Preserved Glucoregulation but Attenuation of the Vascular Actions of Insulin in Mice Heterozygous for Knockout of the Insulin Receptor

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Type 2 diabetes is preceded by years of insulin resistance and is characterized by reduced bioavailability of the antiatherosclerotic signaling molecule nitric oxide (NO) and premature atherosclerosis. The relationship between resistance to the glucoregulatory actions of insulin and its effects on the vasculature (in particular NO-dependent responses) is poorly characterized. We studied this relationship in mice heterozygous for knockout of the insulin receptor (IRKO), which have a mild perturbation of insulin signaling. Male heterozygous IRKO mice aged 8–12 weeks were compared with age- and sex-matched littermates. IRKO mice had fasting blood glucose, insulin, free fatty acid, and triglyceride levels similar to those of wild-type mice. Intraperitoneal glucose and insulin tolerance tests were also similar in the two groups. Insulin levels in response to a glucose load were approximately twofold higher in IRKO compared with wild-type mice (1.08 ± 0.11 vs. 0.62 ± 0.13 ng/ml; P = 0.004). Despite this mild metabolic phenotype, IRKO mice had increased systolic blood pressure (124 ± 4 vs. 110 ± 3 mmHg; P = 0.01). Basal NO bioactivity, assessed from the increase in tension of phenylephrine preconstricted aortic rings in response to the NO synthase inhibitor N_G-monomethyl-l-arginine, was reduced in IRKO (61 ± 14 vs. 152 ± 30%; P = 0.005). Insulin-mediated NO release in aorta, assessed as the reduction in phenylephrine constrictor response after insulin preincubation, was lost in IRKO mice (5 ± 8% change vs. 66 ± 9% reduction in wild-type; P = 0.03). Insulin-stimulated aortic endothelial NO synthase phosphorylation was also significantly blunted in IRKO mice (P < 0.05). These data demonstrate that insulin-stimulated NO responses in the vasculature are exquisitely sensitive to changes in insulin-signaling pathways in contrast to the glucoregulatory actions of insulin. These findings underscore the importance of early intervention in insulin-resistant states, where glucose homeostasis may be normal but substantial abnormalities of the vascular effects of insulin may already be present. Diabetes 53:2645–2652, 2004

The major cause of death and disability in individuals with type 2 diabetes is cardiovascular atherosclerosis. Type 2 diabetes is often preceded by years of insulin resistance, during which normal blood glucose levels are preserved by compensatory increases in pancreatic β-cell function to produce hyperinsulinemia (1). Insulin resistance itself is now recognized as an independent risk factor for the development of coronary heart disease (2), and a substantial proportion of patients with type 2 diabetes have established coronary heart disease at presentation (3). Understanding the mechanisms by which insulin resistance per se leads to premature atherosclerosis is therefore an important aim.

The insulin-resistant conditions of obesity (4,5) and type 2 diabetes (6) are characterized by endothelial dysfunction, a pivotal early event in the development of atherosclerosis (7). Insulin-resistant first-degree relatives of patients with type 2 diabetes have endothelial dysfunction (8). Furthermore, the degree of endothelial dysfunction is associated with the severity of insulin resistance (9). While the term endothelial dysfunction encompasses several potential abnormalities, of particular focus in this context is a reduction in the bioavailability of the signaling molecule nitric oxide (NO), which has potent vasodilatory and antiatherosclerotic properties. It has recently emerged that the endothelium is a target tissue of insulin, and insulin resistance can therefore exist at the level of the endothelial cell. Insulin stimulates the production of NO through activation of phosphatidylinositol-3 kinase (PI3K) and protein kinase B (PKB), resulting in phosphorylation of endothelial NO synthase (eNOS) (10–13). This signaling pathway has some similarities to the pathway that mediates insulin-stimulated glucose uptake in tissues involved in glucoregulation, such as skeletal muscle (14).

In this study, we used an integrated in vivo and ex vivo approach to study the relationship between the effects of a mild perturbation of insulin signaling on glucoregulation and vascular endothelial function in mice heterozygous for knockout for the insulin receptor (IRKO mice) (15,16). These animals provide a useful nonobese nondiabetic model of impaired insulin signaling in which insulin-stimulated PI3K activity in liver and skeletal muscle is reduced by 30% compared with control mice (17).
TABLE 1
Characteristics of wild-type and IRKO mice

<table>
<thead>
<tr>
<th></th>
<th>Wild-type</th>
<th>IRKO</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>25.9 ± 0.8</td>
<td>24.6 ± 0.8</td>
<td>0.31</td>
</tr>
<tr>
<td>Organ weights (% of body wt)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heart</td>
<td>0.49 ± 0.01</td>
<td>0.49 ± 0.01</td>
<td>0.67</td>
</tr>
<tr>
<td>Thoracic aorta</td>
<td>0.062 ± 0.008</td>
<td>0.059 ± 0.007</td>
<td>0.63</td>
</tr>
<tr>
<td>Pancreas</td>
<td>0.59 ± 0.01</td>
<td>0.59 ± 0.02</td>
<td>0.86</td>
</tr>
<tr>
<td>Liver</td>
<td>4.92 ± 0.22</td>
<td>4.87 ± 0.13</td>
<td>0.88</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.29 ± 0.03</td>
<td>0.33 ± 0.01</td>
<td>0.29</td>
</tr>
<tr>
<td>Lungs</td>
<td>0.71 ± 0.05</td>
<td>0.63 ± 0.04</td>
<td>0.25</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.62 ± 0.03</td>
<td>0.64 ± 0.01</td>
<td>0.68</td>
</tr>
<tr>
<td>Perigonadal fat (% of body wt)</td>
<td>1.66 ± 0.22</td>
<td>1.83 ± 0.11</td>
<td>0.54</td>
</tr>
<tr>
<td>Plasma triglycerides (mmol/l)</td>
<td>1.48 ± 0.2</td>
<td>1.44 ± 0.2</td>
<td>0.57</td>
</tr>
<tr>
<td>Plasma free fatty acids (mmol/l)</td>
<td>0.9 ± 0.1</td>
<td>1.2 ± 0.2</td>
<td>0.23</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>110 ± 3</td>
<td>124 ± 4</td>
<td>0.01</td>
</tr>
</tbody>
</table>

Data are means ± SE (n = 8 per group).

RESEARCH DESIGN AND METHODS
IRKO mice (15,16) were obtained from the Medical Research Council Mammalian Genetics Unit, Harwell, Oxfordshire, U.K. Animals were bred on a C57BL/6J background in a conventional animal facility with a 12-h light/dark cycle and received standard laboratory diet. Male IRKO mice aged 8–12 weeks were compared with age- and sex-matched wild-type littermates. Genotyping was performed using PCR on tail genomic DNA, with primers specific for the gene-targeting cassette.

Metabolic assessment. Intrapерitoneal glucose and insulin tolerance tests were performed in conscious, fasted animals (18). Blood glucose was measured at 30-min intervals following intraperitoneal glucose (1 mg/g body wt) or insulin injection (0.75 units/kg; Actrapid, Novo Nordisk, Bagsvaerd, Denmark) using a glucometer (Hemocue, SheffIELD, U.K.). Plasma insulin was measured by enzyme-linked immunosassay (CrystalChem, Downers Grove, IL) using rat insulin standards. Fasting plasma free fatty acids and triglycerides were measured by colorimetric assays (Roche, Mannheim, Germany and ThermoTrace, Victoria, Australia, respectively).

Blood pressure. 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 (19). Animals were habituated to the restraining apparatus and tail cuff inflation on three occasions before measurements were taken. The mean of six recordings on each occasion was taken, and mean data from three separate recording periods were compared between groups.

Aortic ring studies. Vasomotor function was assessed in ex vivo thoracic aortic rings mounted in organ baths 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 (19–21). Rings were equilibrated at a resting tension of 3 g for 45 min before the experiments. The cumulative dose response to the constrictor phenylephrine (PE) (1 nM to 10 μM) was first assessed. After washing and re-equilibration, relaxation responses to acetylcholine (1 nM to 10 μM) or sodium nitroprusside (0.1 nM to 1 μM) were assessed in separate rings preconstricted to ~70% of their maximal PE-induced tension. Relaxation was expressed as the percentage of preconstricted tension. Basal NO bioactivity was assessed in rings maximally constricted with PE by measuring the further increase in tension induced by exposure to the NOS inhibitor Nω-nitro-L-arginine (L-NMMA) (0.1 mM) for 30 min.

To assess the effect of insulin on NO production, we compared the cumulative dose response to PE (1 nM to 10 μM) before and after a 2-h incubation with insulin (100 μU/ml Actrapid; Novo Nordisk) (22, 23). This dose of insulin was found to be optimal in preliminary experiments. To evaluate the contribution of NO to the effect of insulin, this experiment was also performed in the presence of L-NMMA (0.1 mM) (24) or in endothelium-denuded rings.

Norepinephrine-induced hypertension. To investigate the effects of blood pressure per se on vascular responsiveness to insulin, additional experiments were undertaken in 8-week-old male wild-type C57BL/6J mice treated with norepinephrine (4.2 mg·kg−1·day−1·s.c.) or 0.9% saline by osmotic minipump (Alzet Model 1002) for 14 days (25). Systolic blood pressure was measured at days 1–3, 5, 9, and 12. Blood glucose and plasma insulin were measured after 13 days of infusion, and aortic ring studies were performed after 14 days of infusion.

RESULTS

Morphometric data. IRKO mice were morphologically indistinguishable from their wild-type littermates, with no significant differences in total body, organ, or perigonadal fat depot weight (Table 1).

Metabolic homeostasis in IRKO mice. Blood glucose levels were similar in IRKO and wild-type mice both in the fasting state and after a glucose challenge (Fig. 1A). Glucocompetence, assessed by the response to an intraperitoneal glucose load, was also similar in IRKO and wild-type mice (Fig. 1B). Insulin levels were similar in the fasting state (0.60 ± 0.17 vs. 0.34 ± 0.14 ng/ml for IRKO and wild-type mice, respectively) but were higher in IRKO mice after a carbohydrate challenge (1.08 ± 0.11 vs. 0.62 ± 0.13 ng/ml) (Fig. 1C). The hypoglycemic response to an intraperitoneal insulin load was not significantly different in IRKO and wild-type mice (Fig. 1D). Fasting free fatty acid levels were similar in IRKO and wild-type mice (Table 1).
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**Blood pressure.** Systolic blood pressure was significantly higher in IRKO (124 ± 4 mmHg) than wild-type (110 ± 3 mmHg; *P* = 0.01) mice (Table 1).

**Aortic vasomotor responses.** Basal NO bioactivity, assessed by the increment in tension induced by L-NMMA in PE-preconstricted rings, was significantly lower in IRKO than wild-type mice (Fig. 2). Relaxation in response to the endothelial-dependent vasodilator acetylcholine was similar in IRKO and wild-type groups (Fig. 3A). Likewise, endothelium-independent relaxation to sodium nitroprusside was no different between groups (Fig. 3B).

Preincubation of wild-type aortic rings with insulin significantly attenuated the contractile response to PE (Fig. 3C), with the maximal contractile response (E\textsubscript{max}) reduced to 66 ± 9% of control levels (*P* < 0.01) but with no change in the half-maximal effective concentration (not shown). This effect of preincubation was completely abrogated in aortic rings from IRKO mice (Fig. 3D). Consistent with the insulin-induced decrease in PE response due to insulin-stimulated NO release, the decrement in PE response was abolished by coincubation of wild-type rings with l-NMMA (Fig. 3E). Moreover, endothelial denudation also abolished this response in wild-type rings (Fig. 3F).

**Expression of eNOS mRNA.** There was no significant difference in aortic eNOS mRNA expression between IRKO and wild-type mice. (The relative expression of eNOS mRNA, normalized to β-actin mRNA, in IRKO mice compared with wild-type controls, was 1.07 ± 0.19 [*P* = 0.74].)

**Expression of eNOS and phospho-eNOS.** Expression of aortic eNOS protein, normalized for expression of smooth muscle α-actin, was similar in IRKO and wild-type mice (Figs. 4A and 5). In the unstimulated state, eNOS phosphorylation assessed using an antibody specific for phosphorylation of Ser\textsuperscript{1177} was at the limit of detection in both groups (Fig. 5). However, after insulin stimulation, there was a robust increase in phospho-eNOS in wild-type aorta, which was significantly attenuated in the IRKO group (Figs. 4B and 5).

**Norepinephrine-induced hypertension.** To exclude the possibility that the vascular abnormalities observed in IRKO mice may be related to increased blood pressure per se, we also studied wild-type mice receiving chronic subcutaneous infusion of norepinephrine. Norepinephrine infusion led to a significant rise in systolic blood pressure within 3 days and maintained for 2 weeks (Fig. 6A), which was of a similar magnitude to that observed in IRKO mice in the resting state (Table 1). Blood glucose and plasma insulin levels were similar in norepinephrine- and vehicle-infused mice (Fig. 6B and C). In aortic ring studies, insulin

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**FIG. 1.** Glucoregulation in wild-type (WT) and IRKO mice. A: Blood glucose was measured following an overnight fast and then 30 min after glucose challenge (1 mg/g i.p.). ■, fasting; □, glucose challenge. B: Glucocompetence was assessed by an intraperitoneal glucose tolerance test (1 mg/g i.p.). C: Plasma insulin levels were measured following an overnight fast and then 30 min after glucose challenge (1 mg/g i.p.). D: Insulin sensitivity was assessed by an intraperitoneal insulin tolerance test (0.75 units/kg i.p.). *P* < 0.05 compared with fasted animals; †P < 0.01 compared with wild-type (n = 8 per group).

**FIG. 2.** Effect of NO synthase inhibition on aortic contractile response. Aortic rings were preconstricted with PE (10 μmol/l) before incubating with l-NMMA (0.1 mmol/l for 30 min) in order to assess the contribution of basal NO bioactivity to the contractile response. *P* < 0.01 compared with wild-type (n = 8 per group).
preincubation attenuated the contractile response to PE to a similar degree in both norepinephrine- and vehicle-infused mice (Fig. 7). Basal NO bioactivity was also similar in both groups of mice. (The increment in tension induced by L-NMMA in PE-preconstricted rings was 175 ± 20% in norepinephrine-infused mice compared with 187 ± 20% in vehicle-infused animals \(P = 0.36\).)

FIG. 3. Aortic vasomotor responses in wild-type (WT) and IRKO mice. Vascular relaxations to acetylcholine (A) and sodium nitroprusside (B) were determined in aortic rings from wild-type and IRKO mice. The effect of preincubation with insulin (100 mU/ml for 120 min) on the contractile response to PE was assessed in aortic rings from wild-type (C) and IRKO (D) mice. To determine the contribution of endothelial NO to the vascular effects of insulin, insulin preincubation was repeated in wild-type aortic rings in the presence of L-NMMA (E) and following endothelial denudation (F). *\(P < 0.01\) by two-way ANOVA (\(n = 8\) per group).

DISCUSSION

The principal findings of the present study are that mice heterozygous for knockout of the insulin receptor have substantial abnormalities of vascular function despite very mild metabolic dysfunction. Heterozygous IRKO mice are glucometabolically, with hyperinsulinemia in response to a
involving its phosphorylation has been described (10–13). However, a complementary pathway for eNOS activation such as acetylcholine, act via G-protein coupled receptors, such as insulin and IGF-I, induce the activation of PE3K and PKB (Akt), leading to eNOS phosphorylation and enhanced production of NO. Interestingly, shear stress, which may be the major determinant of “basal” NO production in vivo, also activates eNOS at least in part through a similar pathway (28). These data suggest at least one mechanism through which abnormalities of insulin signaling or homeostasis may impact endothelial function.

In the present study, we found that acetylcholine-mediated vasodilatation was not different between IRKO and wild-type animals. However, the IRKO animals had a selective abnormality of basal NO bioactivity and insulin-independent vasodilatation. These results strongly suggest that there may be a selective abnormality of insulin-dependent pathways for eNOS activation. Indeed, we found that while there was no alteration in eNOS mRNA or protein expression, insulin-stimulated eNOS phosphorylation was significantly reduced in the IRKO group. It is unlikely that the defect seen in the IRKO mice is simply due to reduced receptor density per se. Since the insulin receptor itself has intrinsic activity (29), it is highly plausible that it regulates the downstream signaling cascade described above. This hypothesis is consistent with our finding of reduced basal NO bioactivity, the signaling pathway for which shares a number of components of the downstream insulin-signaling pathway. However, the precise nature of the defects leading to blunted eNOS activation in the present report requires further investigation.

In endothelial cells, insulin can also influence the expression levels of eNOS through the PI3K pathway. For example, two structurally different PI3K inhibitors, wortmannin (100 nmol/l) and LY294002 (50 nmol/l), blunt the effect of physiological concentrations of insulin to increase eNOS mRNA and protein expression (rev. in 13). Consistent with these studies, we have previously reported that in mice overexpressing IGF binding protein-1, which have postglucose hyperinsulinemia but normal whole-body insulin sensitivity, eNOS mRNA expression and basal NO bioactivity are increased (19). In the same model, there was also other evidence of increased NO production in response to hyperinsulinemia, manifested as blunted PE-mediated vasoconstriction and a substantial fall in postprandial blood pressure. An elegant study by Vicent et al. (30) recently showed that targeted knockout of the insulin receptor in endothelial cells resulted in reduction of eNOS expression, although the functional effects of insulin-mediated NO

glucose load as the only detectable metabolic abnormality. Nevertheless, the animals have increased blood pressure, evidence of reduced basal NO bioactivity, and marked blunting of insulin-mediated NO release from aorta. At least some of the vascular abnormality may be related to defective insulin-mediated activation of eNOS through phosphorylation of PKB-sensitive sites (10–13). Thus, even during relatively mild metabolic dysfunction (when glucose homeostasis is maintained by hyperinsulinemia), in this model there is a dramatic reduction in insulin-mediated and basal bioactive NO production in conduit vessels that is associated with an increase in arterial blood pressure. These results may have important implications for our understanding of the pathophysiology of vascular disease and accelerated atherosclerosis in insulin-resistant patients and patients presenting with type 2 diabetes.

**Role of endothelial dysfunction.** Compelling evidence supports endothelial cell dysfunction, in particular a reduction in the bioactivity of NO, as a key early event in atherogenesis (7). NO is generated in endothelial cells by the enzyme eNOS. Classical agonists for eNOS activation, such as acetylcholine, act via G-protein–coupled receptors to raise cytosolic Ca$^{2+}$ and activate eNOS. Recently, however, a complementary pathway for eNOS activation involving its phosphorylation has been described (10–13), which may be of particular relevance to the insulin-resistant state. Agonists that act through tyrosine kinase receptors, such as insulin and IGF-I, induce the activation of PE3K and PKB (Akt), leading to eNOS phosphorylation and enhanced production of NO. Interestingly, shear stress, which may be the major determinant of “basal” NO production in vivo, also activates eNOS at least in part through a similar pathway (28). These data suggest at least one mechanism through which abnormalities of insulin signaling or homeostasis may impact endothelial function.

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release were not assessed. The present report provides the important finding that in the presence of a global reduction of insulin receptors, mice have preserved glucose homeostasis but substantial disruption of basal- and insulin-mediated vascular NO release, secondary to impaired eNOS phosphorylation and activation rather than reduced expression. These results also suggest that insulin-mediated effects on eNOS transcription may not be as sensitive to impaired signaling as eNOS phosphorylation and activation.

Are hyperinsulinemia or hypertension per se responsible for the changes demonstrated in the present study? The IRKO mice studied in the present report had hyperinsulinemia in response to a glucose load. It has been suggested that hyperinsulinemia per se leads to endothelial dysfunction (31), although studies in subjects with diabetes treated with insulin do not support this hypothesis (32). Previous work from our laboratory in mice overexpressing IGF binding protein-1 in fact suggests that hyperinsulinemia in the presence of normal insulin signaling may have favorable effects on vasodilator function (19), as discussed above. These findings support a favorable effect of hyperinsulinemia when its signaling pathway is intact.

Hypertension has been shown to be associated with endothelial dysfunction (33) and insulin resistance (34). To explore the possibility that the increased blood pressure demonstrated in the IRKO mice resulted in impaired insulin-mediated NO release, we performed studies in normal mice rendered hypertensive by norepinephrine infusion. However, we found no change in insulin-mediated NO release in the norepinephrine-treated mice, suggesting that the vascular abnormalities found in IRKO mice were unlikely to be a result of increased blood pressure in these animals. The mechanisms responsible for the blood pressure rise in these animals remain to be established, although it is possible that the effect of insulin on vasopressor pathways, such as the sympathetic nervous system (35) or the endothelin system (36), unopposed by NO may at least in part contribute. This warrants further study.

Whether the vascular actions of insulin in experimental studies reflect a physiologic or a pharmacologic effect of insulin is a subject of ongoing debate (37,38). This controversy is not readily addressed by the results of this study, in which vascular function was assessed ex vivo, although the demonstration of increased blood pressure in IRKO mice suggests that the vascular actions of insulin in vivo are of physiological importance.

Clinical implications. Accelerated atherosclerosis is the principal cause of death in type 2 diabetes, and it is well appreciated that much of the risk may be related to the long period of compensated insulin resistance before the onset of overt hyperglycemia in these patients. Insulin receptor defects alone are a rare cause of insulin resistance in humans (39). However, defects in PI3K-mediated signaling are detectable in insulin-resistant subjects before the onset of diabetes (40); therefore, studying the influence of impaired insulin signaling on vascular function is important. Using IRKO mice allows the relationship between the metabolic and vascular effects of impaired insulin signaling to be investigated without the influence of hyperglycemia, obesity, or abnormal growth, which confound some models of insulin resistance (41). The findings of the present study indicate that despite adequate metabolic compensation there can be substantial disruption of pathways stimulating the release of the antiatherosclerotic molecule NO in states of mild insulin resistance. These findings support an aggressive and early approach to detecting abnormal vascular function in individuals at high risk for developing type 2 diabetes as well as in those who already have diabetes.

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