Insulin Resistance, Hyperlipidemia, and Hypertension in Mice Lacking Endothelial Nitric Oxide Synthase

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**Background**—Insulin resistance and arterial hypertension are related, but the underlying mechanism is unknown. Endothelial nitric oxide synthase (eNOS) is expressed in skeletal muscle, where it may govern metabolic processes, and in the vascular endothelium, where it regulates arterial pressure.

**Methods and Results**—To study the role of eNOS in the control of the metabolic action of insulin, we assessed insulin sensitivity in conscious mice with disruption of the gene encoding for eNOS. eNOS−/− mice were hypertensive and had fasting hyperinsulinemia, hyperlipidemia, and a 40% lower insulin-stimulated glucose uptake than control mice. Insulin resistance in eNOS+/+ mice was related specifically to impaired NO synthesis, because in equally hypertensive 1-kidney/1-clip mice (a model of renovascular hypertension), insulin-stimulated glucose uptake was normal.

**Conclusions**—These results indicate that eNOS is important for the control not only of arterial pressure but also of glucose and lipid homeostasis. A single gene defect, eNOS deficiency, may represent the link between metabolic and cardiovascular disease. (Circulation. 2001;104:342-345.)

**Key Words:** insulin ⋄ muscles ⋄ blood flow ⋄ glucose ⋄ hypertension, renal

**Glucose Clamp Studies**

Glucose turnover during the glucose clamp was measured in freely moving mice after a 5-hour fast.9,10 Three to 4 days before study, mice were anesthetized with halothane, and an indwelling catheter to be used for insulin and substrate infusion was inserted into the vena cava through the femoral vein, sealed under the back skin, and exteriorized and glued at the back of the neck.11,12 Homozygote eNOS−/− female mice generated as previously described were used.4 Hypertensive 1-kidney/1-clip female mice (C57/BL6) were generated as described before13 and studied 8 to 10 weeks after surgery. On the day of the clamp, after a 5-hour fast, 3-glucose-3 H (NEN Life Science, 30 μCi · kg−1 · min−1) and insulin (18 mU · kg−1 · min−1) were infused into the femoral vein for 3 hours. Throughout the infusion, blood samples (3.5 μL) were collected every 10 minutes from the tip of the tail vein for the determination of the blood glucose concentration, and euglycemia was maintained by periodic adjustment of a variable infusion of 33% glucose. During the last hour of infusion, additional blood samples were collected at 20-minute intervals for the measurement of plasma 3-glucose-3 H enrichment. The glucose infusion rate was calculated as the mean of the values obtained every 10 minutes. The glucose turnover rate was determined isotonically and calculated by dividing the 3-glucose-3 H infusion rate by the plasma glucose specific activity. Mice showing variations of these 2 parameters >15% during the last hour of the 3-hour infusion were not included in the calculations. Endogenous glucose production was calculated by subtracting the glucose infusion rate from the glucose turnover rate. Whole-body glucose clearance was calculated by dividing the mean whole-body glucose turnover rate by the mean steady-state plasma glucose concentration.
At time 180 minutes, a blood sample was obtained for determination of the insulin plasma concentration (ELISA kit, Linco), and the mice were euthanized. Nine eNOS+/−, 7 eNOS−/−, and 7 1-kidney/1-clip mice were studied.

Muscle Blood Flow
Muscle blood flow was measured in anesthetized mice (4% to 5% halothane inhalation for the induction, followed by 1% to 1.5% for the maintenance of anesthesia) with a laser Doppler probe (Perimed, Probe 403) inserted directly into the hindlimb skeletal musculature. Six eNOS+/−, 6 eNOS−/−, and 4 1-kidney/1-clip mice were studied. During the entire study, the probe was kept stabilized with a micromanipulator. The blood flow signal was recorded on a personal computer with a specific data acquisition software (Perisoft for Windows 1.13). A 90-minute euglycemic glucose clamp was performed as described above.

Measurement of Glucose Utilization in Isolated Muscle
After cervical dislocation, the soleus muscles were rapidly isolated, tied separately by silk threads to the tendons, and immersed for 15 minutes into an incubation medium (Kreb’s-Ringer bicarbonate [pH 7.3] supplemented with 1% BSA [fraction V, pH 7.0] and 2 mmol/L sodium pyruvate). Under an atmosphere containing 5% CO2 and 95% O2, the muscles were then incubated in the medium with or without a scintillation buffer. Sample aliquots were used for 30 minutes, and then dissolved in NaOH 1 mol/L at 55°C for 60 minutes. An aliquot of the extract was neutralized with HCl 1 mol/L and spun down, and the 3H-labeled radioactivity was counted in the presence of a scintillation buffer. Sample aliquots were used for protein determination. Soleus muscles of 7 eNOS−/− and control mice were studied.

Blood Chemical Analysis
Blood glucose (Trinder kit, Sigma) and plasma insulin concentrations were measured between 1 and 3 PM after a 6-hour fast in conscious mice (n=6 for each group) housed with an inverted dark-light cycle. No2 was measured in plasma samples obtained by cardiac punctation by chemiluminescence with an NO analyzer (Sievers 280 NOA) after reduction of NO to NO with VCl3. Data were obtained from ≥6 animals in each group. Total cholesterol, triglyceride, and free fatty acids were measured after an 8-hour fast in ≥9 mice of each group by colorimetric enzymatic determination (Unimate 5 CHOL and 5 TRIG, Roche; NEFA-C, Wako).

Measurement of Arterial Blood Pressure
Arterial pressure was measured in awake, partially restricted, 10- to 14-week-old mice (n=6 for each group) with a fluid-filled PE-10 tubing connected to a pressure transducer. The catheter had been inserted into the carotid artery 3 to 5 hours before the measurement under halothane anesthesia and tunneled subcutaneously to exit at the back of the neck.

Statistical Analysis
Data were analyzed with the JMP software package (SAS Institute Inc). Statistical analysis was done with ANOVA for between-group comparisons and with the 2-tailed t test for single comparisons. Relations between variables were analyzed by calculating Pearson’s product-moment correlation coefficient. All data are presented as mean±SEM. A value of P<0.05 was considered to indicate statistical significance.

Results
Body weight was comparable in eNOS−/− (20.3±1.2 g) and control (19.7±1.1 g) mice. During the clamp studies, the glucose infusion rate (64.0±4.5 versus 107.0±3.0 mg · kg−1 · min−1), the glucose turnover rate (57.8±4.5 versus 102.6±4.2 mg · kg−1 · min−1), and the glucose clearance rate (0.69±0.08 versus 1.00±0.07 dL · min−1 · kg−1) were 30% to 40% lower in eNOS−/− than in wild-type mice (P<0.0001; Figure 1a). The fasting plasma insulin concentration was elevated almost 2-fold in eNOS−/− mice (Figure 1b). During the clamp studies, the glucose (5.9±0.2 versus 5.7±0.2 mmol/L) and insulin (742±104 and 767±120 μU/mL) concentrations were comparable in eNOS−/− and wild-type mice, and hepatic glucose production was completely suppressed in both strains (data not shown).

To determine the impact of eNOS deficiency on vascular NO production, we measured the plasma concentration of...
nitrite and nitrate (NO$\textsubscript{3}$). It was $\approx 60\%$ lower in the knockout than in the wild-type mice ($24.4 \pm 5.5$ versus $58.6 \pm 5.9$ $\mu$mol/L, $P < 0.05$, Figure 1c). This defect of vascular NO production in eNOS$^{-/-}$ mice was associated with arterial hypertension. Baseline mean arterial blood pressure was 106$ \pm $1 mm Hg in wild-type mice and 142$ \pm $3 mm Hg in eNOS$^{-/-}$ mice ($P < 0.001$, Figure 1d). To examine whether insulin resistance was related to hypertension, we measured insulin-stimulated glucose uptake in 1-kidney/1-clip mice, a mouse model of renovascular hypertension. One-kidney/1-clip mice were equally hypertensive as eNOS$^{-/-}$ mice (mean arterial pressure 156$ \pm $8 mm Hg, Figure 1d) and had an almost 2 times larger NO$\textsubscript{3}$ plasma concentration ($39.4 \pm 4.2$ $\mu$mol/L, $P < 0.05$ versus knockout) than the knockout mice and had normal insulin-stimulated glucose uptake (Figure 1a) and glucose clearance ($1.01 \pm 0.08$ $\text{dL} \cdot \text{min}^{-1} \cdot \text{kg}^{-1}$). In humans, insulin resistance is often associated with dyslipidemia. To study the effects of insulin resistance on lipid metabolism in mice, we measured total cholesterol, triglycerides, and free fatty acids. Insulin-resistant eNOS$^{-/-}$ mice had 50$\%$ higher plasma levels of cholesterol ($1.81 \pm 0.05$ versus $1.22 \pm 0.06$ $\mu$mol/L, $P < 0.01$) and a 2-fold elevation of triglyceride ($0.78 \pm 0.12$ versus $0.35 \pm 0.02$ $\mu$mol/L, $P < 0.01$) and free fatty acid ($1.89 \pm 0.14$ versus $0.85 \pm 0.11$ $\mu$mol/L, $P < 0.01$) plasma concentrations (Figure 1e).

To test whether eNOS deficiency alters the insulin stimulation of skeletal muscle perfusion, we measured hindlimb muscle blood flow during clamp studies. Throughout the clamp, the increase in muscle blood flow was smaller in eNOS$^{-/-}$ than in wild-type mice ($P < 0.01$, Figure 2a). In contrast, hindlimb muscle blood flow increased normally in the equally hypertensive 1-kidney/1-clip mice (Figure 2a). In addition, we found a direct relationship between the insulin stimulation of muscle blood flow and muscle glucose uptake during the clamp studies ($r=0.87$, $P<0.0001$, Figure 2b).

To study the effects of eNOS deficiency on glucose uptake in the absence of confounding effects of muscle perfusion, we measured glucose uptake in isolated skeletal muscle preparations. The basal and the insulin-stimulated glucose transport were $\approx 40\%$ lower in eNOS$^{-/-}$ than in wild-type mice ($P<0.01$, Figure 2c).

**Discussion**

Studies using pharmacological inhibition of NO$\textsubscript{3}$ to examine the role of NO in the regulation of the metabolic action of insulin have provided conflicting results. We therefore used a transgenic animal model to study the interaction between eNOS and insulin in the regulation of glucose metabolism. We found that eNOS$^{-/-}$ mice were insulin resistant, as evidenced by fasting hyperinsulinemia and glucose infusion rates during euglycemic clamp studies that were roughly 40$\%$ lower than in wild-type mice. These findings indicate that eNOS plays a major role in the regulation of insulin sensitivity.

During the clamp studies, insulin concentration was comparable in both groups, and hepatic glucose production was completely suppressed in both strains, indicating that the lower glucose infusion rate in eNOS$^{-/-}$ mice is accounted for by decreased glucose uptake in peripheral tissues. The NO, plasma concentration was $\approx 60\%$ lower in the knockout than in the wild-type mice, and as expected, eNOS$^{-/-}$ mice had elevated arterial blood pressure. To examine whether insulin resistance was related to hypertension, we measured insulin-stimulated glucose uptake in a mouse model of renovascular hypertension. One-kidney/1-clip mice were equally hypertensive as eNOS$^{-/-}$ mice, but they had normal insulin-stimulated glucose uptake. These findings indicate that in eNOS$^{-/-}$ mice, metabolic insulin resistance is not related to hypertension but rather to impaired NO synthesis.

Insulin resistance in eNOS$^{-/-}$ mice could be related to a vascular and/or cellular defect. In cultured vascular endothelial cells, insulin activates l-arginine transport and NO$\textsubscript{3}$ and stimulates NO release by a wortmannin-dependent mechanism. In humans, insulin-induced vasodilation is mediated by stimulation of NO release. It has been suggested that insulin stimulation of muscle blood flow promotes substrate delivery to skeletal muscle tissue and thereby may regulate insulin sensitivity. Here, we show that insulin stimulation of muscle blood flow was $\approx 40\%$ smaller in eNOS$^{-/-}$ than in wild-type or 1-kidney/1-clip mice. Moreover, insulin stimulation of muscle blood flow and stimulation of muscle glucose uptake were strongly related. Alternatively, NO may have effects on glucose uptake that are independent of its vascular action. eNOS is expressed in skeletal muscle tissue, where NO regulates metabolic and contractile processes. In

![Figure 2](http://circ.ahajournals.org/)

**Figure 2.** a, Hindlimb muscle blood flow during a 90-minute euglycemic hyperinsulinemic clamp. Data are mean$\pm$SEM for 6 eNOS$^{-/-}$ (○), 6 control (■), and 4 hypertensive 1-kidney/1-clip (1K/1C) (×) mice. P<0.01, eNOS$^{-/-}$ vs both control and 1K/1C mice. b, Relationship between insulin-induced increase in muscle blood flow and glucose infusion rate at end of clamp in eNOS knockout, control, and hypertensive 1K/1C mice. During clamp, increase in muscle blood flow was directly correlated with glucose infusion rate ($r=0.87$, $P<0.0001$). c, Basal and insulin-stimulated 2-deoxyglucose uptake in soleus muscle of knockout and control mice. Data are mean$\pm$SEM for 7 mice in each group. P<0.01, eNOS$^{-/-}$ vs control mice; §P<0.001 for comparison with corresponding baseline.

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rat skeletal muscle preparations in the short term, pharmacological NOS inhibition decreased basal glucose transport in some but not all studies, whereas it did not appear to alter insulin-stimulated glucose uptake. In the present studies, both the basal and the insulin-stimulated glucose transport were ~40% lower in isolated skeletal muscle preparations of genetically eNOS-deficient mice than in wild-type mice. Taken together, these findings suggest that defects in insulin stimulation of muscle perfusion and insulin signaling in the skeletal muscle cell contribute to metabolic insulin resistance in eNOS−/− mice.

In humans, insulin resistance is often associated with dyslipidemia. Here, we found that insulin-resistant eNOS−/− mice had elevated fasting plasma levels of cholesterol, triglycerides, and free fatty acids. Increased triglyceride and free fatty acid levels could be secondary to insulin resistance, as has been shown in other animal models. Alternatively, eNOS deficiency may directly alter lipid metabolism. Finally, it is possible that in eNOS−/− mice, substrate competition between free fatty acids and glucose may contribute to insulin resistance.

Essential hypertension in humans is associated with metabolic insulin resistance and dyslipidemia. Persistence of the metabolic defects after normalization of the blood pressure by pharmacological agents and their absence in secondary forms of human hypertension suggest a common cause for the metabolic and hemodynamic anomalies. Essential hypertension is characterized by a defect of endothelial NO synthesis, and it is associated with an eNOS gene polymorphism. Here, we show that eNOS deficiency results in hypertension, metabolic insulin resistance, and hyperlipidemia in mice. A defect of NO synthesis may therefore represent a candidate mechanism linking metabolic and cardiovascular disease in humans.

**Note Added in Proof**

Consistent with the present findings, during the review process of the manuscript, Shankar et al showed, in euglycemic hyperinsulinemic clamp studies, decreased glucose infusion and glucose turnover rates in eNOS−/− mice.

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**References**

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