced responses to insulin during insulin tolerance testing and elevated serum glucose and insulin levels when fed ad libitum.

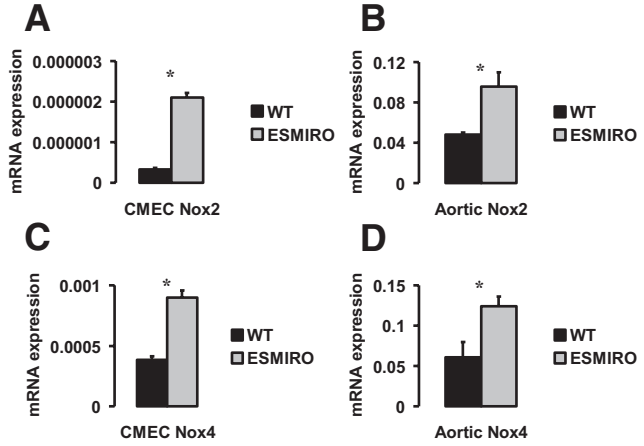

FIG. 6. Real-time RT-PCR examination of Nox2 and Nox4 mRNA expression in CMECs and whole aortae (relative to β-actin). *A* and *C*: ESMIRO mice demonstrate significantly increased Nox2 and Nox4 mRNA expression in CMECs. *B* and *D*: A similar pattern of expression is noted in aortae. CMECs, $n = 6$; aortae, $n = 5$. $^*P < 0.05$. WT, wild type.

Characterization of an endothelial cell–specific model of insulin resistance (ESMIRO mice). ESMIRO mice expressed mutant human insulin receptors in the endothelium, as confirmed by immunohistochemistry. Functional impairment of endothelial insulin signaling was confirmed in two ways. First, the endothelium-dependent, NO-mediated vasorelaxant action of insulin (to reduce the vasoconstrictor response to phenylepherine) (20) was significantly impaired in aortic rings from ESMIRO mice compared with wild type. Second, and consistent with this finding, insulin-stimulated phosphorylation of eNOS at Ser^{1177} was also blunted in the aortae of ESMIRO mice. However, the ESMIRO had similar metabolic responses to intraperitoneal boluses of insulin and glucose when compared with wild-type littermates, indicating that there was no decline in global insulin sensitivity. Moreover, we detected no differences in body weight, adiposity, plasma lipids, fasting insulin, or HOMA. Despite no difference in metabolic homeostasis, ESMIRO mice had a striking defect in endothelium-dependent relaxation in aorta, attributable to an unfavorable imbalance between the production of NO and ROS. Therefore, there was a significant divergence between the impact of impaired endothelial insulin signaling on vascular function versus glucose homeostasis.

Endothelial insulin resistance and NO bioavailability. Insulin resistance and type 2 diabetes are now established as important risk factors for the development of cardiovascular atherosclerosis (3,8,38). The alarming increase in type 2 diabetes in children and young adults and the fact that a substantial proportion of patients with type 2 diabetes have coronary artery disease at presentation makes understanding the mechanisms underlying accelerated atherosclerosis in insulin resistance of particular importance. Compelling evidence supports endothelial cell dysfunction as a key early event in the pathogenesis of atherosclerosis (9).

The bioavailability of NO is dependent on the balance between its production by eNOS and its inactivation by ROS. Classical activation of eNOS (e.g., by ACh) involves a rise in intracellular Ca^{2+} and binding of Ca^{2+}/calmodulin to the enzyme. Recently, a Ca^{2+}-independent regulatory pathway for eNOS stimulated by shear stress or insulin has also been described (31). Both shear stress and insulin increase endothelial NO production via the activation of PI 3-kinase and protein kinase B (PKB/Akt), which phosphorylates eNOS on Ser^{1177} (37). In addition, insulin upregulates eNOS transcription in endothelial cells (39). In the present study, impaired endothelial insulin signaling was accompanied by reduced insulin-mediated vasodilation as might be expected. Interestingly, ACh- and calcium ionophore–stimulated vasodilation were also significantly impaired despite eNOS expression being unaffected. The mechanism underlying this observed endothelial dysfunction appears to be an increase in endothelial cell and aortic superoxide production. In a recent report, we demonstrated that mice with global haploinsufficiency of the insulin receptor (IRKO mice) have blunted insulin-dependent vasodilator responses in aortic rings but preserved vasorelaxant responses to ACh at a young age (20). However, as IRKO mice age, they develop blunting of ACh-mediated aortic relaxation secondary to increased superoxide production (24). The present report indicates a similar mechanism of endothelial dysfunction and sug-

gests that sustained or severe perturbation of insulin signaling at the level of the endothelial cell may lead to increased superoxide generation and therefore a reduction in NO bioavailability.

Although we used a Tie2 promoter construct to direct endothelial cell–specific expression, there is now some evidence that Tie2 may also be expressed within a small subset (2–7%) of circulating blood monocytes (40). This raises the possibility that the increased superoxide demonstrated in whole ESMIRO aortae could result from circulating hematopoietic cells. However, this is unlikely in view of the small population of cells involved and the fact that endothelial dysfunction was confirmed in isolated aorta in the organ bath. Furthermore, both DHE fluorescence and lucigenin-enhanced chemiluminescence performed on isolated endothelial cells from ESMIRO mice confirmed increased NADPH-dependent superoxide production by these cells.

Interestingly, in contrast with the data from Kahn and colleagues (14) and the vascular endothelium insulin receptor knockout (VENIRKO) mouse, total eNOS expression was not downregulated in our model either at mRNA or protein level. There are a number of potential explanations for this difference. The VENIRKO mouse is a conditional knockout model generated using a Cre-lox approach, whereas the ESMIRO mouse presented in the current report demonstrates overexpression of mutant insulin receptors with a dominant-negative action. This mutant receptor has been demonstrated previously to bind insulin normally but to lack tyrosine kinase activity. These fundamental differences between the models may explain the differences observed. It is also unclear from the VENIRKO paper at which age the mice were studied. This may be important because we have recently demonstrated that mice with haploinsufficiency of the insulin receptor are prone to an age-related decline in endothelial function (24).

Three different methods were used to confirm the increased superoxide production in the ESMIRO mouse. Perhaps most notably, a SOD mimetic restored endothelial function through conversion of superoxide to hydrogen peroxide. The source of increased endothelial superoxide in the study does, however, remain to be fully defined. NADPH oxidases are recognized as major sources of ROS in the vasculature (41) and are dysregulated in models of type 2 diabetes, insulin resistance, and obesity (42–45). Several isoforms of these enzymes have been described previously (46), among which Nox2 and Nox4 have been implicated in increased oxidase activity in obesity (47,48) and in insulin signaling in nonendothelial tissues (49). An interesting finding in the current study was the increase in Nox2 and Nox4 mRNA levels in aortae and CMECs of ESMIRO mice, which would be in keeping with a role for NADPH oxidases in the increased superoxide generation. However, in the absence of available good selective inhibitors of these enzymes, definitive evidence of their involvement would require additional studies probably involving appropriate gene-modified models.

The mechanism of Nox2 and Nox4 upregulation in the endothelium requires further elucidation. Recent studies have suggested an association between insulin signaling via the PI 3-kinase pathway and Nox4 expression (49). It has been hypothesized that Nox4 activity may maintain insulin sensitivity. Therefore, in our model Nox4 may be upregulated to compensate for the impaired endothelial insulin signaling. Future studies would also clarify whether Nox2 and Nox4 are upregulated in aortic tissues other than the endothelium.

Finally, the ESMIRO demonstrates a reduced phospho-eNOS–to–eNOS ratio compared with wild type both after stimulation with insulin and at baseline. The latter difference was small. This does raise the possibility that reduced eNOS activity may contribute to the endothelial dysfunction in the ESMIRO irrespective of preserved eNOS expression. This requires further investigation.

Endothelial insulin resistance and glucose homeostasis. Despite a substantial defect of insulin signaling in vascular endothelial cells, we found no demonstrable abnormality in the metabolic phenotype of ESMIRO mice. In fact, consistent with the findings in VENIRKO mice (14), there was a tendency for an improvement in insulin sensitivity during the later stages of insulin tolerance testing. Although a euglycemic insulin clamp may have revealed more subtle abnormalities in glucose homeostasis, our data and those from Kahn and colleagues (14) do not support a nonredundant role for insulin-induced endothelial NO release in blood glucose homeostasis.

Conclusions. The observed phenotype of ESMIRO mice indicates that abnormal endothelium-dependent vasodilatation in the context of insulin resistance may be attributable to impaired insulin signaling at the level of the endothelial cell per se, independent of other abnormalities that are the result of global insulin resistance. Therefore, the integrity of endothelial cell insulin signaling plays a key role in determining conduit vessel endothelial function and NO bioavailability in response to conventional (non-insulin receptor–mediated) agonists.

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REFERENCES

1. James PT, Rigby N, Leach R: The obesity epidemic, metabolic syndrome and future prevention strategies. *Eur J Cardiovasc Prev Rehabil* 11:3–8, 2004
2. Reaven GM: Banting Lecture 1988: Role of insulin resistance in human disease. *Diabetes* 37:1595–1607, 1988
3. Hanley AJ, Williams K, Stern MP, Haffner SM: Homeostasis model assessment of insulin resistance in relation to the incidence of cardiovascular disease: the San Antonio Heart Study. *Diabetes Care* 25:1177–1184, 2002
4. Haffner SM, D'Agostino R Jr, Mykkanen L, Tracy R, Howard B, Rewers M, Selby J, Savage PJ, Saad MF: Insulin sensitivity in subjects with type 2 diabetes. Relationship to cardiovascular risk factors: the Insulin Resistance Atherosclerosis Study. *Diabetes Care* 22:562–568, 1999
5. Sinaiko AR, Steinberger J, Moran A, Prineas RJ, Vessby B, Basu S, Tracy R, Jacobs DR Jr: Relation of body mass index and insulin resistance to cardiovascular risk factors, inflammatory factors, and oxidative stress during adolescence. *Circulation* 111:1985–1991, 2005
6. Haffner SM, Mykkanen L, Festa A, Burke JP, Stern MP: Insulin-resistant prediabetic subjects have more atherogenic risk factors than insulin-sensitive prediabetic subjects: implications for preventing coronary heart disease during the prediabetic state. *Circulation* 101:975–980, 2000
7. Prior JO, Quinones MJ, Hernandez-Pampaloni M, Facta AD, Schindler TH, Sayre JW, Hsueh WA, Schelbert HR: Coronary circulatory dysfunction in insulin resistance, impaired glucose tolerance, and type 2 diabetes mellitus. *Circulation* 111:2291–2298, 2005
8. Haffner SM, Lehto S, Ronnemaa T, Pyorala K, Laakso M: Mortality from coronary heart disease in subjects with type 2 diabetes and in nondiabetic subjects with and without prior myocardial infarction. *N Engl J Med* 339:229–234, 1998

9. Ross R: Atherosclerosis–an inflammatory disease. *N Engl J Med* 340:115–126, 1999
10. Schachinger V, Britten MB, Zeiher AM: Prognostic impact of coronary vasodilator dysfunction on adverse long-term outcome of coronary heart disease. *Circulation* 101:1899–1906, 2000
11. Steinberg HO, Chaker H, Leaming R, Johnson A, Brechtel G, Baron AD: Obesity/insulin resistance is associated with endothelial dysfunction: implications for the syndrome of insulin resistance. *J Clin Invest* 97:2601–2610, 1996
12. Kim JA, Montagnani M, Koh KK, Quon MJ: Reciprocal relationships between insulin resistance and endothelial dysfunction: molecular and pathophysiological mechanisms. *Circulation* 113:1888–1904, 2006
13. Zeng G, Nystrom FH, Ravichandran LV, Cong LN, Kirby M, Mostowski H, Quon MJ: Roles for insulin receptor, PI3-kinase, and Akt in insulin-signaling pathways related to production of nitric oxide in human vascular endothelial cells. *Circulation* 101:1539–1545, 2000
14. Vicent D, Ilany J, Kondo T, Naruse K, Fisher SJ, Kisanuki YY, Bursell S, Yanagisawa M, King GL, Kahn CR: The role of endothelial insulin signaling in the regulation of vascular tone and insulin resistance. *J Clin Invest* 111:1373–1380, 2003
15. Moller DE, Yokota A, White MF, Pazianos AG, Flier JS: A naturally occurring mutation of insulin receptor alanine 1134 impairs tyrosine kinase function and is associated with dominantly inherited insulin resistance. *J Biol Chem* 265:14979–14985, 1990
16. Moller DE, Benecke H, Flier JS: Biologic activities of naturally occurring human insulin receptor mutations: evidence that metabolic effects of insulin can be mediated by a kinase-deficient insulin receptor mutant. *J Biol Chem* 266:10995–11001, 1991
17. Chang PY, Goodyear LJ, Benecke H, Markuns JS, Moller DE: Impaired insulin signaling in skeletal muscles from transgenic mice expressing kinase-deficient insulin receptors. *J Biol Chem* 270:12593–12600, 1995
18. Cusi K, Maezono K, Osman A, Pendergrass M, Patti ME, Pratipanawatr T, DeFronzo RA, Kahn CR, Mandarino LJ: Insulin resistance differentially affects the PI 3-kinase- and MAP kinase-mediated signaling in human muscle. *J Clin Invest* 105:311–320, 2000
19. Alp NJ, Mussa S, Khoo J, Cai S, Guzik T, Jefferson A, Goh N, Rockett KA, Channon KM: Tetrahydrobiopterin-dependent preservation of nitric oxide-mediated endothelial function in diabetes by targeted transgenic GTP-cyclohydrolase I overexpression. *J Clin Invest* 112:725–735, 2003
20. Wheatcroft SB, Shah AM, Li JM, Duncan E, Noronha BT, Crossey PA, Kearney MT: Preserved glucoregulation but attenuation of the vascular actions of insulin in mice heterozygous for knockout of the insulin receptor. *Diabetes* 53:2645–2652, 2004
21. Wheatcroft SB, Kearney MT, Shah AM, Grieve DJ, Williams IL, Miell JP, Crossey PA: Vascular endothelial function and blood pressure homeostasis in mice overexpressing IGF binding protein-1. *Diabetes* 52:2075–2082, 2003
22. Li JM, Wheatcroft S, Fan LM, Kearney MT, Shah AM: Opposing roles of p47phox in basal versus angiotensin II-stimulated alterations in vascular O2- production, vascular tone, and mitogen-activated protein kinase activation. *Circulation* 109:1307–1313, 2004
23. Li JM, Mullen AM, Shah AM: Phenotypic properties and characteristics of superoxide production by mouse coronary microvascular endothelial cells. *J Mol Cell Cardiol* 33:1119–1131, 2001
24. Duncan ER, Walker SJ, Ezzat VA, Wheatcroft SB, Li JM, Shah AM, Kearney MT: Accelerated endothelial dysfunction in mild prediabetic insulin resistance: the early role of reactive oxygen species. *Am J Physiol Endocrinol Metab* 293:E1311–E1319, 2007
25. Noronha BT, Li JM, Wheatcroft SB, Shah AM, Kearney MT: Inducible nitric oxide synthase has divergent effects on vascular and metabolic function in obesity. *Diabetes* 54:1082–1089, 2005
26. Schlaeger TM, Bartunkova S, Lawitts JA, Teichmann G, Risau W, Deutsch U, Sato TN: Uniform vascular-endothelial-cell-specific gene expression in both embryonic and adult transgenic mice. *Proc Natl Acad Sci U S A* 94:3058–3063, 1997
27. Clerk LH, Vincent MA, Jahn LA, Liu Z, Lindner JR, Barrett EJ: Obesity blunts insulin-mediated microvascular recruitment in human forearm muscle. *Diabetes* 55:1436–1442, 2006
28. Laakso M, Edelman SV, Brechtel G, Baron AD: Impaired insulin-mediated skeletal muscle blood flow in patients with NIDDM. *Diabetes* 41:1076–1083, 1992
29. Laakso M, Edelman SV, Brechtel G, Baron AD: Decreased effect of insulin to stimulate skeletal muscle blood flow in obese man: a novel mechanism for insulin resistance. *J Clin Invest* 85:1844–1852, 1990
30. Goff DC Jr, Zaccaro DJ, Haffner SM, Saad MF: Insulin sensitivity and the risk of incident hypertension: insights from the Insulin Resistance Atherosclerosis Study. *Diabetes Care* 26:805–809, 2003
31. Fulton D, Gratton JP, McCabe TJ, Fontana J, Fujio Y, Walsh K, Franke TF, Papapetropoulos A, Sessa WC: Regulation of endothelium-derived nitric oxide production by the protein kinase Akt. *Nature* 399:597–601, 1999
32. Mugge A, Elwell JH, Peterson TE, Hofmeyer TG, Heistad DD, Harrison DG: Chronic treatment with polyethylene-glycolated superoxide dismutase partially restores endothelium-dependent vascular relaxations in cholesterol-fed rabbits. *Circ Res* 69:1293–1300, 1991
33. Guzik TJ, West NE, Black E, McDonald D, Ratnatunga C, Pillai R, Channon KM: Vascular superoxide production by NAD(P)H oxidase: association with endothelial dysfunction and clinical risk factors. *Circ Res* 86:E85–E90, 2000
34. Cai H, Harrison DG: Endothelial dysfunction in cardiovascular diseases: the role of oxidant stress. *Circ Res* 87:840–844, 2000
35. Li JM, Shah AM: Endothelial cell superoxide generation: regulation and relevance for cardiovascular pathophysiology. *Am J Physiol Regul Integr Comp Physiol* 287:R1014–R1030, 2004
36. Tornqvist HE, Gunsalus JR, Nemenoff RA, Frackelton AR, Pierce MW, Avruch J: Identification of the insulin receptor tyrosine residues undergoing insulin-stimulated phosphorylation in intact rat hepatoma cells. *J Biol Chem* 263:350–359, 1988
37. Montagnani M, Chen H, Barr VA, Quon MJ: Insulin-stimulated activation of eNOS is independent of Ca2+ but requires phosphorylation by Akt at Ser(1179). *J Biol Chem* 276:30392–30398, 2001
38. Howard G, O'Leary DH, Zaccaro D, Haffner S, Rewers M, Hamman R, Selby JV, Saad MF, Savage P, Bergman R, The Insulin Resistance Atherosclerosis Study (IRAS) Investigators: Insulin sensitivity and atherosclerosis. *Circulation* 93:1809–1817, 1996
39. Li H, Wallerath T, Forstermann U: Physiological mechanisms regulating the expression of endothelial-type NO synthase. *Nitric Oxide* 7:132–147, 2002
40. Venneri MA, De Palma M, Ponzoni M, Pucci F, Scielzo C, Zonari E, Mazzieri R, Doglioni C, Naldini L: Identification of proangiogenic TIE2-expressing monocytes (TEMs) in human peripheral blood and cancer. *Blood* 109:5276–5285, 2007
41. Cave AC, Brewer AC, Narayanapanicker A, Ray R, Grieve DJ, Walker S, Shah AM: NADPH oxidases in cardiovascular health and disease. *Antioxid Redox Signal* 8:691–728, 2006
42. Picchi A, Gao X, Belmadani S, Potter BJ, Focardi M, Chilian WM, Zhang C: Tumor necrosis factor-alpha induces endothelial dysfunction in the prediabetic metabolic syndrome. *Circ Res* 99:69–77, 2006
43. Delbosc S, Paizanis E, Magous R, Araiz C, Dimo T, Cristol JP, Cros G, Azay J: Involvement of oxidative stress and NADPH oxidase activation in the development of cardiovascular complications in a model of insulin resistance, the fructose-fed rat. *Atherosclerosis* 179:43–49, 2005
44. Guzik TJ, Mussa S, Gastaldi D, Sadowski J, Ratnatunga C, Pillai R, Channon KM: Mechanisms of increased vascular superoxide production in human diabetes mellitus: role of NAD(P)H oxidase and endothelial nitric oxide synthase. *Circulation* 105:1656–1662, 2002
45. Kim YK, Lee MS, Son SM, Kim IJ, Lee WS, Rhim BY, Hong KW, Kim CD: Vascular NADH oxidase is involved in impaired endothelium-dependent vasodilation in OLETF rats, a model of type 2 diabetes. *Diabetes* 51:522–527, 2002
46. Lambeth JD: NOX enzymes and the biology of reactive oxygen. *Nat Rev Immunol* 4:181–189, 2004
47. Furukawa S, Fujita T, Shimabukuro M, Iwaki M, Yamada Y, Nakajima Y, Nakayama O, Makishima M, Matsuda M, Shimomura I: Increased oxidative stress in obesity and its impact on metabolic syndrome. *J Clin Invest* 114:1752–1761, 2004
48. Roberts CK, Barnard RJ, Sindhu RK, Jurczak M, Ehdaie A, Vaziri ND: Oxidative stress and dysregulation of NAD(P)H oxidase and antioxidant enzymes in diet-induced metabolic syndrome. *Metabolism* 55:928–934, 2006
49. Mahadev K, Motoshima H, Wu X, Ruddy JM, Arnold RS, Cheng G, Lambeth JD, Goldstein BJ: The NAD(P)H oxidase homolog Nox4 modulates insulin-stimulated generation of H2O2 and plays an integral role in insulin signal transduction. *Mol Cell Biol* 24:1844–1854, 2004