The effect of endothelium specific insulin resistance on endothelial function *in vivo*

Edward Duncan¹, Paul Crosseý¹, Simon Walker¹, Narayana Anilkumar¹, Lucilla Poston³, Gillian Douglas³, Vivienne Ezzat¹, Stephen Wheatcroft¹,², Ajay M Shah¹, Mark Kearney¹,².

¹Cardiovascular Division, Department of Cardiology, ²Division of Cardiovascular and Diabetes Research, Multidisciplinary Cardiovascular Research Centre and ³Maternal & Fetal Research Unit, Division of Reproduction & Endocrinology, King’s College London, United Kingdom

², Current address: The Leeds Institute for Genetics Health & Therapeutics, Leeds, United Kingdom

⁴, Current address: School of Biomedical and Molecular Sciences, University of Surrey, Guildford, UK

Address for Correspondence:
Professor Mark Kearney, Division of Cardiovascular and Diabetes Research, Multidisciplinary Cardiovascular Research Centre, The LIGHT Laboratories, Clarendon Way, University of Leeds, LS2 9JT
Tel: 44-113-3437764; Fax: 44-113-2423811; email: m.t.kearney@leeds.ac.uk
or
Professor Ajay Shah, Department of Cardiology, King’s College London BHF Centre of Excellence, The James Black Centre, 125 Coldharbour Lane, London SE5 9NU, UK. Email: ajay.shah@kcl.ac.uk

ABSTRACT

**Objective:** Insulin resistance (IR) 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). IR is associated with endothelial dysfunction; however, the mechanistic relationship between these abnormalities and the role of impaired endothelial insulin signalling versus global IR remains unclear.

**Research design and Methods:** To examine the effects of IR 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 (Alanine-Threonine\(^{1134}\)) in its tyrosine kinase domain which disrupts insulin signalling. Humans with the Thr\(^{1134}\) mutation are IR. We performed metabolic and vascular characterisation 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 and calcium ionophore. Furthermore, the vascular action of insulin was lost in ESMIRO mice and insulin induced eNOS phosphorylation was blunted. Despite this phenotype, ESMIRO mice demonstrate similar levels of eNOS mRNA and protein expression to wild type.

Acetylcholine-induced relaxation was normalised by the superoxide dismutase mimetic, MnTMPyP. Endothelial cells of ESMIRO mice showed increased superoxide generation as well as increased mRNA expression of the NADPH oxidase isoforms Nox2 and Nox4.

**Conclusion:** Selective endothelial IR is sufficient to induce a reduction in NO bioavailability and endothelial dysfunction, secondary to increased generation of reactive oxygen species. This arises independent of a significant metabolic phenotype.
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, adipose tissue) is a central pathogenic feature of these disorders (2). Insulin resistance (IR) 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 anti-atherosclerotic signalling 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). IR is associated with endothelial dysfunction (11) and this may account at least in part for its ultimately deleterious consequences. Indeed, a number of studies have suggested a reciprocal relationship between insulin sensitivity and endothelial cell function (12).

The mechanistic relationship between IR 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 IR 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 IR at the level of the endothelium might be expected to be associated with reduced insulin-stimulated NO. Recently, the Kahn laboratory generated a mouse with endothelium-targeted deficiency of the insulin receptor in order to study the impact of IR specific to the endothelium on metabolic homeostasis. Endothelial cell function and NO bioavailability were not addressed in this study (14).

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\textsuperscript{1134}, resulting in markedly impaired insulin signaling and severe IR in homozygous human subjects (15). Thr\textsuperscript{1134} 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 IRS-1 and PI3-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 IR 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**

**Animals.** Endothelial specific transgene overexpression was achieved using the murine Tie2 promoter and intronic enhancer as previously described (19). A Thr\textsuperscript{1134} mutation was introduced into cDNA encoding the human insulin receptor by site-directed mutagenesis (15). The plasmid pHHNS was a kind gift from Keith Channon, University of Oxford. Following excision of the 

\textit{LacZ} sequence with SbfI and MluI (19), the Thr\textsuperscript{1134} mutant human
Endothelial specific insulin resistance

insulin receptor cDNA was cloned into pHHNS (see figure 1A). Plasmid fidelity and orientation were confirmed using standard DNA sequencing and PCR analysis.

The transgene Tie2-hIR was excised with SalI, purified and microinjected into fertilised eggs from superovulated C57BL/6 x CBA mice. Potential founders were screened by genotyping of genomic DNA from tail lysates using primers specific for the human insulin receptor (F: 5‘GGT GGC AGC TTT CCC CAA CAC T; R: 5’AGC CTT GGC TTC AGG CAT GGT C3’).

Animals were backcrossed 8 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 littersmates. Morphological assessment was performed in 8-10 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.

Semi-quantitative and real-time RT-PCR. Total RNA was extracted from a variety of tissues using an RNeasy Mini Kit, (Quiagen). 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 (F: 5’GGT GGC AGC TTT CCC CAA CAC T; R: 5’AGC CTT GGC TTC AGG CAT GGT C3’).

Real-time PCR analysis of eNOS, NOX2, NOX4 and β-actin expression was performed (eNOS F- GGGAAAGCTGCAGGTATTTGAT, R- CACTGTGATGGCTGAACGAAGA; Nox2 F- ACTCCTTGGGTGACTGCG, R- GTCCCCGGTCCAGTTGCTCTCG; Nox4 F- TGAACCTACGTGAAGATTTCTCTTGAAC, R-GACACCCTCGACCCAGGAAT; β-actin F-CGTGAAAAGATGACCCAGATCA, R- TGGTACGACCAGAGGCACAG) (20).

The standard curve method was used and results normalised to β-actin expression. Immunohistochemistry. Thoracic aorta was excised and mounted vertically in OCT embedding matrix (CellPath, Powys, UK) before freezing in cold isopentane. 8μm transverse sections were fixed in paraformaldehyde (4% in PBS, 30 min). A human-specific anti-insulin receptor antibody (E5844, Spring Bioscience, USA; 1 in 200, 30 min) was used, which does not crossreact with the murine insulin receptor. A Vector M.O.M immunodetection kit (Vector Laboratories, USA) reduced background staining of endogenous mouse immunoglobulins. Antibody staining was visualised using a secondary antibody conjugated to fluorophores (Alexa Fluor Dyes, Molecular Probes Inc, USA). Aortic sections were also probed with anti-von Willebrand factor antibody (Sigma-Aldrich, UK).

eNOS and phospho-eNOS (ser1177) protein expression. Fasted mice were anaesthetised. An injection of either human recombinant insulin (Actrapid, NovoNordisk Bagsvaerd, Denmark 5units) or saline vehicle (0.9% NaCl) was administered via the inferior vena cava. After 5 minutes, 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 βactin (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).

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 (20;21). Cumulative dose
response to phenylephrine (PE) (1nmol/L to 10μmol/L) were measured. Phenylephrine constriction responses were re-assessed after incubation with insulin (Actrapid, 100mU/ml, 2 hours) (20). Relaxation responses to acetylcholine (Ach: 1nmol/L to 10μmol/L), A23187 (1nmol/L to 1μmol/L) and sodium nitroprusside (SNP: 0.1nmol/L to 1μmol/L) were then measured. Ach relaxation experiments were repeated following incubation with the SOD mimetic Mn(III)tetrakis(1-methyl-4-pyridyl) porphyrin pentachloride (MnTMPyP, 10μmol/L, 30 minutes) (22).

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

Vascular superoxide production. Three different methods were used to detect superoxide.

Firstly, in-situ ROS generation by CMEC was assessed using DHE fluorescence (2μM, 5minutes) as previously reported (24;25). CMEC were grown to 70% confluence before exposure to DHE. Peak fluorescence intensity per cell was quantified microscopically using a computerised image analysis system (Improvision) from at least 30 cells per group. n=6 mice per CMEC isolation.

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

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

Statistics. Data expressed as means±SEM. In vascular studies, concentration-response relationships were compared using 2-way repeated measures ANOVA. EC50 and Emax were compared using a student t-test. 1-way ANOVA was used to analyse 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. Semi-quantitative 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 (Figure 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 (Figure 1D). Positive staining was found at the luminal endothelial surface of ESMIRO mouse aorta but not WT. The transgene protein co-localised with endothelial cells as labelled 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 behaviour were observed. Whole body weight, mean organ weights, and markers of adiposity were similar in ESMIRO and WT mice (data not shown).

Metabolic homeostasis. It has been suggested that endothelial cell IR might promote global metabolic IR secondary to decreased insulin-stimulated blood flow to insulin sensitive tissues (27-29). However, metabolic
characterisation of the ESMIRO mice did not support such a mechanism. ESMIRO and WT 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 towards 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 WT 0.72±0.06 ng/ml). HOMA-R were similar in ESMIRO and WT mice (2.1±0.2 and 2.4±0.6, Table 1). Fasting serum triglycerides (0.80±0.08 and 0.98±0.14 mmol/L) and free fatty acids (0.92±0.1 and 1.0±0.2 mmol/L) were similar in both groups.

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

Effect of endothelial specific mutant receptor expression on vasoconstrictor responses and the actions of insulin in conduit vessels. Isolated aortic rings of ESMIRO mice and WT mice had similar constrictor responses to KCl (40mmol/L; Emax ESMIRO 0.68±0.04g and WT 0.73±0.06g,) and phenylepherine (Figure 2A: ESMIRO Emax 0.84±0.06g, EC50 227±43nmol/L and WT Emax 0.87±0.05g, EC50 242±26nmol/L).

Acute exposure to insulin blunts the vasoconstrictor response to phenylepherine through an endothelial- and NO-dependent mechanism (20). Consistent with this, exposure of WT aorta to insulin caused a significant blunting of maximal phenylepherine-induced constriction (compare Figure 2A and B). However, this effect was lost in ESMIRO mice (Figure 2B: Emax Phenylepherine + insulin ESMIRO 0.83±0.07g and WT 0.59±0.06g; 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 WT mice exposed to insulin in-vivo. Basal levels of eNOS protein expression were similar in ESMIRO and WT mice (Figure 2 C&D). Following administration of insulin WT mice demonstrated a significant increase in the phospho-eNOS/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, p=NS). Therefore, overexpression of the mutant Thr1134 insulin receptor leads to impaired endothelial insulin signaling. A similar result was demonstrated in complimentary 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 to WT (Figure 3A: Emax ESMIRO 68±6%, WT 102±6%, p<0.01; EC50 ESMIRO 180±73nmol/L, WT 114±37nmol/L, p=NS). Similarly, vasodilator responses to the calcium ionophore A23187 were also blunted (Figure 3B: Emax ESMIRO 51±12%, WT 79±8%, p<0.05; EC50 ESMIRO 119±29nmol/L, WT 39±8nmol/L, p<0.01). However, responses to SNP were similar in ESMIRO and WT mice (Figure 3C: Emax ESMIRO 109±1%, WT 110±2%, p=NS; EC50 ESMIRO 23±6nmol/L, WT 17±2nmol/L, p=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.
Endothelial specific insulin resistance

following 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 acetylcholine-induced relaxation in aortae. MnTMPyP restored vasorelaxation responses to acetylcholine in ESMIRO aortae (Figure 4A: Emax ESMIRO 83±5, WT 86±7, p=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 to WT (Figure 4B, 12.6.0±0.5(x106) vs 8.1±0.4(x106) integrated light units, p<0.001). ESMIRO and WT superoxide production were almost completely abolished by the antioxidant tiron (p<0.001) or the flavoprotein inhibitor DPI (p<0.001). The nitric oxide synthase inhibitor L-NAME had no effect on NADPH-dependent superoxide production in either group.

**CMEC superoxide production.** DHE fluorescence of cultured CMEC confirmed that the ESMIRO has increased superoxide at the level of the endothelial cell compared to WT (2819±151 and 1439±129, p<0.001, n=6 mice per group, Figure 5A). This was confirmed by lucigenin-enhanced chemiluminescence performed on CMEC homogenates (Figure 5B). As in experiments in whole aorta, NADPH-dependent superoxide production in CMEC was inhibited by tiron and DPI but not by L-NAME.

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

**DISCUSSION**

The present study demonstrates a number of important findings adding to our understanding of the role of IR in endothelial cell homeostasis: 1) The induction of endothelial cell-specific IR 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 IR 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-specific IR 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\textsuperscript{1134} is replaced by Thr in the tyrosine kinase domain of the \(\beta\)-subunit have been found in humans (15). In an affected family, heterozygotes were severely insulin resistant. In \textit{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\textsuperscript{1134} insulin receptors had blunted insulin-stimulated glucose uptake (16). Moller \textit{et al} went on to produce tissue-specific transgenic mice overexpressing Thr\textsuperscript{1134} insulin receptors in skeletal muscle (17). These mice were a model of non-diabetic insulin resistance, had reduced responses to insulin during insulin tolerance testing and elevated serum glucose and insulin levels when fed \textit{ad libitum}. 

**Characterisation 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 2 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 to WT. Secondly, and consistent with this finding, insulin-stimulated phosphorylation of eNOS at Ser\textsuperscript{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 to wild-type littermates indicating that there was no decline in \textit{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 unfavourable 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.** IR 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 IR 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 endothelial nitric oxide synthase (eNOS) and its inactivation by ROS. Classical activation of eNOS (e.g. by acetylcholine) involves a rise in intracellular Ca\textsuperscript{2+} and binding of Ca\textsuperscript{2+}/calmodulin to the enzyme. Recently, a Ca\textsuperscript{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 phosphatidylinositol 3-kinase (PI3K) and protein kinase-B (PKB/Akt), which phosphorylates eNOS on Ser\textsuperscript{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, acetylcholine- 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 acetylcholine at a young age (20). However, as IRKO mice age, they develop blunting of acetylcholine-mediated aortic relaxation secondary to increased superoxide production (24). The present report indicates a similar mechanism of endothelial dysfunction and suggests 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-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’s laboratory and the VENIRKO mouse (14), 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, an SODmimetic 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, IR and obesity (42-45). Several isoforms of these enzymes are described (46), among which Nox2 and Nox4 have been implicated in increased oxidase activity in obesity (47;48) and in insulin signalling in non-endothelial tissues (49). An interesting finding in the current study was the increase in Nox2 and Nox4 mRNA levels in aortae and CMEC 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 signalling via the PI3kinase pathway and Nox4 expression (49). Indeed it has been hypothesised that Nox4 activity may maintain insulin sensitivity. Therefore, in our model...
Nox4 may be upregulated to compensate for the impaired endothelial insulin signalling. Future studies would also clarify if Nox2 and Nox4 are upregulated in aortic tissues other than the endothelium.

Finally, the ESMIRO demonstrates a reduced phospho-eNOS/eNOS ratio compared to WT 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 IR 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. While a euglycaemic insulin clamp may have revealed more subtle abnormalities in glucose homeostasis, our data and those from Kahn’s lab (14) do not support a non-redundant 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.

**ACKNOWLEDGEMENTS**

Drs Duncan and Ezzat were British Heart Foundation (BHF) Clinical PhD Fellows. Dr Crossey was supported by Diabetes UK. Dr Kearney was a BHF Intermediate Fellow. Professor Shah holds the BHF Chair of Cardiology at King’s College London.
**Table 1.** Metabolic characteristics and Systolic Blood Pressure of ESMIRO mice compared to their wild-type littermates (n=8). No significant differences were noted between the two groups.

<table>
<thead>
<tr>
<th></th>
<th>Wild Type</th>
<th>ESMIRO</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fasting blood sugar (mmol/L)</td>
<td>6.7±0.4</td>
<td>6.5±0.2</td>
<td>0.68</td>
</tr>
<tr>
<td>Post glucose blood sugar (mmol/L)</td>
<td>16.2±1.0</td>
<td>14.4±0.7</td>
<td>0.17</td>
</tr>
<tr>
<td>Random blood glucose (mmol/L)</td>
<td>11.1±1.1</td>
<td>13.3±1.1</td>
<td>0.19</td>
</tr>
<tr>
<td>Fasting insulin (ng/mL)</td>
<td>0.30±0.03</td>
<td>0.34±0.03</td>
<td>0.27</td>
</tr>
<tr>
<td>Post glucose insulin (ng/mL)</td>
<td>0.72±0.06</td>
<td>0.78±0.10</td>
<td>0.61</td>
</tr>
<tr>
<td>HOMA-R</td>
<td>2.4±0.6</td>
<td>2.1±0.2</td>
<td>0.63</td>
</tr>
<tr>
<td>Triglycerides (mmol/L) - fasted</td>
<td>0.98±0.14</td>
<td>0.8±0.08</td>
<td>0.28</td>
</tr>
<tr>
<td>Free Fatty Acids (mmol/L) - fasted</td>
<td>1.0±0.2</td>
<td>0.92±0.1</td>
<td>0.49</td>
</tr>
<tr>
<td>Systolic Blood Pressure</td>
<td>131±4</td>
<td>129±4</td>
<td>0.68</td>
</tr>
</tbody>
</table>
Figure 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 TG into genomic DNA in both lines of ESMIRO mice. (C) Transgene expression in different organs from ESMIRO mice. ESMIRO mice demonstrated increased TG expression in Lung (Lu) and Kidney (Ki) compared to 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 Wildtype mice (WT). Staining with an antibody specific for the human insulin receptor demonstrated transgene protein expression in ESMIRO but not WT endothelium (magnification x400). The transgene co-localised in the endothelium with Von Willebrand factor (data not shown).
Figure 2. The action of insulin on the aorta of ESMIRO and WT mice. (A) WT (black squares) and ESMIRO (open triangles) had similar aortic vasoconstrictor responses to phenylepherine. (B) Incubation with insulin (100mU/mL) for 2 hours significantly blunted this response in WT but had no effect in ESMIRO mice (n=8). (C) & (D) Immunoblot of eNOS and phospho-eNOS protein expression in WT and ESMIRO aortae (n=5). There was no difference in eNOS protein expression between the two groups (corrected for β-actin). However, WT phospho-eNOS expression was significantly greater than that of ESMIRO mice at baseline. Furthermore, a significant increment in eNOS phosphorylation at ser1177 was seen in WT mice following insulin injection. No such increment was seen in ESMIRO mice. (*p<0.05 WT vs ESMIRO; #p<0.05 WT vs WT+insulin).
Figure 3. Aortic relaxation responses. ESMIRO mice (open triangles) demonstrated significantly impaired relaxation responses to Acetylcholine (A) and Calcium ionophore A23187 (B) compared to WT littermates (closed squares). Relaxation responses to SNP were similar in ESMIRO and WT mice (C). n=8. *p<0.05
Figure 4. Assessment of aortic superoxide production in ESMIRO and WT mice. ESMIRO mice demonstrate impaired relaxation responses to acetylcholine at baseline. (see figure 3). Importantly, ESMIRO acetylcholine responses (open triangles) were normalized following incubation with the SOD mimetic, MnTMPyP (10 μmol/L) providing physiological evidence that increased superoxide production in ESMIRO aortae contributes to the impaired relaxation responses seen (A).

Lucigenin-enhanced chemiluminescence was performed on aortic homogenates (B). ESMIRO aortae demonstrated increased superoxide production compared to WT. 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. This data suggests a role for NADPH oxidase as the major source of the increased superoxide in the ESMIRO aorta. *p<0.001 with inhibitor compared to WT baseline #p<0.001 with inhibitor compared to ESMIRO baseline.
Figure 5. Assessment of CMEC superoxide production.
(A) In-situ ROS generation in CMEC was assessed through measurement of peak fluorescence of CMEC exposed to dihydroethidium (DHE, 2μM, 5minutes). CMEC from ESMIRO mice showed significantly greater fluorescence than WT, n=6 mice, *p<0.05.
(B) Lucigenin (5 μM)-enhanced chemiluminescence was repeated using CMEC homogenates. Similar to the results observed in whole aortae, this suggested increased NADPH-dependent superoxide production in ESMIRO CMEC at baseline. Superoxide levels were reduced by tiron and DPI, but not by L-NAME. *p<0.001 with inhibitor compared to WT baseline #p<0.001 with inhibitor compared to ESMIRO baseline.
Figure 6. Real time RT-PCR examination of Nox2 and Nox4 mRNA expression in CMEC and whole aortae (relative to β actin). ESMIRO mice demonstrate significantly increased Nox2 and Nox4 mRNA expression in CMEC (A)&(C). A similar pattern of expression is noted in aortae (B)&(D). CMEC n=6, aorta n=5. *p<0.05
REFERENCES


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


