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-Thr¹¹³₄) in its tyrosine kinase domain that disrupts insulin signaling. Humans with the Thr¹¹³₄ 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

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 Thr¹¹³₄, resulting in markedly impaired insulin signaling and severe insulin resistance in heterozygous human subjects (15). Thr¹¹³₄ receptors display normal ligand binding but are devoid of detectable insulin-stimulated tyrosine kinase activity and...
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 Thr1134 mutation was introduced into cDNA encoding the human insulin receptor by site-directed mutagenesis (15). The plasmid pHINS was a gift from Keith Channon (University of Oxford, Oxford, U.K.). After excision of the Lox2 sequence with Sh/Em and MfuI (19), the Thr1134 mutant human insulin receptor cDNA was cloned into pHINS (Fig. 1A). Plasmid fidelity and orientation were confirmed using standard DNA sequencing and PCR analysis.

The transgene Tie2-hIR was excised with Sall, 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 GCC AGC TTC CCC CAA CAC T; reverse, 5′-AGG CTG GCC 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 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 GCC AGC TTC CCC CAA CAC T; reverse, 5′-AGG CTG GCC 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, GGGAAGCTGTGAGTATTTGTAG; and reverse, CACGTTGACTGCTGAAGAGA; Nox2 forward, ACTCCCTTGTCGACATCGG; and reverse, GTCCTGCTGAACTTGCTTCG; Nox4 forward, TGAATACGTAAGACAGTTTCTTGAGAC; and reverse, GAACCCCTGACACCGAAAT; and β-actin forward, CAGTGAAGATATGACCCAGATCA; and reverse, TGGTACGACAGGACCTACAG) (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 fixed in paraformaldehyde (4% in PBS, 30 minutes). A human-specific anti-insulin receptor antibody (1 in 200, 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 (Ser1177) 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 (Ser1177) 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 immunomassay 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–response curves to acetylcholine (ACh; 1 nmol/l to 100 μmol/l), phenylepherine (1 nmol/l to 10 μmol/l), and sodium nitroprusside (SNP; 0.1 nmol/l to 1 μmol/l) were used to assess endothelial nitric oxide synthase (eNOS) activity. Vascular relaxation responses to aortic ring are reported as a percentage of the response to phenylepherine (maximal response = 100%). Relaxation responses to ACh (1 nmol/l to 100 μmol/l) were assessed 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.).

** 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) in 8- to 10-week-old male mice (20). Dihydroethidium-construction 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 responses to SNP were added after incubation with the SOD mimetic Mn(III)tetrakis(1-methyl-4-pyridyl) porphyrin pentachloride (MnTMPyP; 10 μmol/l; 30 min) (22).

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

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 (EC50) 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 Thr1134 insulin receptor. Endothelial cell–specific expression of mutant Thr1134 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. 1C). 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. 1D). 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 phenylephrine (Fig. 2A; ESMIRO, Emax 0.84 ± 0.06 g, EC50 227 ± 43 nmol/l; and wild-type, Emax 0.87 ± 0.05 g, EC50 242 ± 26 nmol/l).

Acute exposure to insulin blunts the vasoconstrictor response to phenylephrine through an endothelial- and NO-dependent mechanism (20). Consistent with this, exposure of wild-type aorta to insulin caused a significant blunting of maximal phenylephrine-induced constriction (compare Fig. 2A and B). However, this effect was lost in ESMIRO mice (Fig. 2B; phenylephrine 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 Ser1177, 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 Ser1177 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. 2C 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. 3A; Emax 68 ± 6 and 102 ± 6%, ESMIRO and wild type, respectively, P < 0.01; EC50 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. 3B; Emax 51 ± 12 and 79 ± 8%, ESMIRO and wild type, respectively, P < 0.05; EC50 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. 3C; Emax 109 ± 1 and 110 ± 2%, ESMIRO and wild type, respectively, NS; EC50 23 ± 6 and 17 ± 2 nmol/l, ESMIRO and wild type, respectively, NS). These data
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. 4A; Emax 83 ± 5 and 86 ± 7%, ESMIRO and wild type, respectively, NS). 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.

**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.
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 Ala1134 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 Thr1134 insulin receptors had blunted insulin-stimulated glucose uptake.
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 phenylephrine) (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 Ser1177 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 Ca2+ and binding of Ca2+/calmodulin to the enzyme. Recently, a Ca2+-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 Ser1177 (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 vasodilation 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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