Recent changes in human lifestyle have led to a global epidemic of type 2 diabetes mellitus. A type 2 diabetes mellitus is characterized by hyperinsulinemic insulin resistance, a situation whereby insulin is unable to activate its intricate intracellular signaling network in an appropriate temporospatial fashion. Insulin resistance results in a loss of protection of the human insulin receptor (hIRECO) using the Tie2 promoter–enhancer. Insulin-stimulated phosphorylation of protein kinase B was increased in hIRECO EC as was Nox2 NADPH oxidase generation of superoxide, whereas insulin-stimulated and shear stress–stimulated eNOS activations were blunted. Phosphorylation at the inhibitory residue Y657 of eNOS and expression of proline-rich tyrosine kinase 2 that phosphorylates this residue were significantly higher in hIRECO EC. Inhibition of proline-rich tyrosine kinase 2 improved insulin-induced and shear stress–induced eNOS activation in hIRECO EC.

Conclusions: Enhancing insulin sensitivity specifically in EC leads to a paradoxical decline in endothelial function, mediated by increased tyrosine phosphorylation of eNOS and excess Nox2-derived superoxide. Increased EC insulin sensitivity leads to a proatherosclerotic imbalance between NO and superoxide. Inhibition of proline-rich tyrosine kinase 2 restores insulin-induced and shear stress–induced NO production. This study demonstrates for the first time that increased endothelial insulin sensitivity leads to a proatherosclerotic imbalance between NO and superoxide. (Circ Res. 2017;120:784-798. DOI: 10.1161/CIRCRESAHA.116.309678.)

Key Words: diabetes mellitus ■ insulin ■ insulin resistance ■ reactive oxygen species ■ superoxide
Novelty and Significance

What Is Known?
- Insulin binds to its tyrosine kinase receptor to activate downstream signaling molecules including Akt/PKB leading to activation of endothelial NO synthase, which stimulates production of the antiatherosclerotic signaling radical NO.
- Endothelial insulin resistance, a well-established feature of type 2 diabetes mellitus, leads to a proatherosclerotic imbalance between NO and superoxide.
- Mice with endothelium-specific insulin resistance develop accelerated atherosclerosis.

What New Information Does This Article Contribute?
- Overexpression of insulin receptors in the endothelium increases insulin-mediated activation of Akt.
- Overexpression of insulin receptors in the endothelium leads to PYK2-mediated inhibition of endothelial NO synthase and increased generation of superoxide, derived from the Nox2 isoform of NADPH oxidase.
- Overexpression of insulin receptors in the endothelium accelerates atherogenesis in apolipoprotein E-deficient mice.

Nonstandard Abbreviations and Acronyms

<table>
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<tr>
<th>Abbreviation</th>
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<tr>
<td>Akt</td>
<td>protein kinase B</td>
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<td>EC</td>
<td>endothelial cells</td>
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<td>eNOS</td>
<td>endothelial nitric oxide synthase</td>
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<td>hiRECO</td>
<td>endothelial cell (EC)-specific overexpression of the human insulin receptor</td>
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<td>IR</td>
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<td>PE</td>
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<td>PEC</td>
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<td>PYK2</td>
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have confirmed the hypothesis that insulin resistance per se leads to a reduced level of NO, excessive generation of superoxide, and accelerated atherosclerosis.

Although we appreciate the effects of whole-body and cell-specific insulin resistance on the development of atherosclerosis, the local and systemic consequences of prolonged insulin signaling in the endothelium are poorly understood. Therefore, to improve our mechanistic understanding of the impact of selectively enhancing insulin action in the endothelium on the development of atherosclerosis, from its earliest biochemical manifestation of reduced NO availability to lipid deposition and structural changes in the arterial wall, we have generated a transgenic mouse model overexpressing the human insulin receptor (IR) specifically in the endothelium. For the first time, we demonstrate that EC overexpression of IR enhances insulin-stimulated activation of the kinase protein kinase B (Akt), which increases activity of the superoxide-generating enzyme Nox2 NADPH oxidase. Additionally, overexpression of IR also increases expression of proline-rich tyrosine kinase (PYK2), a negative regulator of endothelial NO synthase (eNOS). In the presence of enhanced insulin signaling, crosstalk between Nox2 and PYK2 increases their expression further, thus amplifying the deleterious effect of prolonged enhancement of EC insulin signaling on the critical balance between NO and superoxide levels. Taking these findings further, we crossed transgenic mice with endothelium-specific expression of the IR onto an atherosclerosis-prone apolipoprotein E-deficient background. Interestingly, these mice developed accelerated atherosclerosis. Our findings demonstrate that enhanced and prolonged insulin signaling specifically in the endothelium establishes a deleterious feed-forward circuit involving IR, Akt, Nox2, PYK2, and eNOS, leading to endothelial dysfunction and accelerated atherosclerosis.

Methods

Mice Overexpressing the Human IR Specific to the Endothelium

To examine the local and systemic consequences of prolonged enhancement of insulin sensitivity in the endothelium, we generated a transgenic mouse with endothelium-specific overexpression of the type A isoform of the human IR (Online Figure IA). Mice were bred onto a C57BL/6J background to at least 10 generations in a conventional animal facility with a 12-hour light/dark cycle. Male mice aged 3 to 4 months were used in all experiments, which were conducted in accordance with accepted ethical standards of humane animal care (UK Animals Scientific Procedure Act 1986) under United Kingdom Home Office Project License No 40/2988.

To overcome the limitations seen in standard transgensics, we used the hypoxanthine phosphoribosyl transferase (Hprt) targeting system that we have used previously applying Quick Knock-in (GenOway) technology to generate genetically modified embryonic stem cells. This approach uses homologous recombination to target a single copy of a transgene (in this case the human IR) driven by a promoter (in this case the Tie2 promoter) into the Hprt locus on the X chromosome.

As previously described, the model was developed with E15Tx2a (E14) cells derived from the strain 129P2/OlaHsd (12901a). Tissue-specific promoters including Tie2 were inserted into the Hprt locus to maintain their expression properties. For detailed methods, see Online Data Supplement.
Myeloid Cell Enumeration, Isolation, and Transcriptional Profiling
Peripheral blood mononuclear cells were isolated using density-gradient centrifugation (Histopaque 1083; Sigma) of heparinized venous blood collected during terminal anesthesia. 2×10⁶ peripheral blood mononuclear cells were used for labeling with an allophycocyanin-conjugated CD11b antibody (AbD Serotec) or allophycocyanin-conjugated isotype control, after blocking nonspecific antibody binding with Fc receptor block (BD Biosciences). Monocytes were enumerated using an LSR-Fortessa cytometer (BD Biosciences) based on either typical light scatter properties or separately based on CD11b expression, with normalization to total mononuclear cell count (Online Figure IA through IF). Remaining peripheral blood mononuclear cells were incubated with CD11b microbeads (Miltenyi Biotec) before undergoing 2 rounds of magnetic purification using MS columns (Miltenyi Biotec), achieving purity exceeding 90%. CD11b+ cells were immediately lysed using TRIzol reagent (Life Technologies), and then, total RNA isolated according the manufacturer’s protocol. RNA was then cleaned (RNeasy MiniElute; Qiagen), amplified, and reverse transcribed to cDNA (Quantitect; Qiagen), and then, total RNA isolated according the manufacturer’s protocol. RNA was then cleaned (RNeasy MiniElute; Qiagen), amplified, and reverse transcribed to cDNA (Quantitect; Qiagen), and then, total RNA isolated according the manufacturer’s protocol. RNA was then cleaned (RNeasy MiniElute; Qiagen), amplified, and reverse transcribed to cDNA (Quantitect; Qiagen), and then, total RNA isolated according the manufacturer’s protocol. RNA was then cleaned (RNeasy MiniElute; Qiagen), amplified, and reverse transcribed to cDNA (Quantitect; Qiagen), and then, total RNA isolated according the manufacturer’s protocol. RNA was then cleaned (RNeasy MiniElute; Qiagen), amplified, and reverse transcribed to cDNA (Quantitect; Qiagen), and then, total RNA isolated according the manufacturer’s protocol. RNA was then cleaned (RNeasy MiniElute; Qiagen), amplified, and reverse transcribed to cDNA (Quantitect; Qiagen), and then, total RNA isolated according the manufacturer’s protocol. RNA was then cleaned (RNeasy MiniElute; Qiagen), amplified, and reverse transcribed to cDNA (Quantitect; Qiagen), and then, total RNA isolated according the manufacturer’s protocol. RNA was then cleaned (RNeasy MiniElute; Qiagen), amplified, and reverse transcribed to cDNA (Quantitect; Qiagen), and then, total RNA isolated according the manufacturer’s protocol.

Metabolic Tests and Plasma Circulating Levels of Insulin, TNF-α, IL-6, and Nitrite
Blood was sampled from the lateral saphenous vein. Glucose and insulin tolerance tests were performed by blood sampling after an intraperitoneal injection of glucose (Sigma; 1 mg/g) or human recombinant insulin (0.75 U/kg; Actrapid; Novo Nordisk, Bagsvaerd, Denmark) as previously described. Glucose concentrations were determined in whole blood by a portable glucometer (Roche Diagnostics, Burgess Hill, UK). Plasma insulin concentrations were determined by enzyme-linked immunossay (Ultrasmouse sensitive mouse ELISA; CrystalChem, Downers Grove, IL). Free fatty acids and triglycerides were measured in fasting plasma using colorimetric assays (AbCam). Plasma levels of TNF-α, IL-6, and nitrite were measured using colorimetric assays (AbCam).

In Vivo Blood Pressure Measurement
Systolic blood pressure was measured using tail-cuff plethysmography in conscious mice, as previously described. Mice were prewarmed for 10 minutes in a thermostatically controlled restrainer (CODA2 System, XBP1000; Kent Scientific). Three training sessions were performed during the week before measurements were taken. The mean of at least 5 separate recordings on 3 occasions was taken to calculate mean systolic blood pressure.

Studies of Vasomotor Function in Aortic Rings
Vasomotor function was assessed ex vivo in aortic rings as previously described. Rings were mounted in an organ bath containing Krebs–Henseleit buffer (composition [in mmol/L]: NaCl 119, KCl 4.7, KH2PO4 1.18, NaHCO3 25, MgSO4 1.19, CaCl2 2.5, and glucose 11.0) gassed with 95% O2/5% CO2. Rings were equilibrated at a resting tension of 3 g for 45 minutes before the experiments. A cumulative dose response to the constrictor phenylephrine (1 mM/L to 10 μM/L) was first performed. Relaxation responses to cumulative addition of acetylcholine (1 mM/L to 10 μM/L) and sodium nitroprusside (0.1 mM/L to 1 μM/L) were performed. Insulin-mediated vasorelaxation was assessed in 2 different ways: (1) direct vasorelaxation responses in response to incremental doses of Actrapid insulin (0.1–4 μM/L) in aortic segments preconstricted maximally with phenylephrine and (2) examining the effect of insulin to reduce phenylephrine-mediated vasoconstriction as we previously reported. Relaxation responses are expressed as percentage decrement in preconstricted tension. Bioavailable NO in aortic segments subjected to isometric tension was measured by recording the change in tension elicited by the nonselective eNOS inhibitor, L-NMMA (N^ω-monomethyl-L-arginine, monooctate; 0.1 mM/L), in aortic segments preconstricted maximally with phenylephrine. (Percentage change calculated as percentage change from maximal constriction to phenylephrine after L-NMMA). The effects of gp91ds-tat (50 μM/L for 30 minutes; GenScript) and Mn(III) tetrakis (1-methyl-4-pyrydil) porphyrin pentachloride (MnTmPyP; 10 μM/L for 30 minutes; Calbiochem) on aortic relaxation were examined, as previously reported.

NO Synthase Activity in ECs
On activation, eNOS produces NO and l-citrulline in a stoichiometric reaction. The effect of insulin on eNOS activity in ECs was determined by conversion of [14C]-l-arginine to [14C]-l-citrulline as we previously described. ECs (1×10⁶ cells) were incubated at 37°C for 20 minutes in HEPES buffer pH 7.4 (in mM/L): 10 HEPES, 145 NaCl, 5 KCl, 1 MgSO4, 10 glucose, and 1.5 CaCl2 containing 0.25% BSA. 0.5 μCi/mL l-[14C]-arginine (Amersham) was then added for 5 minutes, and tissues stimulated with insulin (150 μM/L) for 45 minutes after the reaction was stopped with cold PBS containing 5 mM/L-l-arginine (Sigma Aldrich) and 4 mM/L EDTA after which tissue was denatured in 95% ethanol. After evaporation, the soluble cellular components were dissolved in 20 mM/L HEPES-Na+ (pH 5.5) and applied to a well-equilibrated DOWEX (Na+-form) column (Sigma Aldrich). The l-[14C] citrulline content of the eluate was quantified by liquid scintillation counting and normalized against the total cellular protein used.

Immunoprecipitation of Phosphorylated IR
Tyrosine phosphorylation of IR was quantified using Western blotting. Immunoprecipitation of protein for the quantification of receptors was achieved by incubating equal amounts of protein lysates (50 μg) and 50 μL of protein A-DynaBeads (Invitrogen) precoated with indicated rabbit anti-IR antibody for 20 minutes. After 3 rounds of washing with PBS–0.01% Tween-20, the beads were resuspended in 2× Laemmli loading buffer supplied with the NOVEX Gel Electrophoresis system. Cellular protein was separated by SDS-PAGE and blots were probed with a mouse Phosphotyrosine 4G10 (Millipore) and IR-β (C-19) antibodies for receptor expression (Santa Cruz Biotechnologies). This experiment was performed after overnight serum deprivation. Because basal levels would be decreased during this period, insulin-induced tyrosine phosphorylation of IR has been quantified and compared with vehicle control.

Cell Lysis, Immunoblotting
Primary ECs were cultured in 20% FCS-containing media for analyzing basal protein levels. Cells were lysed in extraction buffer containing 50 mM/L HEPES, 120 mM/L NaCl, 1 mM/L MgCl2, 1 mM/L CaCl2, 10 mM/L Na2PO4, 20 mM/L NaF, 1 mM/L EDTA, 10% glycerol, 1% NP40, 2 mM/L sodium orthovanadate, 0.5 μg/mL leupeptin, 0.2 mM/L PMSF (phenylmethylsulfonyl fluoride), and 0.5 μg/mL aprotinin. Insulin-stimulated ECs were serum deprived overnight with 1% FCS-containing EC media before stimulation and harvesting in lysis buffer for Western blot analysis. Cell extracts were sonicated in an ice bath and centrifuged for 15 minutes at 13000 rpm, before protein measurements were performed by BCA assay (Pierce Protein Quantification Kit) using the supernatant. Equal amounts of cellular protein were resolved on SDS polyacrylamide gels (Invitrogen) and transferred to polyvinylidene difluoride membranes and immunoblotted with appropriate antibodies (for details, see Online Data Supplement). The assays are run on the same blot for both wild-type (WT) and transgenic mice, whenever possible to fit all experimental conditions onto one gel, thereby negating issues with exposure time. Our approach using the Syngene densitometry (Syngene Gel Documentation) system also avoids issues with exposure time and blotting efficiency. Whenever stimulation has been performed after overnight serum deprivation, the result has been calculated as percentage of vehicle control.
Assessment of Superoxide Generation

Lucigenin-Enhanced Chemiluminescence
We used lucigenin (5 μmol/L)-enhanced chemiluminescence to measure NAD(P)H-dependent superoxide production in pulmonary ECs, as previously described. All experiments were performed in triplicate. Pulmonary EC monolayer was trypsinized and resuspended in PBS containing 5% FCS, 0.5% BSA, and 50 μmol/L gp91ds-tat (gptat; GenScript) or scrambled peptide (Scr; GenScript) and incubated at 37°C for 30 minutes. Luminescence was then measured on addition of a nonredox cycling concentration of lucigenin (5 μmol/L) and NADPH (100 μmol/L), using an autotodispenser (VarioSkas 96-well microplate luminometer; Thermo Scientific). Wortmannin (50 nmmol/L; Calbiochem) and LY294002 (25 μmol/L; Calbiochem) were used to inhibit phosphatidylinositol 3-kinase (PI3-K) and MK-2206 (10 μmol/L; Calbiochem) to inhibit Akt in pulmonary ECs to study the role of PI3-K and Akt in NADPH oxidase–induced superoxide production. Where wortmannin, LY294002, or MK-2206 were used for the measurement of reactive oxygen species production, EC suspension was prepared from both WT and hIRECO mice in equal cell concentration. The inhibitors were incubated for 30 minutes before measuring free noninhibited cells.

High-Performance Liquid Chromatographic Measurement of Conversion of Dihydroethidium to Oxyethylidium
To measure Nox2-dependent aortic superoxide production, we used high-performance liquid chromatography–based detection of the oxidation products of dihydroethidium (Oxyethylidin), that is, 2-hydroxyethylidine (EOH) and ethidium (E), as previously described25 in aortic rings from hIRECO and WT mice exposed to the gp91ds-tat inhibitor gp91ds-tat (for detailed methods, see Online Data Supplement).

Amplex Red Assay for Hydrogen Peroxide in Aorta
H2O2, the downstream dismutation product of superoxide, was measured using an Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Molecular Probes, Life Technologies) according to the manufacturer’s protocol (for detailed methods, see Online Data Supplement).

Gene Expression
mRNA was isolated using a commercial kit (Sigma Aldrich), and the levels of eNOS, Nox2, Nox4, SOD, catalase, VE-cadherin, IL-6, TNF-α, and IR mRNA quantified using real-time quantitative polymerase chain reaction (SYBR Green-based or Taqman Probe assay in AB Systems 7900HT machine (Online Table 1)). A range of housekeeping genes were screened for variability and stability. Nonspecific primers and those specific for human and mouse IR were designed and validated in human umbilical vein ECs and mouse pulmonary ECs (Online Table 1).

EC Isolation and Culture
Primary endothelial cells were isolated from lungs by immunoselection (Online Figure IIIB) with CD146-antibody–coated magnetic beads (Miltenyi Biotechnology) as previously reported. (for detailed methods, see Online Data Supplement).

Exposure of ECs to Flow-Mediated Shear Stress
Pulmonary ECs were seeded onto fibronectin-coated 6-well plates. Once confluent, monolayers were placed onto an orbital rotating platform (Grant Instruments) housed inside the incubator. The radius of the orbital shaker was 10 mm, and the rotation rate was set to 210 rpm for 10 minutes, which caused swirling of the culture medium over the cell surface volume. The movement of fluid because of orbital motion represents a free surface flow at the liquid–air interface at 12 dyne/cm².

Chronic Pharmacological Inhibition of NADPH Oxidase
To examine the effect of inhibition of Nox2 NADPH oxidase on EC function in hIRECO mice, we performed chronic treatment studies using the peptidic Nox2 inhibitor, gp91ds-tat. hIRECO mice were anesthetized, and osmotic mini-pumps (Alzet 2004) containing gp91ds-tat or control scrambled peptide implanted, as previously reported. The pump delivered 0.25 μL of drug per hour for 28 days. The concentration of the scrambled and gp91ds-tat peptide was adjusted (50 mg/mL) to deliver 10 mg/kg/d. Animals were euthanized after 28 days, and organ bath experiments were performed to assess vasomotor function.

siRNA-Mediated Knockdown of Nox2 NADPH Oxidase and Proline-Rich Tyrosine Kinase
Silencing of PYK2 and Nox2 NADPH oxidase gene expression in pulmonary ECs was performed by transfection of mouse siRNA duplexes (Stealth RNAi; Invitrogen) with Lipofectamine RNAiMax Transfection Reagent (Invitrogen) exactly according to manufacturer’s established protocol and as we previously reported. Scrambled siRNA was used as negative control (Invitrogen) for transfection. Briefly, isolated pulmonary ECs from lung tissues were rendered 70% confluent and transfected with 25 pmol siRNA for 72 hours before cell lysates were prepared for real-time quantitative polymerase chain reaction and western blotting analysis.

Quantification and Characterization of Atherosclerosis
To study the effects of endothelial IR overexpression on the development of atherosclerosis, we crossbred hIRECO mice with mice holoinsufficient for ApoE (ApoE/) Mice (ApoE/and ApoE/) hIRECO) were fed a Western diet (21% fat from lard supplemented with 0.15% wt/wt cholesterol, #829100; SDS, Witham, Essex, UK) for 12 weeks from 4 weeks of age to induce atherosclerosis. Mice were surgically anesthetized by intraperitoneal injection of sodium pentobarbitone before thorough terminal exsanguination by arterial perfusion via the abdominal aorta with PBS at a constant pressure of 100 mm Hg with outflow through the severed jugular veins. The heart was removed to study the aortic sinus. In other animals, the thoracic and abdominal aorta was dissected to allow en face quantification of plaque development.

Plaque Quantification in En Face Sections of Aorta
For en face analysis, aortas were analyzed, as previously described. Briefly, aortas were removed, fixed in PBS-buffered 4% formalin, cut longitudinally, stained with Oil Red O, and photographed (Olympus digital camera QICAM) under dissection microscope (Olympus SZ61). Percentage coverage of plaque area was measured using Image-Pro Plus 6.0 (Media Cybernetic, Bethesda, MD) software.

Statistics
Results are expressed as mean±SEM. Comparisons within groups were made using paired Student t tests and between groups using unpaired Student t tests, as appropriate; where repeated t tests were performed, a Bonferroni correction was applied. P<0.05 was considered statistically significant.

Results
Generation and Characterization of Transgenic Mice Overexpressing the Human IR in the Endothelium
To examine the effect of enhancing insulin sensitivity in the endothelium, we generated a novel transgenic mouse overexpressing the human type A IR directed to the endothelium
under control of the Tie2 promoter–enhancer (henceforth, described as human IR EC overexpressing mice, hIRECO; Online Figure IA through ID). hIRECO mice were born with the same frequency as wild-type (WT, n=12) littermates. B, mRNA expression of human insulin receptor in heart, lungs, and spleen from hIRECO and WT littermate controls (n=7–8 per group). C, mRNA expression of endogenous insulin receptor in organs from hIRECO and WT littermates (n=4). D, mRNA expression of human insulin receptor (IR) in pulmonary endothelial cells (PEC) and nonendothelial cells from hIRECO compared with WT littermates (n=4). E, mRNA VE-cadherin expression in endothelial cells from hIRECO with no expression in nonendothelial cells (n=4). F, No change in mRNA expression of native insulin receptor expression in endothelial cells from hIRECO (n=4). G, Western blot (left) showing greater insulin receptor protein expression (right) in endothelial cells from hIRECO (n=6) compared with WT littermates (n=4). Data normalized to WT animals experienced in pairs. (Data presented as mean±SEM. *P<0.05; WT vs hIRECO or hIRECO PEC.)

To examine this possibility, we isolated circulating CD11b+ myeloid cells from hIRECO and WT littermates, as previously described.22 There was no difference in total circulating CD11b+ or monocyte count (Online Figure IIA through IIC), gene expression of TNF-α, IL-1β, and IL-6 in hIRECO CD11b+ cells (Online Figure IID through IIF) or concentrations of the circulating inflammatory cytokines TNF-α and IL-6 in hIRECO (Online Figure IIIC and IIID) compared with WT littermates. We could not detect human IR mRNA in monocytes from hIRECO.

Enhanced EC Insulin Sensitivity in hIRECO Mice
Consistent with enhanced insulin action at the IR signaling node, basal and insulin-stimulated levels of tyrosine-phosphorylated IR were substantially higher in ECs from hIRECO compared with their WT littermates (Figure 2A). Basal serine-473 phosphorylation of Akt in ECs from hIRECO was markedly increased (Figure 2B, right) as was basal eNOS serine-1777 (Figure 2B, left). In dose–response experiments, Akt phosphorylation in response to insulin was also significantly enhanced in hIRECO ECs (Figure 2C).
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hIRECO Mice Have Normal Glucose Tolerance and Blood Pressure and Elevated Plasma Nitrite

Overexpression of the IR in the endothelium had no effect on fasting glucose, (Figure 3A) fasting insulin levels (Figure 3B), or blood pressure levels at the age of 3 months (Figure 3C). hIRECO had similar responses in glucose tolerance tests (Figure 3D) and insulin tolerance tests (Figure 3E) to WT littermates. Plasma triglycerides (Figure 3F) and free fatty acid levels (Figure 3G) were not different between hIRECO and WT littermates. Consistent with increased basal eNOS serine phosphorylation, plasma nitrite was also increased (Online Figure IIIE).

hIRECO Mice Have Reduced NO Availability

We examined the effect of excessive insulin signaling on aortic vasomotor responses in an organ bath. Aortic rings from hIRECO mice had blunted responses to the endothelium- and NO-dependent vasodilator, acetylcholine (Figure 4A). Vasorelaxation to phenylephrine remained unchanged in hIRECO compared with WT littermates (Figure 4B), as did vasorelaxation in response to the endothelium-independent vasodilator sodium nitroprusside (Figure 4C). Basal NO production in response to isometric tension assessed by measuring the constrictor response to the NO synthase inhibitor L-NMMA was reduced in hIRECO mice (Figure 4D; Online Figure IVB). In aortic rings from hIRECO animals, we assessed insulin-mediated NO-dependent vasorelaxation by 2 methods. Insulin pretreatment blunts phenylephrine-induced vasoconstriction in an NO- and endothelium-dependent fashion. We demonstrated that this vasodilatory response to insulin was blunted in hIRECO mice aortae (Figure 4E), as was acute vasorelaxation of phenylephrine preconstricted aortic rings in response to varying doses of insulin (Figure 4F, left). Moreover, L-NMMA significantly blunted insulin-mediated vasodilatation (Figure 4F, right), demonstrating that insulin-mediated vasodilatation is NO dependent. There was also a significant reduction in eNOS activity in response to insulin in hIRECO ECs compared with ECs from WT littermates (Figure 4G). Therefore, increased expression of the IR in the endothelium leads to resistance to insulin downstream of Akt. In response to chemical cues (eg, insulin) and mechanical stimuli (eg, shear stress), Akt is activated leading to serine-1177 phosphorylation of eNOS to stimulate generation of NO. Therefore, we assessed whether phosphorylation of Akt and eNOS were impaired in the hIRECO ECs, leading to decreased NO and vasodilation observed in these hIRECO

Figure 2. Enhanced endothelial cell insulin sensitivity in mice with endothelium-specific overexpression of the human insulin receptor (hIRECO). Representative Western blots show basal levels of tyrosine-phosphorylated insulin receptor (IR-Y) with equal loading (60 µg), basal eNOS, and protein kinase B (Akt) phosphorylation and dose response to varying doses of insulin (50–150 nmol/L; 15 min). A, Basal (left) and insulin-stimulated (right) tyrosine phosphorylation of insulin receptor was substantially higher in endothelial cells from hIRECO compared with wild-type (WT) littermates (n=4 in each group). B, Basal serine phosphorylation of eNOS (left) and the downstream insulin target, protein kinase B/Akt (Akt; right) in endothelial cells from hIRECO (n=7 in both groups) was enhanced in endothelial cells from hIRECO compared with WT littermates. Both groups of mice were euthanized, and lungs were isolated at the same time, and, therefore, WT was set as 100% as comparison to hIRECO. C, Dose response to insulin in endothelial cells demonstrating increased sensitivity to insulin-mediated serine phosphorylation of Akt in endothelial cells from hIRECO (n=10) compared with WT littermates (n=7. Data presented as mean±SEM. *P<0.05 vs WT control, †P<0.05 vs hIRECO control.)

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mice. We demonstrated that shear stress–mediated serine phosphorylation of Akt was similar between both groups of mice (Figure 4H). Interestingly, shear stress–mediated serine phosphorylation of eNOS (Figure 4I) was blunted in hIRECO when compared with WT littermates.

**Nox2 NADPH Oxidase and Excess Superoxide in hIRECO Mice**

Decreased endothelium-dependent vasorelaxation and reduced NO bioavailability could be mediated by increased superoxide generation. To examine the possibility that the underlying mechanism of the blunted acetylcholine responses in hIRECO mice is at least, in part because of excess superoxide, we exposed aortic rings from hIRECO to the superoxide dismutase mimetic, MnTmPyP, before performing dose-response studies to acetylcholine (Figure 5A). Consistent with excessive generation of superoxide, the maximal relaxation of rings from hIRECO in response to acetylcholine in the presence of MnTmPyP was significantly restored to normal WT level. We have previously demonstrated that mice with whole-body and endothelium-specific insulin resistance have excess generation of superoxide as a result of increased Nox2 NADPH oxidase activity. To examine the possibility of this underlying mechanism in mice with enhanced endothelial insulin signaling, we treated aortic rings from hIRECO mice with gp91ds-tat, a Nox2-specific NADPH oxidase inhibitor, and assessed the response to acetylcholine. Consistent with this hypothesis, gp91ds-tat significantly increased the maximal response to acetylcholine of aortae from hIRECO mice (Figure 5B). We investigated the role of superoxide in endothelial function by direct measurement of superoxide release in isolated ECs, using NADPH-dependent lucigenin-enhanced chemiluminescence assay, confirming the effects seen in the organ bath studies. hIRECO ECs demonstrated a 1.5-fold increase in superoxide release (Figure 5C). This finding was associated with a significant increase in Nox2 protein expression (Figure 5D) in hIRECO ECs. The basal increase in superoxide production in hIRECO ECs was reduced to WT levels, in the presence of gp91ds-tat (Figure 5E, left), and in hIRECO aorta measured using high-performance liquid chromatography (Figure 5E, center), indicating that Nox2 NADPH oxidase was the principal source of the excess
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Amplex Red quantification of hydrogen peroxide demonstrated marked increase in hydrogen peroxide release in hIRECO ECs (Figure 5E, right). To further examine the effect of chronic pharmacological inhibition of Nox2 NADPH oxidase, we administered gp91ds-tat to hIRECO mice via osmotic mini-pump for 28 days, as we recently described. Chronic treatment with gp91ds-tat had no effect on body mass (Figure 5F), organ weight (Online Figure IVA), glucose (Figure 5G, left) and insulin tolerance tests in hIRECO mice (Figure 5G, center). To examine the effect of chronic in vivo pharmacological inhibition of NADPH oxidase on endothelium-dependent vasomotor responses, we studied aortic rings from hIRECO mice after treatment with gp91ds-tat or scrambled peptide to examine acetylcholine-induced vasorelaxation ex vivo. Gp91ds-tat significantly enhanced (albeit to a small extent) maximal acetylcholine-induced aortic relaxation compared with scrambled peptide (Figure 5H). Chronic treatment with gp91ds-tat had no effect on sodium nitroprusside–mediated vasorelaxation in hIRECO (data not shown), indicative of preservation of responses in vascular smooth muscle.

To determine whether the negative effects of excessive endothelial insulin signaling is a consequence of alteration of expression of various protective or compensatory antioxidant enzymes, quantitative real-time polymerase chain reactions were performed in ECs. hIRECO mice had no difference in expression of superoxide dismutase-1, superoxide dismutase-2, superoxide dismutase-3, or catalase (Online Figure IIIA).
Insulin-Stimulated Superoxide Generation in hIRECO Mice

We next examined the involvement of the critical signaling node PI3-K in generation of the excessive superoxide release seen in hIRECO mice using the PI3-K inhibitors, Wortmannin and LY294002. Wortmannin inhibited NADPH-dependent superoxide production assessed using lucigenin-enhanced chemiluminescence (Figure 6A), as did an alternative PI3-K inhibitor LY294002 (Figure 6B), demonstrating that the PI3-K signaling node is potentially important for the increased endothelial superoxide production in hIRECO mice. Previous studies have suggested that downstream of PI3-K increased Akt activation may lead to increased oxidative stress. Because we found that hIRECO had increased Akt activation, we treated hIRECO ECs with the allosteric Akt inhibitor, MK-2206. MK-2206 reduced superoxide generation from hIRECO ECs (Figure 6C). We further examined whether insulin-stimulated superoxide generation in ECs from hIRECO is mediated via AKT and Nox2 activation. Therefore, we assessed the effects of Akt and Nox2 blockers, MK-2206 or gp91ds-tat, respectively. Insulin did not stimulate a detectable increase in superoxide in WT ECs. Insulin, however, did stimulate an increase in superoxide production in hIRECO ECs (Figure 6D). This excess generation of superoxide in response to insulin was blunted by the Akt inhibitor MK-2206 (Figure 6D) and the Nox2 inhibitor gp91ds-tat (Figure 6D), demonstrating that the
insulin-stimulated increment in superoxide was Akt and Nox2 dependent.

**Role of eNOS Tyrosine Phosphorylation in Endothelial Dysfunction in hIRECO Mice**

Previous studies have demonstrated that insulin, by increasing the expression of PYK2 in hIRECO mice, may lead to phosphorylation of eNOS on tyrosine residues that inhibit eNOS activity. To examine this possibility, we measured levels of tyrosine-657 phosphorylated eNOS in ECs from hIRECO. We found that phosphorylation of the inhibitory residue Tyr657 was significantly increased in hIRECO compared with WT littermates (Figure 6E, left), and inhibition of Nox2 by gp91ds-tat significantly reversed this effect (Figure 6E, right). Moreover, expression of PYK2 was also increased (Figure 6F) and the expression of phosphorylated PYK2 was similar when comparing hIRECO to WT littermates (data not shown).

To further examine the role of PYK2 in endothelial dysfunction in hIRECO using siRNA in isolated ECs, we reduced PYK2 expression by ≈50% (Figure 6G). In siRNA-treated cells, we assessed eNOS activity and performed the l-arginine to l-citrulline conversion assay in the presence of insulin and eNOS phosphorylation in response to shear stress. Both insulin-stimulated eNOS activity
Examining Crosstalk Between Nox2 and PYK2

To examine the possibility of crosstalk between Nox2 and PYK2, we performed siRNA experiments knocking down either Nox2 or PYK2. These experiments revealed an intriguing, yet previously unrecognized feed-forward loop. Chronic reduction of Nox2 activity with gp91 ds-tat and inhibiting Akt with MK-2206 reduced PYK2 expression in the hIRECO ECs (Figure 7A). MK-2206 and gp91ds-tat reduced PYK2 expression revealing a new signaling circuit linked to enhanced insulin-stimulated NO bioactivity. To further examine this interaction in hIRECO mice, we used siRNA to knockdown Nox2; this led to reduced PYK2 expression (Figure 7B), indicative of superoxide-mediated regulation of PYK2 expression in hIRECO mice. Knockdown of PYK2 in hIRECO ECs with siRNA significantly reduced Nox2 expression (Figure 7C) and also improved eNOS phosphorylation in the presence of insulin (Figure 7D). However, knockdown of Nox2 failed to reverse the decrease in insulin-stimulated eNOS phosphorylation (Figure 7D), suggesting that the reduction of insulin-stimulated eNOS phosphorylation is mediated via increases in PYK2 activity and not directly through Nox2 activation.

Overexpression of IR in the Endothelium Leads to Accelerated Atherosclerosis

To examine the effect of overexpression of IR in the endothelium on the development of advanced atherosclerosis, we crossed proatherogenic ApoE−/− mice with hIRECO to generate ApoE−/−/hIRECO. ApoE−/−/hIRECO and ApoE−/− littermates were fed a high cholesterol Western diet for 12 weeks from 8 weeks of age. After 12-week feeding, body weights were similar in ApoE−/−/hIRECO and ApoE−/− littermates.
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(Figure 8A). Systolic blood pressure (Figure 8B), plasma cholesterol (Figure 8C), and triglyceride levels (Figure 8D) were not different in ApoE−/−/hIRECO compared with ApoE−/− mice. Fasting glucose (Figure 8E) was similar between ApoE−/−/hIRECO and ApoE−/− mice. Glucose homeostasis assessed in glucose tolerance (Figure 8F) and insulin tolerance tests (Figure 8G) were comparable between ApoE−/−/hIRECO and ApoE−/− mice. H, Increased plaque development in the thoracic aorta in ApoE−/−/hIRECO mice compared with ApoE−/− littermates. I, Increased plaque development at the level of the aortic sinus in ApoE−/−/hIRECO mice compared with ApoE−/− littermates. J, Proposed mechanism of downstream effects of increased insulin sensitivity in the endothelium. (Data presented as mean±SEM. n=15 mice per group, *P<0.05, ApoE−/− vs ApoE−/−/hIRECO; +stimulatory effect −inhibitory effect).

Discussion

Despite intensive efforts to prevent the adverse effects of type 2 diabetes mellitus on cardiovascular morbidity and mortality, the presence of insulin-resistant type 2 diabetes mellitus substantially increases the risk of developing atherosclerosis-related vascular disease. The presence of type 2 diabetes mellitus also significantly worsens the prognosis of most of the adverse sequelae of atherosclerotic arterial disease, including myocardial infarction, left ventricular dysfunction, and peripheral vascular disease. Improving our understanding of mechanisms linking accelerated atherosclerosis and insulin signaling is, thus, of critical importance. Here, we describe the phenotype of a novel transgenic mouse with endothelium-targeted overexpression of the human IR, generated to investigate the local and systemic consequences of prolonged enhancement of insulin sensitivity in the endothelium. This is the first description of a manipulation of the insulin-signaling pathway leading to an increase in atherosclerosis.
pathway leading to specific enhancement of insulin action in the endothelium; a model of particular relevance as insulin sensitization is a strategy actively pursued to treat patients experiencing the cardiovascular complications of type 2 diabetes mellitus. Here, we show that increased insulin sensitivity specifically in the endothelium (a model of excess insulin action independent of systemic influences) at the most proximal node in its signaling pathway has relevant pathophysiological effects on its downstream targets.

The major finding of our study is that specific and prolonged enhancement of insulin signaling in the endothelium in vivo establishes a positive proatherosclerotic signaling loop around the Akt signaling node, involving the protein kinase Akt, the superoxide-generating Nox2 NADPH oxidase, the proline-rich nonreceptor tyrosine kinase, PYK2, and the eNOS isoform. This circuit impacts negatively on NO actions in 2 ways: (1) by increasing Nox2 NADPH oxidase-derived superoxide that reacts with NO to reduce its availability and (2) by increasing expression of PYK2 that reduces NO production by tyrosine phosphorylation of eNOS, a modification shown to attenuate the activity of the enzyme. The convergence of these adverse pathways leads to a significant reduction in a range of downstream actions, fundamental to the homeostatic function of NO that allied to excess concentrations of EC-derived superoxide leads to a proatherosclerotic vascular environment. Indeed, consistent with this, hIRECO mice crossed onto an atherosclerosis-prone apolipoprotein E–deficient background developed significantly more atherosclerosis at the level of the aortic sinus and whole aorta compared with apolipoprotein E–deficient littermates. Interestingly, we did not see an increase in systolic blood pressure in hIRECO mice consistent with our findings in mice with endothelium-specific insulin resistance because of expression of a dominant negative IR. These findings may reflect the relative importance of IRs in arteries of different size. We also saw no effect on glucose homeostasis in hIRECO mice similar to mice with endothelium-specific insulin resistance because of expression of a dominant negative IR and unlike mice with EC-specific deletion of IR substrate-2, suggesting that the effect of EC insulin sensitivity on whole-body glucose homeostasis is complex and may depend on the signaling node involved.

Insulin activates its intracellular signaling pathway by binding to and activating its tyrosine kinase receptor, which leads to activation of IR substrates. Downstream of IR substrates is PI3-K, which activates the serine/threonine kinase Akt. On insulin stimulation, Akt is phosphorylated by phosphoinositide-dependent kinase-1 and the mechanistic target of rapamycin complex-2 to yield a fully activated kinase, which phosphorylates eNOS to increase NO synthesis in response to a range of cues, including mechanical shear stress and insulin. Although deletion of Akt has been shown to promote the development of atherosclerosis and short-term enhancement of Akt activity has been shown to increase NO availability, unrestrained Akt activity may contribute to the development of atherosclerosis and, in the setting of obesity, contribute to the development of oxidative stress. Here, we demonstrate that EC overexpression of IR increases basal and insulin-stimulated activation of Akt, which increases the activity and expression of the superoxide-generating enzyme Nox2 NADPH oxidase. Although a link between PI3-K/Akt and NADPH oxidase has been proposed, this is the first study to unequivocally demonstrate a direct pathway between enhanced insulin sensitivity, PI3-K, Akt activation, and Nox2 NADPH oxidase activity. During normal physiology, the NADPH oxidases generate low levels of reactive oxygen species in a highly regulated fashion for use in redox-dependent signaling. When the generation of reactive oxygen species exceeds their homeostatic threshold, a range of disorders of the human cardiovascular system may ensue. Enhancing insulin sensitivity in the endothelium not only increased basal superoxide generation but also led to the ability of ECs from hIRECO to generate superoxide in response to insulin, unlike cells from WT mice. This insulin-mediated superoxide release was blunted by specific inhibitors of Nox2 and Akt.

We also demonstrated increased EC expression of the cytoplasmic tyrosine kinase PYK2 in hIRECO mice. PYK2 is activated in response to a range of stimuli and is thought to be important in coupling several receptors with downstream effectors. Elegant work by Fisslthaler et al has shown that insulin and shear stress increase expression levels of PYK2, which in turn phosphorylates a tyrosine residue on eNOS, which leads to inhibition of enzyme activity and reduced NO generation. This effect, which was shown to be independent of reactive oxygen species and dependent on absolute expression levels of PYK2, has emerged as a potentially important regulator of NO generation. Here, we show in an in vivo model that selective enhancement of insulin sensitivity in the endothelium leads to increased expression of PYK2 and a concurrent increase in tyrosine phosphorylation of eNOS (Figure 8J). Inhibition of PYK2 reversed the detrimental effect of excess insulin signaling on insulin- and shear stress–induced eNOS activation. We demonstrated no difference in phosphorylated PYK2 in hIRECO ECs. However, consistent with the work of Fisslthaler et al, an increase in the absolute levels of PYK2 expression was sufficient to inhibit eNOS in our system. We took this further by demonstrating that pharmacological blockade of Akt reduced the expression of PYK2, and we also demonstrated for the first time that Nox2-derived superoxide increases the expression of PYK2. This concomitant increase in PYK2 expression and downstream phosphorylation of eNOS at tyrosine residue 657 reduces eNOS activity and NO generation (Figure 8J).

Although other residues on eNOS could be phosphorylated and inhibit enzyme activity, our data clearly demonstrate a key role for tyrosine residue 657 in insulin-mediated adverse effects on eNOS activity. Consistent with our findings in mice with whole-body insulin resistance, we demonstrated an increase in basal serine phosphorylated eNOS, which may itself limit further increments in eNOS activation in response to different stimuli. Although our gain-of-function transgenic model generated using the hprt approach does circumvent some of the problems with standard transgenic models, it will be important in the future to generate and examine complementary gene-modified models with targeted knockout of key downstream proteins to enhance insulin signaling at different signaling nodes in the endothelium.
Our data provides evidence of the integration of 2 discrete but interrelated signaling pathways, impacting NO availability unfavorably. Enhancing insulin sensitivity in the endothelium led to excess generation of superoxide, which in a range of complementary assays we showed to be Nox2 NADPH oxidase dependent. Via increased expression of PYK2, enhancing insulin sensitivity led to attenuation of eNOS activity in response to insulin and shear stress. At the center of this lies Akt, driving the expression of both Nox2 and PYK2. It is important to note that type 2 diabetes mellitus is a progressive disorder and insulin hypersecretion can occur in healthy humans well before the onset of insulin-resistant type 2 diabetes mellitus. Indeed, hyperinsulinemia is a strong independent predictor of the development of atherosclerosis in individuals free from diabetes mellitus. We have demonstrated that metabolic insulin resistance may precede vascular insulin resistance. Whether individuals at early stages of the disease before the onset of hyperglycaemia or indeed individuals with type 1 diabetes mellitus treated with high dose of insulin have evidence of the proatherosclerotic signaling circuit in the endothelium demonstrated here warrants investigation.

Acknowledgments

We would like to thank Luke Brewin, Nuffield Research Foundation Scholar, as well as Joseph Turner, Aneesa Razaq, and Kar Yeun Tang from the University of Huddersfield-LICAMM Work Placement Programme, for their invaluable technical support.

Sources of Funding

This work was supported by British Heart Foundation grant RG09/010. A.M.S. research was supported by BHF grant CH/1999001/11735.

Disclosures

None.

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Selective Enhancement of Insulin Sensitivity in the Endothelium In Vivo Reveals a Novel Proatherosclerotic Signaling Loop


Circ Res. 2017;120:784-798; originally published online December 5, 2016;
doi: 10.1161/CIRCRESAHA.116.309678

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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SUPPLEMENTAL MATERIALS

Selective enhancement of insulin sensitivity in the endothelium in vivo reveals a novel proatherosclerotic signalling loop.

First Author's Surname: Viswambharan
Short Title: Insulin sensitivity and NO availability

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SUPPLEMENTAL MATERIALS

Detailed and Extended Methods

Mice over expressing the human insulin receptor specific to the endothelium.
To overcome the limitations seen in standard transgenics we used the Hypoxanthine Phosphoribosyl transferase (Hprt) targeting system1 applying ‘Quick Knock-inTM (GenOway) technology to generate genetically modified embryonic stem (ES) cells. This approach uses homologous recombination to target a single copy of a transgene (in this case the human IR) driven by a promoter (in this case the Tie2 promoter) into the Hprt locus on the X chromosome (Supplemental Figure IA).

As previously described2 the model was developed with E15Tg2a (E14) cells derived from the strain 129P2/OiaHsd (12901a). In E14 cells, 35kb of the Hprt gene encompassing the 5’ UTR up to intron 2 is deleted. The Hprt gene encodes a constitutively expressed housekeeping enzyme involved in purine synthesis from the degradation products of nucleotide bases (salvage pathway). Cells normally synthesise purines by the salvage and de-novo pathways. In Hprt deleted cell lines, only the de-novo pathway is functional enabling the cells to grow in classical medium. However, in the presence of the aminopterin drug the de-novo pathway is blocked. As a result Hprt deleted cells die in media called HAT (containing hypoxanthine, aminopterin and thymidine substrates). The targeted insertion of a transgenic cassette in E14 ES cells with a functional Hprt gene rescues these cells, which can then be selected using HAT media to identify ES cells showing the correct targeting event. Thus ES cells with the correct insertion can be selected by virtue of their expression of Hprt and the resultant ability to grow in HAT medium. Numerous studies have shown that the functional properties of the Hprt locus protect transgenic constructs inserted in this region against gene silencing and positional or methylation effects. Furthermore, tissue-specific promoters including Tie2 inserted3 into the Hprt locus maintains their expression properties. This sequence has been shown confer uniform and high level of expression of Lac Z reporter gene in endothelial cells in vivo.

Vector construction. Endothelial cell-specific transgene expression was achieved using the mouse Tie2 promoter and intronic enhancer as previously described. In order to reduce the size of
transgene the minimal-enhancer sequence (core-enhancer) was inserted into the final construct. This sequence has been shown confer uniform and high level of expression of Lac Z reporter gene in endothelial cells in vivo. The targeting vector was obtained by inserting human IR cDNA (a kind gift from Dr M Quon NIH Bethesda USA) into the pHHNS plasmid comprising the murine Tie2 promoter, LacZ cDNA and SV40 polyA signal and a 10-kb intronic enhancer from the murine Tie2. The targeting vector was obtained by inserting human IR cDNA (a kind gift from Dr M Quon NIH Bethesda USA) into the pHHNS plasmid comprising the murine Tie2 promoter, LacZ cDNA4 and SV40 polyA signal and a 10-kb intronic enhancer2 from the murine Tie2. The final transgenic vector consisting of Tie2 promoter, human IR, polyadenylation site and core-enhancer was then inserted into the Hprt targeting vector by a gateway reaction between the transgenic vector and the Hprt targeting vector. The final targeting vector had the following features; 1) Isogenic with E14 ES cells favouring homologous recombination, 2) Symmetrical homology arms (5’ short arms-SA: 3.8kb, 3’ long arm –LA: 3.7kb), 3) Transgenic cassette expressing the human IR cDNA under control of the short form of the Tie2 promoter, 4) Wild type Hprt sequences to reconstitute the Hprt gene in the E14 ES cells. In ES cells, this process was highly successful with 3 clones selected for blastocyst injection. The 5’ and 3’ targeting events were unambiguously confirmed by Southern blot analysis. These 3 ES clones were expanded and genetically manipulated agouti ES cells were then injected into C57BL/6J derived blastocysts that were then implanted into the uteri of recipient females.

Breeding of Chimeras and generation of F1 mice heterozygous for the human insulin receptor. Highly chimeric males (displaying 100% chimerism) generated by blastocyst injection of ES clones were mated with 2 wild type C57BL/6J female mice, to examine whether the targeted ES cells contributed to the germ layer. To assess whether ES cells have contributed to the germ layer of chimeras, mouse coat colour markers are used. The ES cells used to develop the model were originally derived from a 129 strain of mice which have an agouti coat colour. This marker is dominant over the black coat of C57BL/6J mice. Therefore, mating of the chimeras with C57BL/6J mice yields agouti coloured pups when the ES cells have contributed to the germ layer. Agouti F1 females were genotyped by Southern blot (Supplemental Figure IB). Southern blot validated the correct heterozygous status of tested F1 females, by detecting the 15.2 kb sized AvrII fragment of the C57BL/6 Hprt wild type allele and the 9.8kb sized AvrII fragment of the reconstituted Hprt allele (females #2, #4, #6, #7, (Supplemental Figure IB). Genotyping protocol validated the multiple copies of transgene (Supplemental Figure IC). Two of the F1 females were backcrossed for at least 10 generations into a C57BL/6J background and genotyped (Supplemental Figure ID) before commencing experiments.

All experiments were carried out comparing with wild type littermate controls. Genotyping PCR primer sequence (5’ to 3’): Primer 1, ACGTCAGTAGTACAGGAACTGCGTGC and Primer 2, TGCCTGATTTACAGGATAGCTGAGG.

High performance liquid chromatographic measurement of conversion of dihydroethidium to oxyethidium. Aortic segments were incubated with scrambled peptide or the Nox2 specific inhibitor, gp91-dstat for 30 minutes in KREBS buffer and then washed twice with PBS before incubating in PBS/DHE at a final concentration of 100 μmol/L for 30 min. Cells were washed thrice with cold PBS, harvested in cold methanol/HCl (0.5 ml/well), vortexed vigorously, centrifuged (12,000 g for 10 min at 4°C), and supernatants recovered for analysis. Pellets were stored at −20°C in the dark until analysis. Samples were injected (30μL) into an HPLC system (Shimadzu), equipped with a photodiode array (SPD-M10AVP) and fluorescence (RF-10AXL) detectors. Chromatographic separation was carried out in a C18-Kromosil column (4.6 x 250 mm, 5 μm particle size) and DHE monitored by ultraviolet absorption at 245 nm and EOH and ethidium by fluorescence detection (excitation 510 nm and emission 595 nm). Quantification is performed by comparison of peak signal between the samples and standard solutions under identical chromatographic conditions. DHE-derived products are expressed as ratios of ethidium generated per DHE consumed (ethidium/DHE) and normalized against total weight of tissue. Data expressed as gp91ds-tat-inhibitable component of superoxide.
Amplex red assay for hydrogen peroxide in aorta. Freshly harvested aortas were collected into a modified Krebs-HEPES buffer, containing 20 mM HEPES, 119 mM NaCl, 4.6 mM KCl, 1 mM MgSO₄·7H₂O, 0.15 mM Na₂HPO₄, 0.4 mM KH₂PO₄, 5 mM NaHCO₃, 1.2 mM CaCl₂ and 5.5 mM glucose, pH 7.4. The aortas were cleaned of peripheral adipose tissue and divided in about 2mm aortic rings. Half of the aortic rings were incubated in 50μL of modified Krebs-HEPES buffer and the remaining aortic rings were incubated in 50μL of modified Krebs-HEPES buffer with 1250 U/mL catalase (free from tymol) for 1h at 37°C. Fifty microlitres of freshly-prepared 100 μM Amplex Red reagent with 0.2U/mL HRP was added to the samples and incubated for 1 hour at 37°C, protected from light. The aortic rings were removed from the samples and fluorescence was measured on VarioSkan (Thermo Scientific) plate reader (excitation 530 nm and emission 590 nm). The average readings with catalase were subtracted from average readings without catalase, and the value was used as input into an H₂O₂ standard curve. The H₂O₂ standard curve was prepared in the same plate simultaneously, with the tissues and was used to determine H₂O₂ concentration released in the samples. Weight of blotted dry tissue was used for normalization.

Pulmonary endothelial cell isolation and culture. Lungs were harvested, washed, finely minced and digested in HBSS containing 0.18U/ml collagenase type 2 (Worthington, USA) and for 45 minutes at 37°C. The digested tissue was filtered through a 70μm cell strainer and centrifuged at 1200 rpm for 5 minutes. The cell pellet was washed with PBS/0.2% BSA, centrifuged, resuspended in 100μl of PBS/0.2%FCS and incubated with 1 x 10⁶ CD146-Ab-coated beads at 4°C for 15 minutes. Bead-bound cells were separated from non-bead bound cells using a magnetic MS Column (Milteny Biotechnology). Bead-bound (CD146-positive) and non-bead-bound cells were resuspended in 2 ml Endothelial growth medium–MVs (PromoCell, Heidelberg, Germany) supplemented with hEGF, hydrocortisone, VEGF, hFGF-B, R3-IGF-1, ascorbic acid, gentamicin, amphotericin-B and 5% FCS and plated out. Only the endothelial cell population tested positive for a range of endothelial markers including eNOS, Tie2, ve-cadherin, WF and CD102 protein measured using immunoblotting.

Characterisation of isolated pulmonary endothelial cells using Dil-Ac-LDL

The endothelial phenotype of PEC was corroborated by demonstrating uptake of acetylated low-density lipoprotein cholesterol (Ac-LDL). Confluent PEC were incubated in standard culture medium containing 10ug/ml Dil-conjugated Ac-LDL (Molecular Probes, ThermoFisher Scientific) for one hour, prior to washing with PBS and fixing with 4% paraformaldehyde in PBS. Imaging was performed using an Olympus CKX-41 microscope using a red fluorescence emission filter. No fluorescence was detected in PEC not exposed to Dil-conjugated Ac-LDL.

Cell lysis, Immunoblotting. Primary endothelial cells were lysed in extraction buffer containing 50mM HEPES, 120mM NaCl, 1mM MgCl₂, 1mM CaCl₂, 10mM NaPO₂7, 20mM NaF, 1mM EDTA, 10% glycerol, 1% NP40, 2mM sodium orthovanadate, 0.5μg/ml leupeptin, 0.2mM PMSF, and 0.5μg/ml aprotinin. Insulin-stimulated endothelial cells were serum-deprived overnight with 1% FCS-containing endothelial cell media before stimulation and harvesting in lysis buffer for western blot analysis. Cell extracts were sonicated in an ice-bath and centrifuged for 15 minutes at 13000 rpm, before protein measurements were carried out by BCA assay (Pierce Protein Quantification Kit) using the supernatant. Equal amounts of cellular protein were resolved on SDS polyacrylamide gels (Invitrogen) and transferred to polyvinylidine difluoride membranes. Immunoblotting was carried out with indicated primary antibodies, diluted as necessary in 5% BSA-TBST buffer. Blots were incubated with appropriate HRP-conjugated secondary antibodies and developed with enhanced chemiluminescence (Millipore). Sixty micrograms of total cell lysate was used for immunoprecipitation and thirty micrograms for western blotting with indicated antibodies: IR beta (C19) and β-Actin, (Santa Cruz Biotechnology), phospho-tyrosine, (pY4G10, Millipore), Nox2, eNOS, phospho-eNOS (BD Biosciences), PYK2, phospho-PYK2, Akt and phospho-Akt, (Cell Signalling), and phospho-tyrosine 657 (ECM Biosciences) and Protein A-Dynabeads (Invitrogen) for 20 minutes at room temperature. Akt inhibitor, MK-2206 10 micromol/L was from SelectChem. Immunoblots were scanned on a Syngene Gel Documentation System and bands quantified using Syngene.
Genetools Image Analysis software. Where bar charts depict percentage of control or wild type, we carried out all experiments for the two groups of mice (wild type and hIRECO) in pairs using the same conditions; from harvesting organs, isolating cells and stimulation of insulin or various inhibitors/siRNA and therefore, set its own wild type or serum-deprived control wells as hundred percent. When responses to insulin or inhibitors were analysed, the experiments were serum-deprived in 0.5% serum containing medium without supplements overnight before carrying out stimulation and harvesting. Chronic inhibition in cells to analyse protein expression was been performed in full growth medium and inhibitors incubated for 8 hours prior to harvest.
References


Supplemental Figure I. A) Construction of targeting vector to generate mice with endothelium-specific transgenic expression of human insulin receptor. B) Southern blot validated the correct heterozygous status of 4 of 7 tested F1 females, by detecting the 15.2 kb sized AvrII fragment of the C57BL/6 Hprt wild type allele and the 9.8 kb sized AvrII fragment of the reconstituted Hprt allele. C) Genotyping protocol validated the multiple copies of transgene. D) PCR product using isolated DNA, showing no human insulin receptor gene expression in wild type (WT) and in hIRECO (TG) ear notches. Neg denotes negative control, using water as template.
Supplemental Figure II. A) No difference in monocyte count in mice with endothelial cell specific over-expression of the human insulin receptor (hIRECO). B) No difference in monocyte as percent of circulating cells in hIRECO. C) No difference in CD11b expression in monocytes from hIRECO. D) No difference in TNF-alpha mRNA expression in monocytes from hIRECO. E) No difference in IL-16 mRNA expression in monocytes from hIRECO. F) No difference in IL-1Beta mRNA expression in monocytes from hIRECO (Data presented as mean ± SEM. All experiments n=5 mice WT denotes wild type).
Supplemental Figure III. A) No difference in mRNA expression of Nox2 NADPH oxidase (Nox2), Nox4 NADPH oxidase (Nox4) in superoxide dismutase 1,2 and 3 (SOD), catalase and endothelial NO synthase in pulmonary endothelial cells from mice with endothelial cell specific over-expression of the human insulin receptor (hIRECO). B) Isolated pulmonary endothelial cells under phase contrast microscopy and upon staining with Dil-conjugated Acetylated LDL (Ac-LDL). C) No difference in circulating plasma TNF-alpha in hIRECO. D) No difference in circulating plasma IL-6 in hIRECO. E) Total nitrite levels are significantly higher in the hIRECO plasma compared to WT (all experiments n=5 mice WT denotes wild type).
Supplemental Figure IV. A) Chronic pharmacological inhibition of Nox2 NADPH oxidase with gp91ds-tat to hIRECO mice via osmotic mini-pump for 28 days had no effect on organ mass. B) Basal NO production in response to isometric tension assessed by measuring the constrictor response to the NO synthase inhibitor, L-NMMA was reduced in hIRECO mice (left). PE constrictor response in the WT with and without L-NMMA (centre). PE constrictor response in hIRECO mice aorta with and without L-NMMA (right). % Change means the change from maximal constriction to PE after L-NMMA (Data presented as mean ± SEM. All experiments n=5 mice WT denotes wild type).
## Supplemental Table I. Primer sequences used for quantitative PCR for mRNA levels

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