Accelerated endothelial dysfunction in mild prediabetic insulin resistance: the early role of reactive oxygen species

Edward R. Duncan,1 Simon J. Walker,1 Vivienne A. Ezzat,1 Stephen B. Wheatcroft,2 Jian-Mei Li,3 Ajay M. Shah,1 and Mark T. Kearney2
1The Cardiovascular Division, King’s College London School of Medicine, King’s College London, London; 2School of Biomedical and Molecular Sciences, University of Surrey, Guilford; and 3The Leeds Institute of Genetics, Health, and Therapeutics, University of Leeds, Leeds, United Kingdom

Submitted 16 May 2007; accepted in final form 3 August 2007

Duncan ER, Walker SJ, Ezzat VA, Wheatcroft SB, Li J-M, Shah AM, Kearney MT. Accelerated endothelial dysfunction in mild prediabetic insulin resistance: the early role of reactive oxygen species. Am J Physiol Endocrinol Metab 293: E1311–E1319, 2007.—Insulin resistance is a central pathogenic feature of type 2 diabetes and cardiovascular atherosclerosis. Most studies have examined atherogenesis in models of severe insulin resistance or diabetes. However, by the time of diagnosis, individuals with type 2 diabetes already demonstrate a significant atheroma burden. Furthermore, recent studies suggest that, even in adolescence, insulin resistance is a progressive disorder that increases cardiovascular risk. In the present report, we studied early mechanisms of reduction in the bioavailability of the antiatherosclerotic molecule nitric oxide (NO) in very mild insulin resistance. Mice with haploinsufficiency for the insulin receptor (IRKO) are a model of mild insulin resistance with preserved glycemic control. We previously demonstrated that 2-mo-old (Young) IRKO mice have preserved vasorelaxation responses to ACh. This remained the case at 4 mo of age. However, by 6 mo, despite no significant deterioration in glucose homeostasis (Adult), IRKO mice had marked blunting of ACh-mediated vasorelaxation [IRKO maximum contraction response \( E_{\text{max}} \) 66 ± 5% vs. wild type 87 ± 4%, \( P < 0.01 \)]. Despite the endothelial dysfunction demonstrated, aortic endothelial nitric oxide synthase (eNOS) mRNA levels were similar in Adult IRKO and wild-type mice, and, interestingly, aortic eNOS protein levels were increased, suggesting a compensatory upregulation in the IRKO. We then examined the potential role of reactive oxygen species in mediating early endothelial dysfunction. The superoxide dismutase mimetic Mn([III]tetraakis(1-methyl-4-pyridyl) porphyrin pentachloride (MnTMPyP) restored ACh relaxation responses in the Adult IRKO \( E_{\text{max}} \) to ACh with MnTMPyP 85 ± 5%. Dihydroethidium fluorescence of aortas and isolated coronary microvascular endothelial cells confirmed a substantial increase in endothelium-derived reactive oxygen species in IRKO mice. These data demonstrate that mild insulin resistance is a potent substrate for accelerated endothelial dysfunction and support a role for endothelial cell superoxide production as a mechanism underlying the early reduction in NO bioavailability.

insulin resistance; endothelial function; superoxide; enos

INSULIN RESISTANCE is a central pathogenic feature of type 2 diabetes mellitus (42), the incidence of which is rising substantially (24). The principal cause of death in type 2 diabetes is premature cardiovascular atherosclerosis (23), and patients with type 2 diabetes have a similar risk of fatal cardiovascular events as nondiabetic patients who have sustained a myocardial infarction (19). We recently demonstrated that, despite contemporary therapies, patients with type 2 diabetes who have sustained an acute myocardial infarction have not benefited from the improvements in outcome seen in nondiabetic patients over the last 10 years (8). This finding highlights the need for better understanding of the pathophysiological mechanisms underlying the progressive vasculopathy seen in insulin-resistant individuals.

Type 2 diabetes is preceded by a long period of insulin resistance during which a compensatory increase in pancreatic \( \beta \)-cell function maintains normoglycemia at the expense of fasting and postprandial hyperinsulinemia (55). Insulin resistance per se is now well established as an independent risk factor for the development of cardiovascular atherosclerosis (18, 20, 21) and type 2 diabetes (57). As a result of this relationship, a significant proportion of patients with type 2 diabetes have evidence of myocardial ischemia at presentation (13). This suggests that future therapies should be targeted earlier at the mild end of the spectrum of insulin resistance. The importance of unraveling the mechanistic link between early insulin resistance and accelerated atherosclerosis is underscored by the recent finding that, even in adolescence, mild insulin resistance is associated with increased cardiovascular risk (47).

To date, studies have predominantly examined animal models of severe insulin resistance or type 2 diabetes characterized by a variety of features of severe insulin resistance, including obesity, hyperglycemia, dyslipidemia, hyperinsulinemia, and hypertension. Examples of such models include the fructose-fed rat (46), the Zucker fatty rat (11), and the \( db/db \) mouse (40). In the present study, we examined a model of “mild” whole body insulin resistance [mice with haploinsufficiency for the insulin receptor (IRKO); 54]. By doing so, we further explored the impact of mild insulin resistance on endothelial function. Notably, the IRKO mouse displays a metabolic phenotype that mirrors the abnormalities of insulin signaling described in insulin-resistant humans, including reduced insulin receptor numbers (56), reduced insulin receptor tyrosine kinase activity (14), and impaired intracellular insulin signaling (9).

A hallmark and key pathophysiological step in the development of atherosclerosis is endothelial cell dysfunction (16, 51). The term endothelial dysfunction encompasses a range of...
abnormalities among which a reduction in the bioavailability of the signaling molecule nitric oxide (NO) is of particular relevance to insulin resistance/type 2 diabetes. Longitudinal studies have established endothelial dysfunction as an independent predictor of progressive coronary disease (4, 44, 48). The bioavailability of NO is dependent on its production by the endothelial isoform of nitric oxide synthase (eNOS) and its inactivation by reactive oxygen species (ROS). We hypothesized that mild whole body insulin resistance is a substrate for accelerated endothelial dysfunction. We chose to study endothelial function in the IRKO mouse at serial time points to examine temporal changes in NO bioavailability as insulin-resistant mice reach adulthood.

The key findings of the present report are that mild insulin resistance is associated with accelerated endothelial dysfunction secondary to a substantial increase in endothelial ROS production that arises despite a compensatory increase in eNOS protein expression.

RESEARCH DESIGN AND METHODS

Animals. A colony of IRKO mice is established within our institution. Animals were bred on a C57BL/6J background in a conventional animal facility with a 12:12-h light-dark cycle and received standard laboratory chow. Male IRKO mice aged 8 wk (young) and their wild-type (WT) littermates were compared with 6-mo-old (adult) IRKO mice and their WT littermates (n = 6–10 animals/group).

Genotyping was performed using PCR on tail genomic DNA, with primers specific for the gene-targeting cassette.

Metabolic assessment. Intrapleuralal glucose and insulin tolerance tests were performed in conscious fasted mice as previously reported (53, 54). Blood glucose was measured at 30-min intervals following intrapleural glucose (1 mg/g body wt) or insulin injection (1 U/kg; Actrapid; Novo Nordisk, Bagsvaerd, Denmark) using a glucometer (Hemocue, Sheffield, UK). Plasma insulin was measured by enzyme-linked immunosassay (Crystalchem, Downers Grove, IL) using mouse insulin standards. The homeostasis model assessment of insulin resistance (HOMA-IR) method was used to assess insulin resistance. HOMA scores were calculated using the formula: HOMA-IR = insulin (mU/l) × glucose (mmol/l) / 22.5 (35). Fasting triglycerides and fasting free fatty acids were measured by colorimetric assays (Thermotrace, Victoria, Australia and Roche applied science, respectively).

Blood pressure measurement. Systolic blood pressure was measured using tail cuff plethysmography (XBP 1000; Kent Scientific, Torrington, CT) in conscious restrained mice at an ambient temperature of 24–26°C (n = 10). Animals were habituated to the restraining apparatus, and tail cuff inflation on three occasions before measurements was taken. The mean of a minimum of eight recordings on each occasion was taken, and mean data were compared between groups (54).

Aortic ring studies. Vasomotor function was assessed ex vivo in aortic rings (38, 53, 54) mounted in an organ bath containing Krebs Henseleit buffer (composition in mmol/l): 119 NaCl, 4.7 KCl, 1.18 KH₂PO₄, 25 NaHCO₃, 1.19 MgSO₄, 2.5 CaCl₂, and 11.0 glucose gassed with 95% O₂-5% CO₂. Rings were equilibrated at a resting tension of 3 g for 45 min before the experiments. To ensure integrity of the contractile apparatus of the aortic ring, rings were exposed to 40 mmol KCl (10 min). Following washing and reequilibration, the cumulative dose response to the constrictor phenylephrine (PE; 1 nmol/l to 10 µmol/l) was first assessed. After washing and reequilibration, relaxation responses to Ach (1 nmol/l to 10 µmol/l) and sodium nitroprusside (SNP; 0.1 nmol/l to 1 µmol/l) were assessed in separate rings preconstricted to ~70% of their maximal PE-induced tension. Relaxation was expressed as the percentage of preconstricted tension. To assess the effect of insulin on vascular function, maximal PE constriction responses (Eₘₐₓ) were assessed in a separate cohort of aortic rings following incubation with insulin (Actrapid, 100 µU/ml, 2 h; see Ref. 54). Aortic vascular responses to PE, Ach, and SNP were studied in cohorts of IRKO and WT mice aged 2, 4, and 6 mo.

Aortic responses to Ach were then reassessed in aortic rings from 6-mo-old mice incubated with the superoxide dismutase (SOD) mimetic Mn(III)tetrakis(1-methyl-4-pyridyl) porphyrin pentachloride (MnTMPyP), 10 µmol/l, 30 min; see Ref. 34) or the tetrahydrodipetin analog sepiapterin (10 µmol/l, 30 min; see Ref. 29) as previously described.

Coronary microvascular endothelial cell isolation. Six mouse hearts were used for each preparation of coronary microvascular endothelial cells (CMEC; see Ref. 32). After ethanol exposure to devitalize epicardial mesothelial cells, ventricular tissue was minced and predigested in collagenase [1 mg/ml in Hanks’ balanced salt solution (HBSS)] to separate cardiomycocytes and other cells. The residual tissue pellet was used for CMEC isolation. The pellet was digested three times (10 min each) with 8 ml isolation buffer containing 0.05% trypsin, 10 mM EDTA, 0.1 mg/ml DNase, 0.1 mM ethylenediaminetetraacetic acid, 0.1% pronase, and 1% collagenase and 1% dispase. Real-time RT-PCR assays for eNOS and β-actin mRNA expression were performed in duplicate and using the ABI Prism 7000 sequence detection system (32). Primers and SYBR Green-labeled probes (Applied Biosystems) specific for these genes were used. cDNA was amplified in the following conditions: 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. The standard curve method (User Bulletin No 2; ABI Systems) was used for quantification, and results were normalized for the expression of β-actin.

Expression of eNOS mRNA in aorta. Total RNA was extracted from aortas (n = 5/group; RNeasy Mini Fibrous Kit; Quiagen). Equal quantities of RNA were reverse transcribed using superscript II RT (Invitrogen) and random decamer oligonucleotides. Real-time RT-PCR assays for eNOS and β-actin mRNA expression were performed in duplicate and using the ABI Prism 7000 sequence detection system (32). Primers and SYBR Green-labeled probes (Applied Biosystems) specific for these genes were used. cDNA was amplified in the following conditions: 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. The standard curve method (User Bulletin No 2; ABI Systems) was used for quantification, and results were normalized for the expression of β-actin.

Expression of eNOS protein in aorta. Mice were euthanized, and the thoracic aorta was excised and snap-frozen in liquid nitrogen. The aorta was then homogenized while in liquid nitrogen, before addition of 400 µl RIPA lysis buffer (25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, and 0.1% sodium deoxycholate) containing protease inhibitors. Following centrifugation at 13,000 rpm at 4°C for 8 min, the supernatant was preserved, and the soluble protein content was determined using a Bio-Rad protein assay kit (Bio-Rad Laboratories). Equal amounts of protein (50 µg/sample) were separated on 10% polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were blocked with 3% BSA in 25 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.1% Tween 20 before probing with a mouse monoclonal antibody against total murine eNOS (BD Biosciences) or a rabbit polyclonal antibody against β-actin overnight at 4°C. Specific bands were detected using an ECL chemiluminescence kit (Amersham) and quantified using densitometry.

Assessment of vascular ROS production. Three different methods were used for the detection of ROS. In situ ROS generation in CMEC was assessed using FACS analysis of CMEC exposed to dihydroethidium (DHE, 2 µM, 5 min) as described previously (52). Cells...
grown to 70% confluency were detached with trypsin and counted. Then CMEC (1 × 10⁶/ml) were incubated for 5 min at 37°C with DHE in a dark chamber while shaking. Cells were then stored on ice to stop the reaction and used immediately for flow cytometric analysis. A minimum of 5,000 events/test was analyzed in a FACSCalibur flow cytometer (Becton-Dickinson).

In situ ROS generation within the thoracic aorta was assessed using DHE fluorescence (2 μM, 5 min) as previously reported (38). Fluorescence intensity at the endothelial surface was quantified microscopically using a computerized image analysis system (Improvevision) from at least 10 aortic sections/mouse and 3 mice/group from aortas exposed to DHE.

Lucigenin (5 μmol/l)-enhanced chemiluminescence was used to assess NADPH-dependent superoxide production by CMEC homogenates in a microplate luminometer (Anthos Lucy 1; 37°C) as previously described (32, 34). Chemiluminescence was reported as arbitrary light units per minute over a period of 20 min. Hearts from six mice were homogenized for each CMEC isolation. Readings were taken from three separate cell cultures, and all readings were undertaken in triplicate. In some experiments, one of the following agents was preincubated with cells or homogenate for 10 min: tiron (20 mmol/l), the flavoprotein inhibitor diphenyleneiodonium (DPI, 10 μmol/l), the NO synthase inhibitor Nω-nitro-L-arginine methyl ester (l-NAME, 100 μmol/l), or the mitochondrial electron transport chain inhibitor rotenone (20 μmol/l).

Statistics. All data are expressed as means ± SE. For isometric tension studies, concentration-response relationships were compared between groups of age-matched littersmates using two-way repeated-measures ANOVA. EC₅₀ and Eₘₓ were calculated for each agonist and compared between groups. One-way ANOVA was used in lucigenin-enhanced chemiluminescence experiments with the Student-Newman-Keuls multiple-comparison test used for post hoc analysis. Other variables were compared using Student’s t-test. P < 0.05 was taken as statistically significant. Data were analyzed using Statview (SAS Institute) and GraphPad (GraphPad Software) software.

RESULTS

Morphological data. There were no differences in solid organ weight, heart-to-body weight ratio, or adiposity between IRKO mice and their WT littersmates at 2 or 6 mo of age (data not shown).

Metabolic comparison of IRKO and WT mice. At both 2 and 6 mo of age, IRKO mice had similar fasting blood glucose, fasting insulin levels, and HOMA-IR scores to their WT littersmates. There was also no difference observed between groups in fasting serum triglycerides or free fatty acids (Table 1). Moreover, there was no difference in glucose tolerance between IRKO and WT mice at 2 or 6 mo of age, with both groups demonstrating similar blood glucose levels 30 min postintraperitoneal glucose challenge (Fig. 1A). However, IRKO mice demonstrated a significantly larger increment in serum insulin in response to a glucose load compared with their age-matched WT littersmates (Fig. 1B). This was apparent at both 2 and 6 mo of age. Both age groups therefore displayed compensatory hyperinsulinemia following glucose challenge. However, no differences were noted between groups in blood glucose responses to an intraperitoneal bolus of insulin (Fig. 1C).

Notably there was no evidence of progressive metabolic dysregulation during aging from 2 to 6 mo.

Baseline vascular assessment of IRKO and WT mice. Vasoinconstriction responses to PE were similar in WT and IRKO at 2 mo of age [EC₅₀ (nmol/l) 118 ± 13 vs. 143 ± 27, Eₘₓ (g) 0.77 ± 0.12 vs. 0.83 ± 0.07], 4 mo of age (data not shown), and at 6 mo of age (EC₅₀ 210 ± 35 vs. 211 ± 31, Eₘₓ 0.75 ± 0.09 vs. 0.83 ± 0.11; Fig. 2A). Non-receptor-mediated contraction responses to KCl were also similar (data not shown).

As we previously reported in 2-mo-old mice (54), incubation with insulin blunted Eₘₓ in WT but not IRKO mice aged 6 mo (Eₘₓ WT 0.71 ± 0.08 vs. WT + insulin 0.47 ± 0.10; Eₘₓ IRKO 0.58 ± 0.06 vs. IRKO + insulin 0.61 ± 0.10; Fig. 2B), thus confirming vascular insulin resistance in the old IRKO mice.

There was no significant difference in ACh-mediated vasorelaxation between young IRKO mice and their WT littersmates aged 2 mo [Eₘₓ 92.0 ± 8 vs. 90.3 ± 8%; P > 0.05, EC₅₀ (nmol/l) 164 ± 35 vs. 130 ± 70, P > 0.05; Fig. 2C]. This remained the case at 4 mo of age (data not shown). In contrast to this, at 6 mo of age, IRKO mice demonstrated significant blunting of ACh-mediated vasorelaxation [Eₘₓ 66.0 ± 5 vs. 87.3 ± 4%; P < 0.01, EC₅₀ (nmol/l) 137 ± 27 vs. 105 ± 27, P > 0.05; Fig. 2C].

No difference was demonstrated in the response to SNP at 2 mo [Eₘₓ 116 ± 7 vs. 112 ± 4%; P > 0.05, EC₅₀ (nmol/l) 8.4 ± 4 vs. 7.4 ± 2, P > 0.05], 4 mo (data not shown), or 6 mo of age [Eₘₓ 110 ± 2 vs. 110 ± 3%; P > 0.05, EC₅₀ (nmol/l) 20.4 ± 4 vs. 15.6 ± 5, P > 0.05, Fig. 2D].

The effect of aging from 2 to 6 mo was also compared in each group. The vascular phenotypes of WT control mice revealed no significant age-related changes in responses to PE, ACh, or SNP. Six-month-old IRKO mice showed no significant change in constriction responses to PE compared with 2-mo-old IRKO mice. However, ACh-induced relaxation was blunted (Eₘₓ 66.0 ± 5% vs. 92.0 ± 8, P < 0.05, EC₅₀ 137 ± 27 vs. 164 ± 35, P > 0.05). Interestingly, 6-mo-old IRKO mice demonstrated a significant increase in EC₅₀ to SNP (20.4 ± 4 vs. 8.4 ± 9 mmol/l, P < 0.05) but no change in Eₘₓ (111 ± 2 vs. 116 ± 7%).

Table 1. Metabolic characteristics

<table>
<thead>
<tr>
<th></th>
<th>Young</th>
<th>Old</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wild type</td>
<td>IRKO</td>
</tr>
<tr>
<td>Fasting blood sugar, mmol/l</td>
<td>5.4±0.4</td>
<td>5.3±0.3</td>
</tr>
<tr>
<td>Fasting insulin, ng/ml</td>
<td>0.33±0.03</td>
<td>0.47±0.05</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>2.02±0.3</td>
<td>2.80±0.6</td>
</tr>
<tr>
<td>Triglycerides, mmol/l (fasted)</td>
<td>1.48±0.2*</td>
<td>1.44±0.2*</td>
</tr>
<tr>
<td>Free fatty acids, mmol/l (fasted)</td>
<td>0.9±0.1*</td>
<td>1.2±0.2*</td>
</tr>
</tbody>
</table>

Values are means ± SE. IRKO, mice with haploinsufficiency for the insulin receptor; HOMA-IR, homeostasis model assessment of insulin resistance. *From Ref. 49.
Systolic blood pressure in IRKO mice was mildly elevated compared with WT littermates at 2 mo of age (124 ± 3 vs. 109 ± 2 mmHg, \(P < 0.05\)); however, at 6 mo of age, this difference did not reach statistical significance (111 ± 3 vs. 102 ± 3 mmHg, \(P = 0.05\); Fig. 3).

**Expression of eNOS mRNA and protein in thoracic aortas.** Real-time RT-PCR examination of whole aortas (n = 5/group; Fig. 4A) demonstrated no significant difference in levels of mRNA expression of eNOS (relative to \(\beta\)-actin) between adult IRKO and their WT littermates. Western blots were performed on protein extracted from aortic tissue from IRKO and WT mice (n = 5; Fig. 4, B and C). IRKO mice demonstrated significantly higher expression of eNOS protein (relative to \(\beta\)-actin) compared with WT (2.5 ± 0.7 and 0.5 ± 0.1, \(P = 0.05\)).

Western blots were performed on protein extracted from aortic tissue from IRKO and WT mice (n = 5; Fig. 4, B and C). IRKO mice demonstrated significantly higher expression of eNOS protein (relative to \(\beta\)-actin) compared with WT (2.5 ± 0.7 and 0.5 ± 0.1, \(P = 0.05\)).

**The role of ROS in mediating endothelial dysfunction in IRKO mice.** To explore the possibility that the impaired vasorelaxation to ACh in 6-mo-old IRKO mice was because of increased production of ROS, rings of thoracic aorta from both

**Fig. 1.** A: blood glucose levels measured both in the fasting state and 30 min following an ip injection of glucose were similar in young and old mice with haploinsufficiency for the insulin receptor (IRKO) and their wild-type (WT) littermates. B: serum insulin levels measured in the fasting state were also similar in IRKO mice and their age-matched WT littermates. However, 30 min following an ip injection of glucose, both young and old IRKO mice demonstrated significant compensatory hyperinsulinemia compared with their age-matched WT littermates; n = 8–10 mice/group. C: WT and IRKO mice showed a similar reduction in blood glucose levels 60 min following an insulin challenge at both 2 and 6 mo of age. \(*P < 0.05\) cf. age-matched WT littermates.

**Fig. 2.** Aortic vasomotor responses in IRKO and WT mice. IRKO and WT mice show similar vasoconstriction responses to phenylepherine (A). However, incubation with insulin reduced the maximum contraction response (Emax) to phenylepherine in 6-mo-old WT mice but had no effect in age-matched IRKO mice (B). This confirms vascular insulin resistance in the old IRKO group. Young IRKO and WT mice demonstrate similar relaxation responses to ACh, but, by 6 mo of age, old IRKO mice show significantly impaired relaxation responses to this agonist (C). Finally relaxation responses to sodium nitroprusside were similar in both groups at both 2 and 6 mo of age (D); n = 8–10. \(*P < 0.05\) cf. age-matched WT littermates.
groups of mice were exposed to the SOD mimetic MnTMPyP before undertaking ACh responses. MnTMPyP had no significant effect on WT ACh relaxation but significantly improved IRKO relaxation responses, completely restoring the vasorelaxation response to ACh in adult IRKO mice ($E_{\text{max}}$ 84.6 ± 5%; Fig. 5).

Vascular and endothelial ROS production. To further study endothelial ROS production, we measured in situ superoxide production using DHE fluorescence in isolated CMEC and in aortic sections. FACS analysis of CMEC loaded with DHE confirmed that IRKO cells had significantly increased mean fluorescence compared with WT (Fig. 6A). In aortic sections, quantitative assessment of endothelial DHE fluorescence confirmed increased ROS levels in the IRKO endothelium compared with WT (174 ± 15 vs. 140 ± 18 units of fluorescence, respectively, $P < 0.05$; Fig. 6B).

The role of eNOS in the production of ROS in the IRKO endothelium. To explore the possibility that the impaired vasorelaxation to ACh in 6-mo-old IRKO mice was because of increased production of ROS by dysfunctional eNOS, rings of thoracic aorta from both groups of mice were exposed to the tetrahydrobiopterin analog sepiapterin before undertaking ACh responses. Sepiapterin did not significantly improve maximal ACh responses in either group of mice aged 6 mo ($E_{\text{max}}$ (%) WT 87 ± 4 vs. WT sepiapterin 93 ± 8; $E_{\text{max}}$ IRKO 66 ± 5 vs. IRKO sepiapterin 75 ± 7; Fig. 7A).

Lucigenin-enhanced chemiluminescence was performed to assess NADPH-dependent superoxide production in CMEC. In CMEC from 6-mo-old WT mice, NADPH-dependent superoxide production was largely inhibited by the flavoprotein inhibitor DPI and the superoxide scavenger tiron ($P < 0.05$). The mitochondrial electron chain inhibitor rotenone and the eNOS inhibitor L-NAME had no significant effect (Fig. 7B). Lucigenin-enhanced chemiluminescence experiments on IRKO CMEC confirmed increased superoxide production by IRKO CMEC (IRKO superoxide 167 ± 14% of WT, $P < 0.05$; Fig. 7C). Similarly to WT CMEC, superoxide production by IRKO CMEC was almost completely inhibited by DPI and tiron, suggesting flavoproteins to be the significant source ($P < 0.05$; Fig. 7C). However, rotenone partially, but significantly, also reduced superoxide production by IRKO CMEC ($P < 0.05$). Notably, the eNOS inhibitor L-NAME had no significant effect, confirming that the increased eNOS protein in the IRKO endothelium is not contributing to the increased superoxide production demonstrated.
In the present study, we demonstrated that, despite very mild metabolic insulin resistance, which remains unchanged, IRKO mice have progressive endothelial cell dysfunction. Indeed, by the time IRKO mice reach 6 mo of age, they have markedly impaired vasorelaxation to ACh despite preserved glucose tolerance. This blunting of the vasorelaxation response to ACh arises secondary to an increase in endothelial cell production of ROS, despite an increase in eNOS protein expression. These data demonstrate that even extremely mild insulin resistance promotes vascular dysfunction. In addition, our data support endothelial production of superoxide as a potential early therapeutic target in patients with mild insulin resistance.

Insulin resistance and type 2 diabetes are now well established as important risk factors for the development of cardiovascular atherosclerosis. The alarming increase in type 2 diabetes in children and young adults and the fact that a substantial proportion of patients with type 2 diabetes have coronary artery disease at presentation makes understanding the mechanisms underlying accelerated atherosclerosis in insulin resistance of particular importance (13).

Compelling evidence supports endothelial dysfunction as a key early event in the pathogenesis of atherosclerosis (16). A reduction in the bioavailability of NO, an antiatherosclerotic signaling molecule released by the vascular endothelium, is a hallmark of endothelial dysfunction and is present in atherosclerotic vessels before vascular structural changes occur. 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 (4, 44, 48).

The bioavailability of NO is dependent on the balance between its production by eNOS and its inactivation by ROS. Classical activation of eNOS (e.g., by ACh) involves a rise in intracellular Ca²⁺ and binding of Ca²⁺/calmodulin to the eNOS enzyme to activate the enzymatic function of eNOS (12, 14). However, an alternative pathway for the activation of eNOS is through the release of NO by the tetrahydrobipterin (BH₄) analog, sepiapterin. The relative contribution of BH₄ and BH₄ analogs to the activation of eNOS is dependent on the availability of BH₄, which is an essential cofactor for eNOS. Low BH₄ may result in impaired NO production and increased production of superoxide.

Fig. 6. Endothelial cell reactive oxygen species production. A: FACS analysis of WT and IRKO coronary microvascular endothelial cells (CMEC) exposed to dihydroethidium (DHE) showed a rightward shift in mean fluorescence intensity and therefore increased superoxide production by IRKO CMEC (dark gray) compared with WT (light gray). B: units of fluorescence of the endothelium from sections of thoracic aorta exposed to DHE. The endothelium of IRKO mice demonstrated greater fluorescence than that of WT littermates; n = 3. *P < 0.05.

Fig. 7. Role of eNOS as a source of superoxide in the old IRKO mouse. A: effect of incubation with the tetrahydrobipterin (BH₄) analog sepiapterin on Ach-mediated relaxation in 6-mo-old WT and IRKO mice. Sepiapterin did not improve maximal Ach-mediated relaxation responses (Emax) in either group of mice; n = 11. *P < 0.05 cf. baseline. B and C: lucigenin-enhanced chemiluminescence in CMEC. B: NADPH-dependent superoxide production in WT cells was inhibited by tiron (20 mmol/l) and diphenyleneiodonium (DPI; 10 μmol/l). C: similarly, in IRKO CMEC, superoxide production was largely prevented by tiron and DPI; however, rotenone also partially but significantly inhibited superoxide production. N⁵-nitro-l-arginine methyl ester (l-NAME) had no effect on superoxide production in either group of mice; n = 3. #P < 0.05 cf. baseline superoxide production in that group.
enzyme. Recently, a Ca$^{2+}$-independent regulatory pathway for eNOS has been described (37). Both shear stress and agonists such as insulin have been shown to increase endothelial NO production via the activation of phosphatidylinositol 3-kinase and protein kinase B, which phosphorylates eNOS (10, 37, 58). We have previously demonstrated in young IRKO mice that the latter pathway is selectively blunted with preservation of ACh-mediated vasorelaxation (54). The current data suggest a progression in endothelial dysfunction such that responses to the classical Ca$^{2+}$-dependent pathway for activation also become dysfunctional.

It is well established that ACh-induced relaxation in the thoracic aorta/conduit vessels of C57BL/6J mice is almost entirely NO mediated, whereas endothelium-derived hyperpolarizing factor (EDHF) plays a significant role in smaller/resistance vessels (45). It is therefore likely that the impaired relaxation demonstrated in the 6-mo IRKO aorta is predominantly because of reduced NO bioavailability secondary to scavenging of NO by superoxide. However, there is also evidence that vascular production of EDHF and prostanooids is blunted by aging and diabetes (12, 36). It is possible therefore that abnormalities of these pathway may contribute to the impaired relaxation seen in older IRKO mice. This warrants further study.

The evidence that production of ROS such as superoxide within the endothelium plays an important role in the development of endothelial dysfunction is compelling (6, 17). Superoxide leads to endothelial and vascular dysfunction in the following several ways (for review, see Ref. 33): 1) it reacts rapidly with NO to inactivate it, 2) the reaction between NO and superoxide produces peroxynitrite, which may itself exert toxic effects through protein nitrosylation, 3) species such as H$_2$O$_2$ and peroxynitrite (and the loss of NO) may activate redox signaling cascades that induce deleterious changes in the endothelial cell phenotype, and 4) overproduction of ROS activates a variety of proinflammatory pathways.

Oxidative stress plays a critical role in the pathogenesis of various diseases. In diabetes, oxidative stress impairs glucose uptake in muscle (50) and fat (43) and decreases insulin secretion from $\beta$-cells (28). In obesity, increased ROS have been shown to contribute to vascular dysfunction, diabetes, hepatic steatosis, and dysregulation of adipokines and lipids (7, 15, 26). Consistent with this and of particular relevance to the present report, in adolescents, a positive association between insulin resistance and systemic oxidative stress was recently reported (47). A recent elegant report by Houstis and colleagues (22) also demonstrated that increased ROS may lead to insulin resistance using in vitro and in vivo models. Our data add to this finding suggesting that mild insulin resistance leads to vascular oxidative stress. This raises the intriguing possibility that a vicious cycle may ensue in the vasculature.

The IRKO mouse represents a model of mild insulin resistance. We and others have previously confirmed that IRKO mice exhibit a metabolic phenotype of preserved glycemic control, compensatory hyperinsulinemia, and impaired insulin signaling in both metabolic and vascular tissues at 2 mo of age (3, 27, 54). Bruning et al. (3) also confirmed metabolic insulin resistance, hyperinsulinemia secondary to $\beta$-cell hyperplasia, but preserved glycemic control in 6-mo-old IRKO mice. In the current report, a similar metabolic phenotype is described. Aged 6 mo, IRKO mice exhibit preserved glycemic control despite hyperinsulinemia, strongly suggesting reduction in the metabolic action of insulin in these mice. Resistance to the vascular action of insulin to blunt PE-induced constriction was also demonstrated at 6 mo of age, confirming that at this time point, IRKO mice remain mildly insulin resistant.

Interestingly, no difference was noted between 6-mo-old WT and IRKO in glucose responses to an intraperitoneal bolus of insulin. We suggest that the supraphysiological doses of insulin used in such testing do not allow differentiation of the mild insulin resistance seen in IRKO mice.

The IRKO aorta demonstrates impaired endothelium-mediated relaxation at 6 mo that is restored to levels seen in the WT by an SOD mimetic. The SOD mimetic had no effect on WT vascular function, suggesting that superoxide is a key mediator of endothelial dysfunction in the IRKO. Three different methods were then used to confirm the significantly increased endothelial ROS production by the IRKO endothelium compared with WT. Moreover, the increased production of ROS by the endothelium promotes accelerated endothelial dysfunction independent of any worsening of the metabolic phenotype. This implies a divergence of the pathologies of metabolic and vascular insulin resistance.

Intriguingly, the IRKO appears to upregulate eNOS protein expression, potentially compensating for the scavenging of NO by ROS. In the literature, eNOS expression has been demonstrated to be both up- and downregulated in responses to a variety of adverse conditions. Indeed, eNOS expression appears to vary between disease states and with duration of those conditions. For example, in hypertension, there may be a temporal pattern in eNOS expression, with young animals mostly demonstrating increased eNOS levels, suggesting a compensatory response, whereas older animals with more advanced disease have reduced expression (31). Consonant with this, human vessels demonstrating advanced atherosclerosis have diminished eNOS levels (5, 39). Conversely, models of hypercholesterolemia (25, 41) and cigarette smoking (1, 2) have shown elevated eNOS expression. Notably, in endothelial cell-specific insulin receptor knockout mice (VENIRKO), a reduction in eNOS mRNA was demonstrated (49). The discrepancy between the current data and that described in the VENIRKO mouse may result from the differences between the models. The IRKO is a model of “global” mild insulin resistance with a 50% reduction in insulin receptor numbers, whereas the VENIRKO mouse is a model of “endothelial cell specific” insulin resistance exhibiting no clear metabolic phenotype and a 95% reduction in insulin receptors. The mechanisms by which eNOS expression is regulated remain an important area for future study.

Further experiments were performed to elucidate whether the increased eNOS protein in the IRKO aorta contributes to the increased ROS production observed. The tetrahydrobiopterin analog sepiapterin did not improve endothelial function in the IRKO. Furthermore, lucigenin-enhanced chemiluminescence experiments confirm that dysfunctional eNOS does not contribute significantly to superoxide production in these mice. Indeed the source of the increased ROS was shown to be flavoproteins with a contribution by the mitochondrial respiratory chain. Importantly, our data suggest that increased endothelial superoxide production precedes any downregulation or dysfunction of eNOS protein in mild insulin resistance.
Both IGF-I and insulin have been shown to stimulate NO production in endothelial cells (59). IGF-I receptors have been demonstrated on endothelial cells and are in fact more abundant than insulin receptors (59). Moreover, IGF-I receptors have been shown to form IGF-I-insulin hybrid receptors in endothelial cells. These hybrid receptors are responsive to high concentrations of insulin (30). It is possible therefore that hybrid receptors may contribute to the vascular phenotype seen in the present study. The potential formation of hybrid receptors in IRKO mice and their contribution to endothelial function warrants further studies.

In summary, the present study provides compelling evidence for a key role for endothelial ROS production in the early endothelial dysfunction characteristic of prediabetic insulin resistance. Moreover, our data suggest that ROS production could be an early target to prevent the accelerated vasculopathy characteristic of this disease state. Finally, our data emphasize that therapy should be targeted at the long prediabetic phase of this illness before the onset of gross metabolic dysregulation, since vascular abnormalities can already be established.

REFERENCES


