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).

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Received for publication 27 March 2004 and accepted in revised form 2 July 2004.

eNOS, endothelial NO synthase; L-NMMA, N_G-monomethyl-L-arginine; PE, phenylephrine; PI3K, phosphatidylinositol-3 kinase; PKB, protein kinase B.

TABLE 1
Characteristics of wild-type and IRKO mice

	Wild-type	IRKO	*P* value
Body weight (g)	25.9 ± 0.8	24.6 ± 0.8	0.31
Organ weights (% of body wt)			
Heart	0.49 ± 0.01	0.49 ± 0.01	0.67
Thoracic aorta	0.062 ± 0.008	0.059 ± 0.07	0.63
Pancreas	0.59 ± 0.01	0.59 ± 0.02	0.86
Liver	4.92 ± 0.22	4.87 ± 0.13	0.88
Spleen	0.29 ± 0.03	0.33 ± 0.01	0.29
Lungs	0.71 ± 0.05	0.63 ± 0.04	0.25
Kidney	0.62 ± 0.03	0.64 ± 0.01	0.68
Perigonadal fat (% of body wt)	1.66 ± 0.22	1.83 ± 0.11	0.54
Plasma triglycerides (mmol/l)	1.48 ± 0.2	1.44 ± 0.2	0.57
Plasma free fatty acids (mmol/l)	0.9 ± 0.1	1.2 ± 0.2	0.23
Systolic blood pressure (mmHg)	110 ± 3	124 ± 4	0.01

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. Intraperitoneal 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 immunoassay (CrystalChem, Downers Grove, IL) using rat insulin standards. Fasting plasma free fatty acids and triglycerides were measured by colorimetric assays (Roche, Mannheim, Germany and Thermo-Trace, 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, KH_2PO_4 1.18, $NaHCO_3$ 25, $MgSO_4$ 1.19, $CaCl_2$ 2.5, and glucose 11.0) gassed with 95%O_2/5%CO_2 (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 nmol/l to 10 μmol/l) was first assessed. After washing and re-equilibration, relaxation responses to acetylcholine (1 nmol/l to 10 μmol/l) or sodium nitroprusside (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. 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 N_G-monomethyl-L-arginine (L-NMMA) (0.1 mmol/l) for 30 min.

To assess the effect of insulin on NO production, we compared the cumulative dose response to PE (1 nmol/l to 10 μmol/l) before and after a 2-h incubation with insulin (100 mU/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 (0.1mmol/l) (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, 6, 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.

Aortic eNOS mRNA expression. Relative eNOS mRNA expression in aorta was measured by real-time RT-PCR. Total RNA was extracted (Mini Fibrous Kit; Quiagen), and equal amounts were reverse transcribed using Superscript II reverse transcriptase (Invitrogen) and random decamer oligonucleotides. PCR analysis was performed in duplicate using an ABI Prism 7000 sequence detection system, using primers and a FAM-labeled probe specific for murine eNOS (Assays-on-Demand; Applied Biosystems). β-Actin mRNA expression, assayed with a specific FAM-labeled probe, was used to control for mRNA loading. cDNA was amplified for 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. eNOS mRNA expression for each sample was calculated using the standard curve method (User Bulletin no. 2; Applied Biosystems) and adjusted for β-actin expression.

Aortic eNOS and phospho-eNOS protein expression. Fasted mice were anesthetized with sodium pentobarbitone before caval injection of insulin (Actrapid 5 units) or vehicle (0.9% NaCl) (26). After 5 min, the thoracic aorta was excised and snap frozen. Immunoblotting was performed on total aortic tissue homogenates extracted on ice with 0.5% Triton X-100, as previously described (27). Soluble protein concentration was determined using a Bio-Rad kit. Equal amounts of protein (25 μg/sample) were separated on 10% polyacrylamide gels and transferred to polyvinylidine fluoride membranes. Membranes were blocked with 5% nonfat milk/PBS/0.2% Tween 20 before probing with monoclonal antibodies against total murine eNOS (BD Biosciences), phospho-eNOS (Ser^{1177}; BD Biosciences), and smooth muscle α-actin (Sigma). Specific bands were detected by enhanced chemiluminescense (Amersham) and quantified by densitometry.

Statistics. All data are expressed as means ± SE. For isometric tension studies, concentration-response relationships were compared between groups by two-way repeated measures ANOVA. Other variables were compared using Student's *t* test. $P < 0.05$ was considered significant.

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. 1*A*). Glucocompetence, assessed by the response to an intraperitoneal glucose load, was also similar in IRKO and wild-type mice (Fig. 1*B*). 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. 1*C*). The hypoglycemic response to an intraperitoneal insulin load was not significantly different in IRKO and wild-type mice (Fig. 1*D*). Fasting free fatty

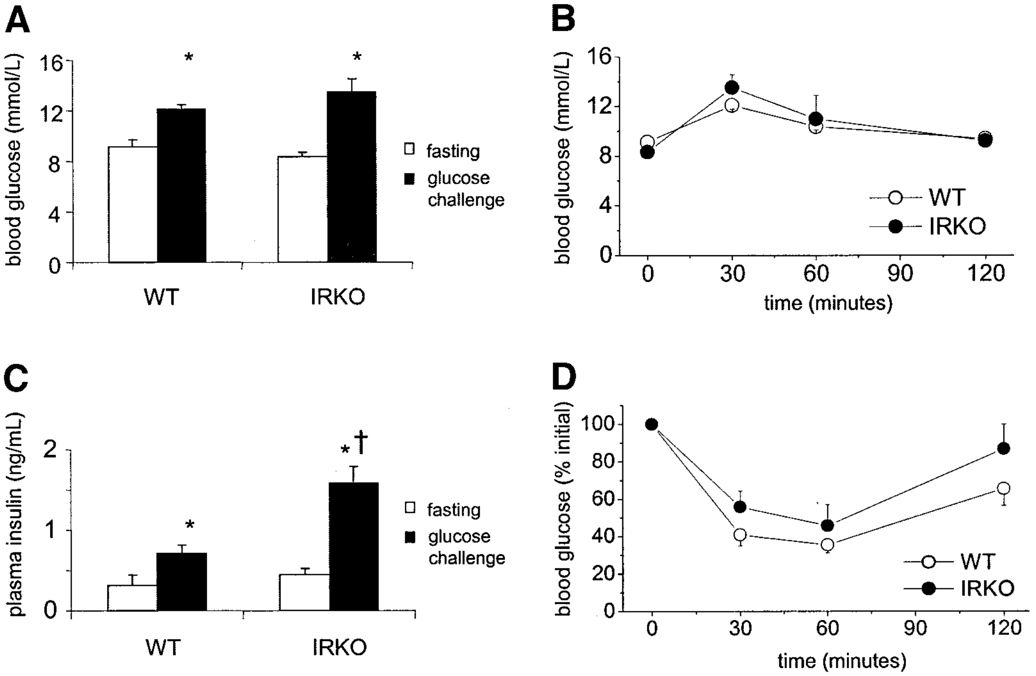

FIG. 1. Glucoregulation in wild-type (WT) and IRKO mice. *A*: Blood glucose was measured following an overnight fast and then 30 min after glucose challenge (1 mg/g i.p.). □, fasting; ■, glucose challenge. *B*: Glucocompetence was assessed by an intraperitoneal glucose tolerance test (1 mg/g i.p.). *C*: 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).

acid and triglyceride levels were also similar in the two groups (Table 1).

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. 3*A*). Likewise, endothelium-independent relaxation to sodium nitroprusside was no different between groups (Fig. 3*B*).

Preincubation of wild-type aortic rings with insulin significantly attenuated the contractile response to PE (Fig. 3*C*), with the maximal contractile response (E_{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. 3*D*). 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. 3*E*). Moreover, endothelial denudation also abolished this response in wild-type rings (Fig. 3*F*).

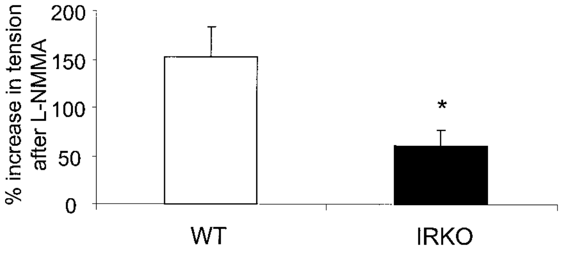

FIG. 2. Effect of NO synthase inhibition on aortic contractile response. Aortic rings were preconstricted with PE (10 μmol/l) before incubating with L-NMMA (0.1 mmol/l for 30 min) in order to assess the contribution of basal NO bioactivity to the contractile response. $*P < 0.01$ compared with wild-type ($n = 8$ per group).

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. 4*A* and 5). In the unstimulated state, eNOS phosphorylation assessed using an antibody specific for phosphorylation of Ser^{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. 4*B* 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. 6*A*), 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. 6*B* and *C*). In aortic ring studies, insulin

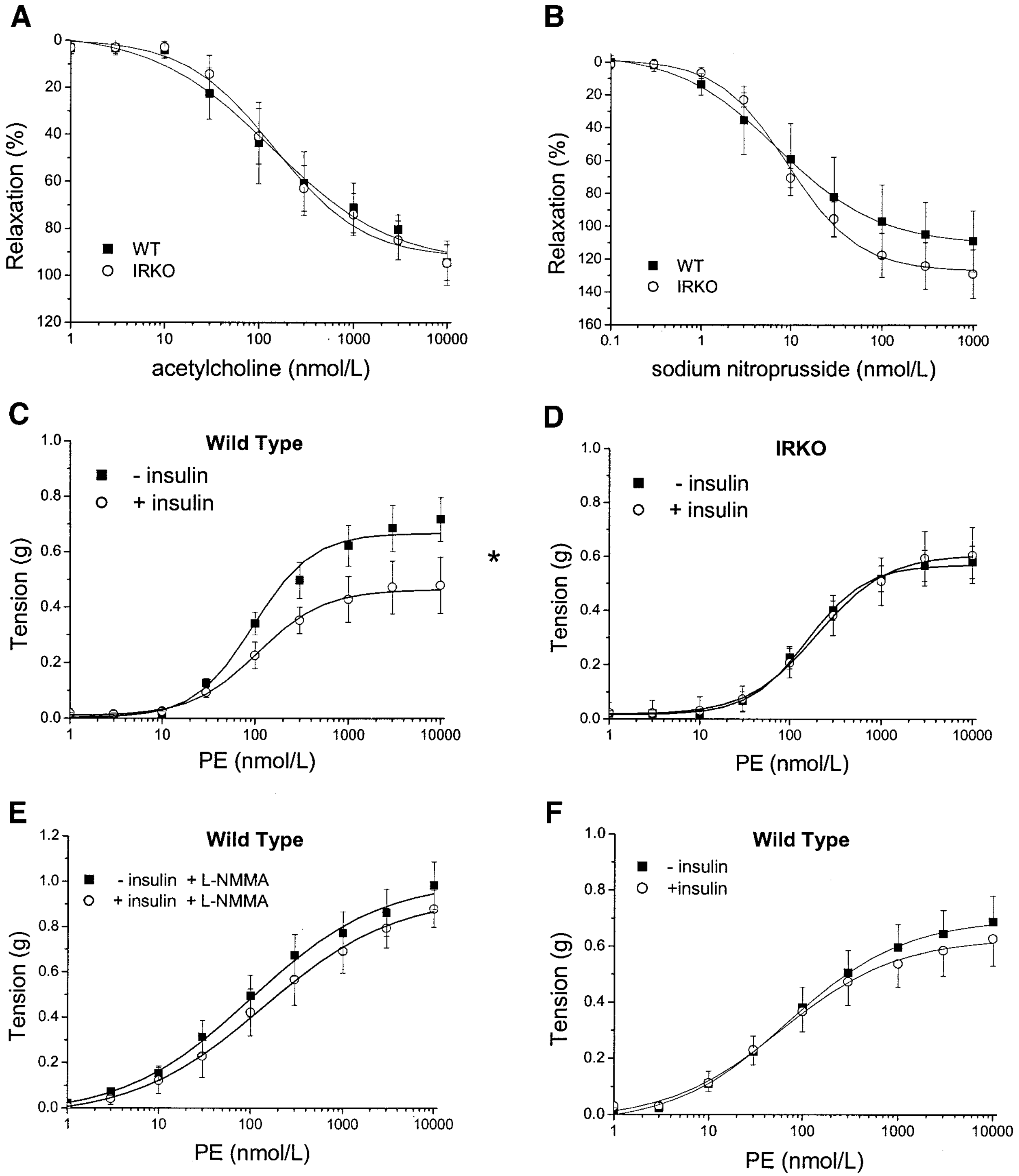

FIG. 3. Aortic vasomotor responses in wild-type (WT) and IRKO mice. Vascular relaxations to acetylcholine (*A*) and sodium nitroprusside (*B*) were determined in aortic rings from wild-type and IRKO mice. The effect of preincubation with insulin (100 mU/ml for 120 min) on the contractile response to 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).

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 [$P = 0.36$].)

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 glucocompetent, with hyperinsulinemia in response to a

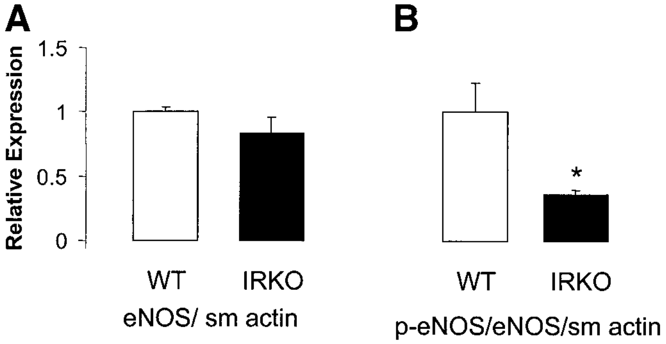

FIG. 4. Aortic expression of eNOS and insulin-stimulated eNOS phosphorylation. Expression of eNOS protein, normalized to expression of smooth muscle α-actin, was determined in aortic homogenates from wild-type (WT) and IRKO mice by Western blotting (*A*). Phosphorylation of eNOS at Ser^{1177} (normalized to expression of eNOS/smooth muscle [sm] α-actin) was assessed in aortic homogenates from wild-type and IRKO mice after stimulation with insulin (*B*). p-eNOS, phospho eNOS. **P* < 0.05 compared with wild-type aorta (*n* = 6 per group).

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 PI3K 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-dependent 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

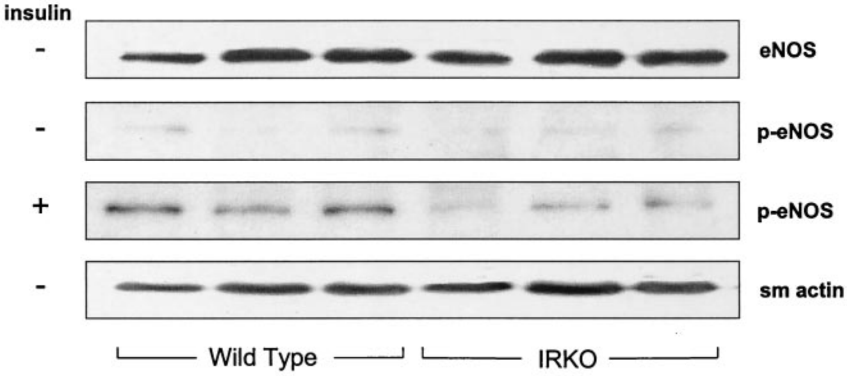

FIG. 5. Aortic expression of eNOS and phospho-eNOS (p-eNOS). Representative Western blot showing eNOS and Ser^{1177} p-eNOS) expression in aortas harvested from wild-type and IRKO mice after caval injection of vehicle or insulin. Expression of smooth muscle (sm) α-actin was determined as a loading control.

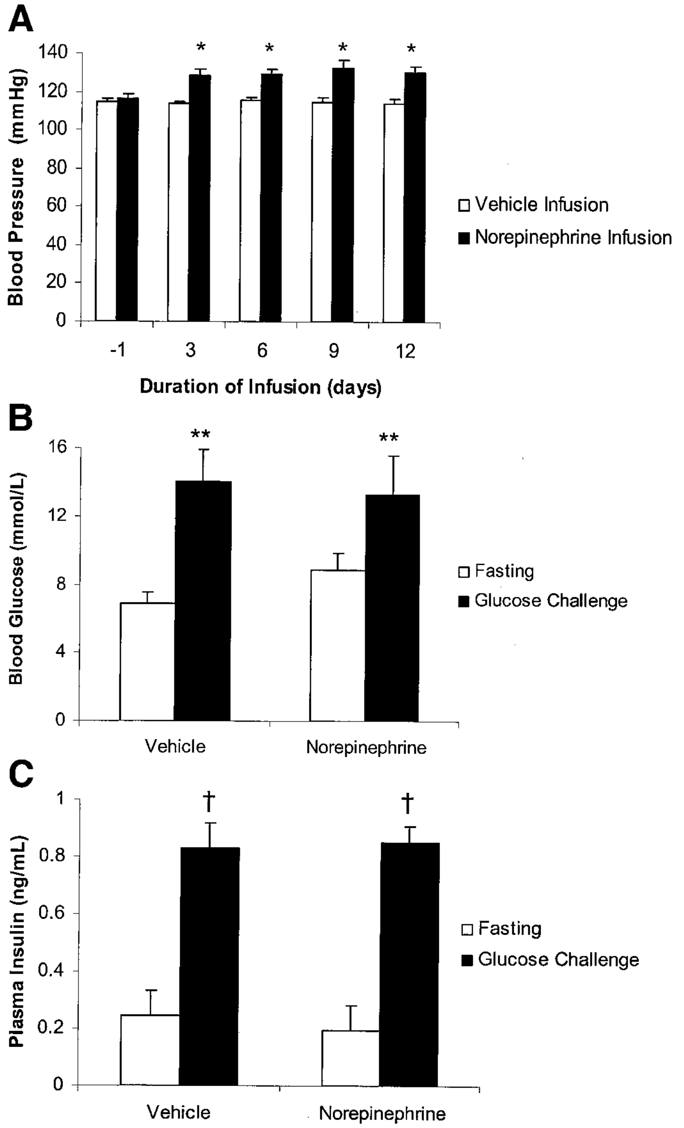

FIG. 6. Norephinephrine infusion in wild-type mice. Norephinephrine ($4.2 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$) or vehicle (0.9% saline) was infused subcutaneously by osmotic minipump in C57BL/6J mice. *A*: Systolic blood pressure was measured at intervals by tail cuff plethysmography. □, vehicle infusion; ■, norepinephrine infusion. Blood glucose (*B*) and plasma insulin (*C*) were assessed after 13 days in fasted animals and after a glucose challenge (1 mg/g i.p.). □, fasting; ■, glucose challenge. *$P < 0.01$ compared with vehicle-infused animals; **$P < 0.05$ compared with fasted animals; †$P < 0.02$ compared with fasted animals ($n = 6$ per group).

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.

ACKNOWLEDGMENTS

This work was funded by the British Heart Foundation (BHF). S.B.W. and E.D. are BHF Clinical PhD Fellows, M.T.K. a BHF Intermediate Fellow, and A.M.S. holds the

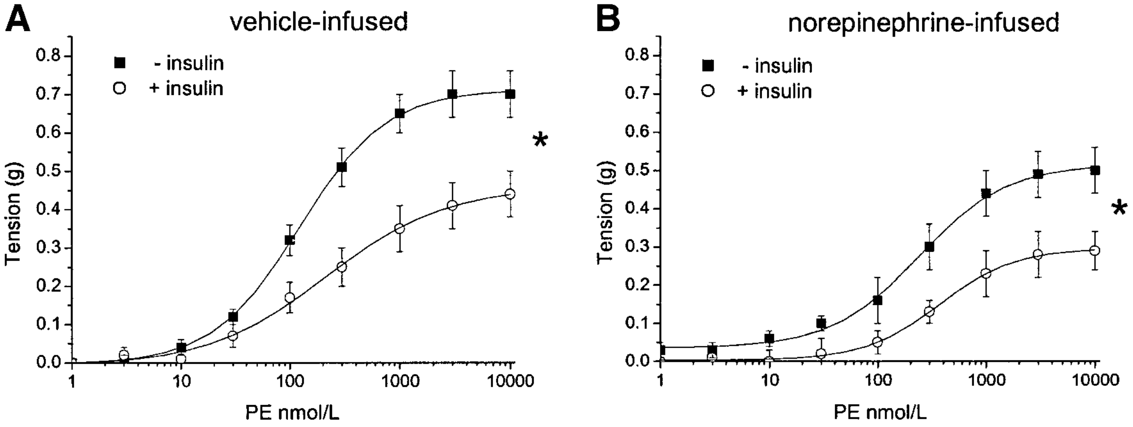

FIG. 7. Aortic vasomotor responses in norepinephrine-infused mice. The effects of insulin preincubation (100 mU/ml for 120 min) on the contractile response to PE were determined in aortic rings from wild-type mice receiving vehicle (*A*) or norepinephrine (*B*) by subcutaneous infusion for 14 days. ■, −insulin; ○, +insulin. *$P < 0.01$ by two-way ANOVA ($n = 6$ per group).

BHF Chair of Cardiology at King's College London. P.A.C. is funded by Diabetes U.K.

REFERENCES

1. DeFronzo RA: Lilly Lecture: the triumvirate: beta-cell, muscle, liver: a collusion responsible for NIDDM. *Diabetes* 37:667–687, 1988
2. Pyorala M, Miettinen H, Laakso M, Pyorala K: Hyperinsulinemia predicts coronary heart disease risk in healthy middle-aged men: the 22-year follow-up results of the Helsinki Policemen Study. *Circulation* 98:398–404, 1998
3. Hurst RT, Lee RW: Increased incidence of coronary atherosclerosis in type 2 diabetes mellitus: mechanisms and management. *Ann Intern Med* 139:824–834, 2003
4. Steinberg HO, Chaker H, Leaming R, Johnson A, Brechtel G, Baron AD: Obesity/insulin resistance is associated with endothelial dysfunction: implications for the syndrome of insulin resistance. *J Clin Invest* 97:2601–2610, 1996
5. Laakso M, Edelman SV, Brechtel G, Baron AD: Decreased effect of insulin to stimulate skeletal muscle blood flow in obese man: a novel mechanism for insulin resistance. *J Clin Invest* 85:1844–1852, 1990
6. Williams SB, Cusco JA, Roddy MA, Johnstone MT, Creager MA: Impaired nitric oxide-mediated vasodilation in patients with non-insulin-dependent diabetes mellitus. *J Am Coll Cardiol* 27:567–574, 1996
7. Ross R: Atherosclerosis: an inflammatory disease. *N Engl J Med* 340:115–126, 1999
8. Balletshofer BM, Rittig K, Enderle MD, Volk A, Maerker E, Jacob S, Matthaei S, Rett K, Haring HU: Endothelial dysfunction is detectable in young normotensive first-degree relatives of subjects with type 2 diabetes in association with insulin resistance. *Circulation* 101:1780–1784, 2000
9. Mather K, Laakso M, Edelman S, Hook G, Baron A: Evidence for physiological coupling of insulin-mediated glucose metabolism and limb blood flow. *Am J Physiol Endocrinol Metab* 279:E1264–E1270, 2000
10. Dimmeler S, Fleming I, Fisslthaler B, Hermann C, Busse R, Zeiher A: Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. *Nature* 399:601–605, 1999
11. Fulton D, Gratton JP, McCabe TJ, Fontana J, Fujio Y, Walsh K, Franke TF, Papapetropoulos A, Sessa WC: Regulation of endothelium-derived nitric oxide production by the protein kinase Akt. *Nature* 399:597–601, 1999
12. Zeng G, Nystrom FH, Ravichandran LV, Cong LN, Kirby M, Mostowski H, Quon MJ: Roles for insulin receptor, PI3-kinase, and Akt in insulin-signaling pathways related to production of nitric oxide in human vascular endothelial cells. *Circulation* 101:1539–1545, 2000
13. Vincent MA, Montagnani M, Quon MJ: Molecular and physiologic actions of insulin related to production of nitric oxide in vascular endothelium. *Curr Diab Rep* 3:279–288, 2003
14. Khan AH, Pessin JE: Insulin regulation of glucose uptake: a complex interplay of intracellular signalling pathways. *Diabetologia* 45:1475–1483, 2002
15. Accili D, Drago J, Lee EJ, Johnson MD, Cool MH, Salvatore P, Asico LD, Jose PA, Taylor SI, Westphal H: Early neonatal death in mice homozygous for a null allele of the insulin receptor gene. *Nat Genet* 12:106–109, 1996
16. Kitamura T, Kahn CR, Accili D: Insulin receptor knockout mice. *Annu Rev Physiol* 65:313–332, 2003
17. Kido Y, Burks DJ, Withers D, Bruning JC, Kahn CR, White MF, Accili D: Tissue-specific insulin resistance in mice with mutations in the insulin receptor, IRS-1, and IRS-2. *J Clin Invest.* 105:199–205, 2000
18. Crossey PA, Jones JS, Miell JP: Dysregulation of the insulin/IGF binding protein-1 axis in transgenic mice is associated with hyperinsulinemia and glucose intolerance. *Diabetes* 49:457–465, 2000
19. Wheatcroft SB, Kearney MT, Shah AM, Grieve DJ, Williams IL, Miell JP, Crossey PA: Vascular endothelial function and blood pressure homeostasis in mice overexpressing IGF binding protein-1. *Diabetes* 52:2075–2082, 2003
20. Bendall JK, Heymes C, Wright TJ, Wheatcroft S, Grieve DJ, Shah AM, Cave AC: Strain-dependent variation in vascular responses to nitric oxide in the isolated murine heart. *J Mol Cell Cardiol* 34:1325–1333, 2002
21. Li JM, Wheatcroft S, Fan LM, Kearney MT, Shah AM: Opposing roles of p47phox in basal versus angiotensin II-stimulated alterations in vascular O2- production, vascular tone, and mitogen-activated protein kinase activation. *Circulation* 109:1307–1313, 2004
22. Verma S, Yao L, Dumont AS, McNeill JH: Metformin treatment corrects vascular insulin resistance in hypertension. *J Hypertens* 18:1445–1450, 2000
23. Walker AB, Dores J, Buckingham RE, Savage MW, Williams G: Impaired insulin-induced attenuation of noradrenaline-mediated vasoconstriction in insulin-resistant obese Zucker rats. *Clinical Science* 93:235–241, 1997
24. Walker AB, Chattington PD, Buckingham RE, Williams G: The thiazolidinedione rosiglitazone (BRL-49653) lowers blood pressure and protects against impairment of endothelial function in Zucker fatty rats. *Diabetes* 48:1448–1453, 1999
25. Bendall JK, Cave AC, Heymes C, Gall N, Shah AM: Pivotal role of a gp91(phox)-containing NADPH oxidase in angiotensin II-induced cardiac hypertrophy in mice. *Circulation* 105:293–296, 2002
26. Mauvais-Jarvis F, Ueki K, Fruman DA, Hirshman MF, Sakamoto K, Goodyear LJ, Iannacone M, Accili D, Cantley LC, Kahn CR: Reduced expression of the murine p85alpha subunit of phosphoinositide 3-kinase improves insulin signaling and ameliorates diabetes. *J Clin Invest* 109:141–149, 2002
27. Li JM, Shah AM: Intracellular localization and preassembly of the NADPH oxidase complex in cultured endothelial cells. *J Biol Chem* 277:19952–19960, 2002
28. Boo YC, Jo H: Flow-dependent regulation of endothelial nitric oxide synthase: role of protein kinases. *Am J Physiol Cell Physiol* 285:C499–C508, 2003
29. Virkamaki A, Ueki K, Kahn CR: Protein-protein interaction in insulin signaling and the molecular mechanisms of insulin resistance. *J Clin Invest* 103:931–943, 1999
30. Vicent D, Ilany J, Kondo T, Naruse K, Fisher SJ, Kisanuki YY, Bursell S, Yanagisawa M, King GL, Kahn CR: The role of endothelial insulin signaling in the regulation of vascular tone and insulin resistance. *J Clin Invest* 111:1373–1380, 2003
31. Arcaro G, Cretti A, Balzano S, Lechi A, Muggeo M, Bonora E, Bonadonna RC: Insulin causes endothelial dysfunction in humans: sites and mechanisms. *Circulation* 105:576–582, 2002
32. Vehkavaara S, Makimattila S, Schlenzka A, Vakkilainen J, Westerbacka J,

Yki-Jarvinen H: Insulin therapy improves endothelial function in type 2 diabetes. *Arterioscler Thromb Vasc Biol* 20:545–550, 2000
33. Higashi Y, Sasaki S, Nakagawa K, Matsuura H, Oshima T, Chayama K: Endothelial function and oxidative stress in renovascular hypertension. *N Engl J Med* 346:1954–1962, 2002
34. Lind L, Reneland R, Andersson PE, Haenni A, Lithell H: Insulin resistance in essential hypertension is related to plasma renin activity. *J Hum Hypertens* 12:379–382, 1998
35. Anderson EA, Hoffman RP, Balon TW, Sinkey CA, Mark AL: Hyperinsulinemia produces both sympathetic neural activation and vasodilation in normal humans. *J Clin Invest* 87:2246–2252, 1991
36. Cardillo C, Nambi SS, Kilcoyne CM, Chocair WK, Katz A, Quon MJ, Panza JA: Insulin stimulates both endothelin and nitric oxide activity in the human forearm. *Circulation* 100:820–825, 1999
37. Yki-Jarvinen H, Utriainen T: Insulin-induced vasodilatation: physiology or pharmacology? *Diabetologia* 41:369–379, 1998
38. Steinberg HO, Baron AD: Insulin-mediated vasodilation: why one's physiology could be the other's pharmacology. *Diabetologia* 42:493–495, 1999
39. Taylor SI: Lilly Lecture: Molecular mechanisms of insulin resistance: lessons from patients with mutations in the insulin-receptor gene. *Diabetes* 41:1473–1490, 1992
40. Cusi K, Maezono K, Osman A, Pendergrass M, Patii ME, Pratipanawatr T, DeFronzo RA, Kahn CR, Mandarino LJ: Insulin resistance differentially affects the PI 3-kinase- and MAP kinase-mediated signaling in human muscle. *J Clin Invest* 105:311–320, 2000
41. Nandi A, Kitamura Y, Kahn CR, Accili D: Mouse models of insulin resistance. *Physiol Rev* 84:623–647, 2004