Partial Gene Deletion of Endothelial Nitric Oxide Synthase Predisposes to Exaggerated High-Fat Diet–Induced Insulin Resistance and Arterial Hypertension

Stéphane Cook,1 Olivier Hugli,1 Marc Egli,1 Barbara Ménard,1 Sébastien Thalmann,1 Claudio Sartori,1 Christophe Perrin,2 Pascal Nicod,1 Bernard Thorens,3 Peter Vollenweider,1 Urs Scherrer,1 and Rémy Burcelin2,3

Nitric oxide (NO) plays a major role in the regulation of cardiovascular and metabolic homeostasis, as evidenced by insulin resistance and arterial hypertension in endothelial NO synthase (eNOS) null mice. Extrapolation of these findings to humans is difficult, however, because eNOS gene deficiency has not been reported. eNOS gene polymorphism and impaired NO synthesis, however, have been reported in several cardiovascular disease states and could predispose to insulin resistance. High-fat diet induces insulin resistance and arterial hypertension in normal mice. To test whether partial eNOS deficiency facilitates the development of insulin resistance and arterial hypertension during metabolic stress, we examined effects of an 8-week high-fat diet on insulin sensitivity (euglycemic clamp) and arterial pressure in eNOS+/- mice. When fed a normal diet, these mice had normal insulin sensitivity and were normotensive. When fed a high-fat diet, however, eNOS+/- mice developed exaggerated arterial hypertension and had fasting hyperinsulinemia and a 35% lower insulin-stimulated glucose utilization than control mice. The partial deletion of the eNOS gene does not alter insulin sensitivity or blood pressure in mice. When challenged with nutritional stress, however, partial eNOS deficiency facilitates the development of insulin resistance and arterial hypertension, providing further evidence for the importance of this gene in linking metabolic and cardiovascular disease. Diabetes 53:2067–2072, 2004

Metabolic insulin resistance is a problem of utmost clinical importance and a major risk factor for cardiovascular morbidity and mortality. Epidemiological studies indicate that insulin resistance and arterial hypertension are related (1,2), suggesting the possibility of a common underlying mechanism. Endothelial nitric oxide (NO) synthase (eNOS)-dependent NO synthesis by the vascular endothelium regulates arterial pressure (3,4) and is defective in human essential hypertension (5). Endothelium-derived NO also mediates insulin-induced stimulation of the perfusion of skeletal muscle (6), its main metabolic target tissue. In insulin-resistant individuals, insulin stimulation of endothelial NO synthesis is impaired and may contribute to defective skeletal muscle glucose uptake (7). In line with this hypothesis, NOS inhibitors reduce insulin-stimulated muscle glucose uptake in rats in vivo (8). Moreover, eNOS is expressed in the skeletal muscle (9), and NO donors stimulate glucose transport in isolated rat muscle preparations in vitro (10–12). Consistent with the concept of an important role of NO in the regulation of insulin sensitivity and arterial pressure, eNOS null mice are insulin resistant (13–15) and hypertensive (3,14,15). Extrapolation of these findings in mice to humans is problematic, because eNOS gene deficiency has not been reported in humans so far. There is evidence, however, that cardiovascular disease states such as hypertension, coronary artery disease, and myocardial infarction are associated with eNOS gene polymorphism (16–21) and impaired NO synthesis (19,20). The impaired NO synthesis, which under some conditions is directly related to the polymorphism (22–23), could predispose to insulin resistance (24,25). We hypothesized that partial eNOS deficiency in mice, when challenged with a metabolic stress, may predispose to insulin resistance and hypertension. To test this hypothesis, we assessed insulin sensitivity and arterial pressure in eNOS+/- and wild-type mice that were fed a normal or a high-fat diet for 8 weeks.

RESEARCH DESIGN AND METHODS

Experiments were carried out under protocols approved by the Institutional Animal Care and Use Committee. eNOS-/- C57BL6 mice, as previously described, were used (14,15). Female heterozygous (eNOS+/-) and control
mice (eNOS+/−) were generated by mating heterozygous animals from our colony. Mice of generations 9–12 were used for our studies. Starting at the age of 6 weeks, the mice were fed a normal diet (NC; UAR, Epinay sur Orge, France; energy content: 12% fat, 28% protein, and 60% carbohydrate, low nitrates). For the high-fat diet (HFD; UAR; energy content: 72% fat [corn oil and lard], 28% protein, and <1% carbohydrate, low nitrates) (26).

Glucose clamp studies. The glucose turnover rate was assayed after 8 weeks of dietary treatment during a euglycemic-hyperinsulinemic clamp in freely moving mice for each group (29 mice per group). Mice of generations 9–12 were used for our studies. Starting at the age of 6 weeks, the mice were fed a normal diet (NC; UAR, Epinay sur Orge, France; energy content: 12% fat, 28% protein, and 60% carbohydrate, low nitrates). For the high-fat diet (HFD; UAR; energy content: 72% fat [corn oil and lard], 28% protein, and <1% carbohydrate, low nitrates) (26).

Throughout the study period, the mice were housed with light on from 7:00 A.M. to 7:00 P.M. with food and water ad libitum.

RESULTS

Body weight was comparable in eNOS+/− and wild-type mice throughout the study period (data not shown). eNOS expression in skeletal muscle tissue was roughly 50% lower in heterozygous than in control mice and remained unchanged during HFD.

Effects of HFD on insulin sensitivity. In mice that were fed NC, the glucose infusion (96.0 ± 6.4 vs. 109.9 ± 5.9 mg · kg−1 · min−1) and glucose clearance rates (0.55 ± 0.06 dl · kg−1 · min−1) were comparable in eNOS+/− and wild-type mice (Fig. 1A and B, Table 1). In contrast, in mice that were fed HFD, the glucose infusion (54.4 ± 2.6 vs. 82.2 ± 3.1 mg · kg−1 · min−1) and glucose clearance rates (0.55 ± 0.03 vs. 0.82 ± 0.03 dl · kg−1 · min−1) were roughly 35% lower in eNOS+/− than in wild-type mice (P < 0.001; Fig. 1A and B). In eNOS+/− mice that were fed HFD, insulin resistance was also more marked during clamps using a physiological insulin infusion rate (glucose infusion rate, 29.1 ± 1.3 vs. 43.6 ± 6.8 mg · kg−1 · min−1 in eNOS+/− and control mice, respectively). During the high-dose insulin clamps, hepatic glucose production was completely suppressed in all groups. During the low-dose insulin clamps, hepatic glucose production in HFD-fed mice was incompletely but equally suppressed in both groups (5.8 ± 1.1 and 4.7 ± 2.5 mg · kg−1 · min−1 in eNOS+/− and control mice, respectively). During the clamp studies, the plasma glucose concentration was comparable in all conditions. In NC-fed mice, the fasting plasma insulin concentration was comparable in both strains (12.9 ± 1.6 and 13.0 ± 1.2 μU/ml), whereas during HFD, it was almost 2.5-fold higher in eNOS+/− than in wild-type mice (P < 0.05; Fig. 1C). The fasting blood glucose concentration was not altered by the HFD (Fig. 1C).

Effects of HFD on vascular NO synthesis, arterial pressure, and heart rate. In humans, fat administration may induce endothelial dysfunction (28). To determine the impact of an HFD on vascular NO production in mice, we measured the plasma concentration of NOx. It was ~60% lower in HFD than in NC-fed mice (P < 0.05; Fig. 1D). This defect of diet-induced vascular NO production was associated with the development of arterial hypertension (Fig. 1E). After 8 weeks of HFD, mean arterial pressure had increased from 97 ± 6 to 130 ± 4 mmHg (P < 0.01) in eNOS+/− and from 99 ± 2 to 117 ± 1 mmHg (P < 0.001) in
wild-type mice. The diet-induced increase in arterial pressure was significantly larger in eNOS−/− than in wild-type mice (P < 0.05). Similarly, during HFD, heart rate was significantly faster in eNOS−/− mice than in control mice (516 ± 31 vs. 395 ± 29 bpm; P = 0.008), whereas during NC, it was comparable in both groups (469 ± 25 vs. 439 ± 41 bpm; P > 0.1). Free fatty acid plasma concentration did not differ during HFD between wild-type and heterozygous mice (1.37 ± 0.21 vs. 1.08 ± 0.34 μmol/l; P > 0.1).

Effects of HFD on insulin stimulation of skeletal muscle blood flow in vivo and muscle glucose utilization in vitro. In eNOS knockout mice, insulin resistance is associated with impaired insulin stimulation of skeletal muscle perfusion (14). To study the effect of HFD on insulin-stimulated muscle perfusion, we measured hind-limb muscle blood flow during clamp studies. During NC, muscle blood flow had increased by ~45% by the end of the clamp in both strains of mice. HFD almost completely abolished the insulin-induced stimulation of muscle blood flow in eNOS−/− and wild-type mice (Fig. 2A). In skeletal muscle, insulin resistance could be constitutive or induced by external factors. To address this point, we studied
isolated muscle glucose utilization. We found that basal and insulin-stimulated glucose transport were comparable in soleus muscles from heterozygous and control mice that were fed NC or HFD (Fig. 2B).

**DISCUSSION**

Recently, we found that eNOS null mice are insulin resistant and hypertensive (13–15). As complete gene deficiencies are seldom found in human disease, we examined whether a partial deletion of eNOS also affects insulin sensitivity and blood pressure homeostasis. We found that when fed an NC, eNOS+/− mice had normal insulin sensitivity and were normotensive. When fed HFD for 8 weeks, however, eNOS+/− mice developed exaggerated insulin resistance at both physiological and maximal insulin-stimulated rates, as evidenced by fasting hyperinsulinemia and glucose infusion rates during euglycemic clamp studies that were ~40% lower than in wild-type mice. Moreover, HFD caused an exaggerated increase of the arterial pressure in eNOS+/− mice. These findings indicate that one eNOS gene provides sufficient insulin sensitivity and arterial blood pressure under usual conditions. During a metabolic stress (HFD), however, eNOS deficiency amplified a pathological mechanism observed under normal conditions and led to exaggerated insulin resistance and arterial hypertension.

In mice that were fed HFD, hepatic glucose production was equally suppressed in both groups, indicating that the lower glucose infusion rate in eNOS+/− mice was mostly accounted for by decreased glucose uptake in peripheral tissues. In eNOS null mice, insulin resistance is associated with impaired stimulation of muscle blood flow (14). In eNOS+/− mice that were fed NC, insulin stimulation of muscle blood flow was comparable to that of wild-type mice, indicating that one allele is sufficient to maintain a normal response to this stimulus. During the HFD, insulin stimulation of muscle blood flow was markedly impaired in both groups, possibly related to impaired vascular NO synthesis (as reflected by the ~60% decrease of NOx plasma concentration in both groups). These findings suggest that impaired insulin stimulation of muscle blood flow and, in turn, substrate delivery contributed to HFD-induced insulin resistance in wild-type and eNOS+/− mice.

The observation that during HFD, for a comparable impairment of insulin stimulation of muscle blood flow, insulin resistance was more marked in eNOS+/− than in wild-type mice, suggests that additional mechanisms contributed to metabolic insulin resistance in eNOS+/− mice. eNOS is expressed in skeletal muscle tissue (9), where NO regulates metabolic and contractile processes (11). In isolated skeletal muscle preparations of eNOS null mice, the basal and insulin-stimulated glucose transport is impaired (14). Here we found that, consistent with normal glucose uptake in vivo, glucose uptake in vitro in response to insulin was also normal in eNOS+/− mice, although soleus muscle eNOS expression was ~50% lower than in control mice. Moreover and consistent with previous results in normal mice, the low-carbohydrate HFD did not alter basal and insulin-mediated glucose uptake in vitro in

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### TABLE 1

**Fasted parameters in control and mutant mice**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Diet</th>
<th>Fasting plasma glucose (mmol/l)</th>
<th>Fasting plasma insulin (μU/ml)</th>
<th>Glucose infusion rate (mg/kg · min⁻¹)</th>
<th>Glucose clearance rate (dl/min · kg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>eNOS+/+</td>
<td>NC</td>
<td>5.4 ± 0.1</td>
<td>12.9 ± 1.6</td>
<td>109.9 ± 5.9</td>
<td>1.08 ± 0.06</td>
</tr>
<tr>
<td>eNOS+/+</td>
<td>HFD</td>
<td>5.3 ± 0.1</td>
<td>12.8 ± 3.1</td>
<td>82.2 ± 3.1</td>
<td>0.82 ± 0.03</td>
</tr>
<tr>
<td>eNOS+/−</td>
<td>NC</td>
<td>5.3 ± 0.1</td>
<td>13.0 ± 1.2</td>
<td>96.0 ± 6.4</td>
<td>0.97 ± 0.10</td>
</tr>
<tr>
<td>eNOS+/−</td>
<td>HFD</td>
<td>5.3 ± 0.2</td>
<td>30.6 ± 3.4*</td>
<td>54.4 ± 2.6†</td>
<td>0.55 ± 0.03†</td>
</tr>
</tbody>
</table>

Data are mean ± SE. *P < 0.05, eNOS+/− HFD vs. eNOS+/+ HFD; †P < 0.01, eNOS+/− HFD vs. eNOS+/+ HFD.

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**FIG. 2.** A: Hindlimb muscle blood flow during 90-min euglycemic-hyperinsulinemic clamp studies in eNOS+/− (n = 6) and wild-type mice (n = 8) fed NC (open symbols, n = 6–8 for each group) or HFD (filled symbols, n = 5 for each group). Data are mean ± SE. P < 0.05, NC vs. HFD, for both strains. B: Basal and insulin-stimulated 2-deoxyglucose uptake in soleus muscle of eNOS+/− (NC, n = 7; HFD, n = 8) and control mice (NC, n = 7; HFD, n = 8). Data are mean ± SE.
control and eNOS\(^+/-\) mice (29). These findings suggest that differences in eNOS expression in skeletal muscle tissue did not contribute importantly to exaggerated metabolic insulin resistance in HFD-fed eNOS\(^+/-\) mice in vivo.

Exaggerated insulin resistance in HFD-fed eNOS\(^+/-\) mice does also not seem to be related to differences in free fatty acid levels, which were found to be comparable in the two groups. Finally, despite marked insulin resistance at the end of the 8-week HFD, the fasting plasma glucose concentration remained normal in eNOS\(^+/-\) mice, suggesting that the compensatory hyperinsulinemia was sufficient to maintain glucose concentration within normal limits.

In addition to metabolic insulin resistance, HFD induced arterial hypertension. Our findings suggest that this was related, at least in part, to impaired vascular NO synthesis. The observation that for comparable values of NOx plasma concentration the arterial hypertension was more pronounced in the eNOS\(^+/-\) mice suggests that additional mechanisms may play a role. Insulin stimulates sympathetic nervous activity in rodents and humans (24,25). Thus, in eNOS\(^+/-\) mice, basal hyperinsulinemia-induced sympathetic overactivity could contribute to exaggerated arterial hypertension during HFD. Consistent with this hypothesis, heart rate was faster in eNOS\(^+/-\) than in control mice during HFD. Parenthetically, sympathetic overactivity could also represent one of the factors facilitating insulin resistance in eNOS\(^+/-\) mice (30).

Recently, vascular endothelial insulin receptor knock-out mice have been generated and were found to have normal insulin sensitivity blood pressure under normal conditions but showed insulin resistance and altered blood pressure control when challenged with changes in dietary salt intake (31). Taken together with the present data, these findings suggest that under normal conditions, either a specific loss of insulin stimulation of vascular endothelial NO release (VENIRKO mice) or a generalized partial defect of eNOS-driven NO synthesis (eNOS\(^+/-\) mice) is largely inconsequential with regard to blood pressure homeostasis and insulin sensitivity, although in the VENIRKO mice, the potential role of insulin-stimulated endothelial NO production in the regulation of whole-body insulin sensitivity might have been overlooked, because the clamp studies were of very short duration and a high insulin infusion rate was used (i.e., it remains possible that a shift in insulin sensitivity may have been detected if lower insulin infusion rates had been used). When hit by an additional challenge, however, normal eNOS function seems to represent a line of defense to maintain normal insulin sensitivity and vascular function.

Our data in mice indicate that there exists an important interaction between genetic and environmental factors in the regulation of vascular NO synthesis and glucose and blood pressure homeostasis. In human populations, the prevalence of eNOS polymorphism ranges from 5 to 35\% (16–21). We speculate that whereas under normal, unstressed conditions a partial defect of NO synthesis may not alter the phenotype, under a metabolic stress, such as the one represented by a Western-type diet, it may facilitate the development of insulin resistance and arterial hypertension.

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REFERENCES