Rapid Publication

Mice With Gene Disruption of Both Endothelial and Neuronal Nitric Oxide Synthase Exhibit Insulin Resistance

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Studies from our laboratory using acute pharmacologic blockade of nitric oxide synthase (NOS) activity have suggested that nitric oxide (NO) has an important role in regulating carbohydrate metabolism. We now report on insulin sensitivity in mice with targeted disruptions in endothelial NOS (eNOS) and neuronal NOS (nNOS) genes compared with their wild-type (WT) counterparts. Mice underwent hyperinsulinemic-euglycemic clamp studies after a 24-h fast, during an insulin infusion of 20 mU · kg^{-1} · min^{-1}. Glucose levels were measured at baseline and every 10 min during the clamp. Insulin levels were measured at baseline and at the end of the clamp study. Glucose infusion rates (GIRs) during the last 30 min of the clamp study were in a steady state. Tritiated glucose infusion was used to measure rates of endogenous glucose output (EGO) both at baseline and during steady-state euglycemia. Glucose disposal rates (GDRs) were computed from the GIR and EGO. Fasting and steady-state glucose and insulin levels were comparable in the 3 groups of mice. No differences in fasting EGO were noted between the groups. GIR was significantly reduced (37%, $P = 0.001$) in the eNOS knockout (KO) mice compared with the WT mice, with values for the nNOS mice being intermediate. EGO was completely suppressed in the nNOS and WT mice during insulin infusion, but not in the eNOS mice. Even so, the eNOS mice displayed significantly reduced whole-body GDRs compared with those of the WT mice (82.67 ± 10.77 vs. 103.67 ± 3.47 mg · kg^{-1} · min^{-1}, $P = 0.03$). eNOS KO mice are insulin resistant at the level of the liver and peripheral tissues, whereas the nNOS KO mice are insulin resistant only in the latter. These data indicate that NO plays a role in modulating insulin sensitivity and carbohydrate metabolism and that the eNOS isoform may play a dominant role relative to nNOS. *Diabetes* 49:XXX–XXX, 2000

Nitric oxide (NO) has emerged as an important molecule with diverse biological functions. In the blood vessels, NO mediates endothelium-dependent vasodilation (1–3) in response to diverse stimuli such as shear stress (4–6), insulin (7), acetylcholine (8,9), and bradykinin (3,10). In the central nervous system (CNS) and peripheral nervous tissue, NO is an unusual neurotransmitter (11–13). NO is generated when the amino acid L-arginine is converted to citrulline by the enzyme NO synthase (NOS) (14,15). Three separate genes encode the known isoforms of NOS (16): endothelial NOS (eNOS or NOS III) and neuronal NOS (nNOS or NOS II) catalyze the constitutive production of NO in a calcium-dependent manner predominantly in the blood vessels and neural tissues, respectively. The third isoform, inducible NOS (iNOS or NOS I) is located in macrophages and catalyzes NO formation in inflammatory cells.

Intravenous administration of N^G-monomethyl-L-arginine (L-NMMA), a competitive inhibitor of all NOS isoforms, acutely induces hypertension and insulin resistance in rats (17). More recently, we reported that acute pharmacologic blockade of NOS activity in the CNS by intracerebroventricular (ICV) administration of L-NMMA resulted in peripheral insulin resistance and insulin secretory defects in unrestrained conscious rats (18). We now report on the studies undertaken to confirm the findings above in eNOS and nNOS knockout (KO) mice. The phenotype and other biological effects noted in these KO animals have been described elsewhere (19–32).

RESEARCH DESIGN AND METHODS

Animals. Breeding colonies of eNOS and nNOS KO mice and their wild-type (WT) counterparts (4 colonies of each type) were obtained from the Cardiovascular Research Center, Massachusetts General Hospital, Boston, Massachusetts, courtesy of Paul Huang and Mark Fishman. The mice were housed and bred in the Indiana University Laboratory Animal Research Center on a 12-h light/dark cycle with standard diet and water available ad libitum. The study protocol was approved by the Indiana University Animal Use Committee.

Insertion of jugular venous catheters. Specially prepared catheters were inserted into the right atrium of each mouse under ketaset (Fort Dodge Laboratories, Fort Dodge, IA) anesthesia as described previously (33).

Hyperinsulinemic clamp studies. These studies were performed 2–3 days after insertion of the jugular catheters to allow the animals to recover from

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CNS, central nervous system; EGO, endogenous glucose output; eNOS, endothelial nitric oxide synthase; GCR, glucose clearance rate; GDR, glucose disposal rate; GIR, glucose infusion rate; ICV, intracerebroventricular; KO, knockout; L-NMMA, N^G-monomethyl-L-arginine; nNOS, neuronal nitric oxide synthase; NO, nitric oxide; NOS, nitric oxide synthase; PCR, polymerase chain reaction; R_a, rate of glucose appearance; WT, wild-type.

the surgery. Evidence that the animals were ready for surgery included a healthy appearance, normal activity, and weight regained after surgery. The animals were studied after a 24-h fast while the animals were awake, unrestrained, and unstressed in their regular cages. All of the mice were studied at an insulin infusion rate of 20 mU · kg^{-1} · min^{-1} as described by our group (33).

Glucose turnover. The rate of glucose appearance (R_a) was determined isotopically in the basal and insulin-stimulated states.

Endogenous glucose output. Endogenous glucose output (EGO) represents residual glucose output from hepatic and renal sources during insulin infusion. EGO was calculated from the R_a and glucose infusion rate (GIR) (EGO = R_a – GIR). In cases in which the R_a was underestimated (i.e., R_a < GIR), EGO was considered to be 0.

Glucose disposal and glucose clearance rates. When R_a > GIR, the glucose disposal rate (GDR) was considered to be equal to the R_a. When R_a < GIR, then the latter was considered to represent the GDR. To adjust for the variation that clamped glucose concentration can have on glucose utilization rates, the glucose clearance rate (GCR) was calculated as follows: GCR (ml · kg^{-1} · min^{-1}) = GDR/steady-state plasma glucose.

Three animals from each group were randomly selected and DNA was obtained and the KO status was confirmed by polymerase chain reaction (PCR) analysis for the eNOS and nNOS gene transcripts.

Data analysis. Data are reported as means ± SE. The results are expressed in the following order: eNOS versus nNOS versus WT animals. Comparisons between these groups were performed with analysis of variance using StatView 5.0 program (Abacus Concepts, Berkeley, CA), followed by a Fisher protected least-significant difference test. A P value <0.05 was considered statistically significant.

RESULTS

PCR analysis of DNA from each group of animals demonstrated that the lines were pure for the gene knocked out, whereas the gene transcripts were intact in the WT animals (data not shown). The characteristics of the 3 groups of animals are described in Table 1. The animals in the 3 groups had comparable body weights, fasting glucose and insulin levels, and EGO.

Euglycemic clamp studies. During the clamp study, plasma glucose concentrations stabilized by 40 min and remained unchanged for the next 30 min. Steady-state glucose levels were comparable in all of the groups (Table 1, Fig. 1). Figure 2 illustrates the GIRs during the 70-min clamp study. Steady-state GIR was achieved by 40 min and remained unaltered during the remainder of the study. Steady-state GIR was highest in the WT mice and lowest in the eNOS mice, and intermediate in the nNOS mice (65.62 ± 5.49 vs. 86.80 ± 4.21 vs. 103.67 ± 3.47 mg · kg^{-1} · min^{-1}, P < 0.0001). EGO was completely suppressed in the nNOS and WT mice, but the eNOS KO mice continued to exhibit residual EGO (20.85 ± 8.60 mg · kg^{-1} · min^{-1}) during steady-state hyperinsulinemia (P = 0.041). GDR (GIR + residual EGO) was 82.67 ± 10.77 mg · kg^{-1} · min^{-1} in the eNOS mice, which was lower than the GDR in the other groups of mice (P = 0.0189). GCR was lowest in the eNOS mice and highest in the WT mice, and intermediate in the nNOS mice (P = 0.0196).

DISCUSSION

The demonstration that mice deficient in eNOS and nNOS activity via gene disruption display insulin resistance confirms our earlier observations obtained with acute pharmacologic antagonism of NOS activity in rats (18,33).

nNOS (or type 1 NOS) activity was originally described in the neurons of the CNS as well as the various peripheral nerve plexi (16,34). It has also been described in skeletal muscle where it is complexed with dystrophin (35). eNOS is highly expressed in the endothelial cells of blood vessels (36), but it is also observed in the epithelial cells of the bronchial tree (37) as well as in the pyramidal cells of the hippocampus (38). Based on this pattern of distribution, it is logical to expect multiple phenotypes when these genes are disrupted.

Thus far, the described phenotypes of mice lacking the nNOS gene have included hypertension (20), pyloric stenosis (20), resistance to vascular stroke (21), impaired recovery from viral encephalitis (24), defective nocturnal motor coordination (26), abnormal neurotransmitter release in the brain (23), aggressive behavior (25,39), and resistance to hypoxic-ischemic injury in the neonatal period (19). Mice congenitally

TABLE 1
Characteristics of the groups of animals

	eNOS	nNOS	WT	P
Weight (g)	26 ± 1 (42)	27 ± 0.3 (56)	27 ± 1 (22)	>0.05*†‡§
Fasting glucose (mg/dl)	89.71 ± 2.39 (42)	90.29 ± 1.95 (55)	95.28 ± 3.16 (21)	>0.05*†‡§
Fasting insulin (μU/ml)	13.48 ± 3.09 (10)	19.00 ± 8.04 (13)	19.46 ± 5.65 (5)	>0.05*†‡§
Basal EGO (mg · kg^{-1} · min^{-1})	37.79 ± 11.67 (4)	35.83 ± 6.84 (6)	43.35 ± 8.96 (3)	>0.05*†‡§
Steady-state glucose (mg/dl)	111.8 ± 6.26 (25)	103.15 ± 5.88 (31)	90.86 ± 5.65 (19)	>0.05*†§ 0.0289‡
Clamp insulin (μU/ml)	307.23 ± 69.12 (22)	484.99 ± 126.06 (22)	507.00 ± 111.68 (14)	>0.05*†‡§
Steady-state GIR (mg · kg^{-1} · min^{-1})	65.62 ± 5.49 (25)	86.80 ± 4.21 (31)	103.67 ± 3.47 (19)	<0.0001* 0.0011† <0.0001‡ 0.0148§
Steady-state GDR (mg · kg^{-1} · min^{-1})	82.67 ± 10.77 (7)	86.80 ± 4.21 (31)	103.67 ± 3.47 (19)	0.0189* >0.05† 0.0331‡ 0.0101§
Steady-state GCR (dl/min)	0.69 ± 0.09 (7)	0.94 ± 0.09 (31)	1.24 ± 0.10 (19)	0.0196* >0.05† 0.0103‡ 0.0346§
Residual EGO (mg · kg^{-1} · min^{-1})	20.85 ± 8.60 (8)	0 (7)	0 (4)	0.041*

Data are means ± SD (n). *Comparison between all groups; †comparison between eNOS and nNOS; ‡comparison between eNOS and WT; §comparison between nNOS and WT.

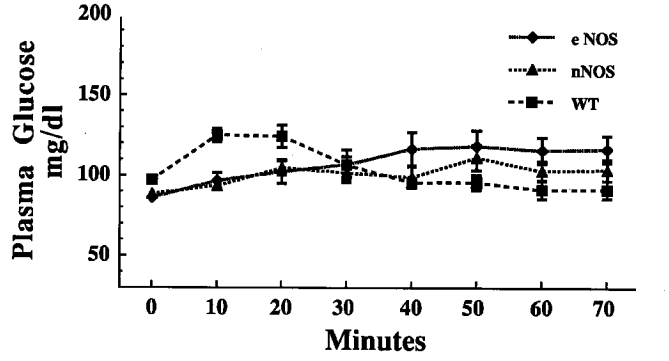

FIG. 1. Blood glucose values during the clamp study. Steady-state glucose levels were achieved by 40 min. The eNOS KO animals were clamped at a much higher glucose level than the other 2 groups (P = 0.0289 compared with the WT animals).

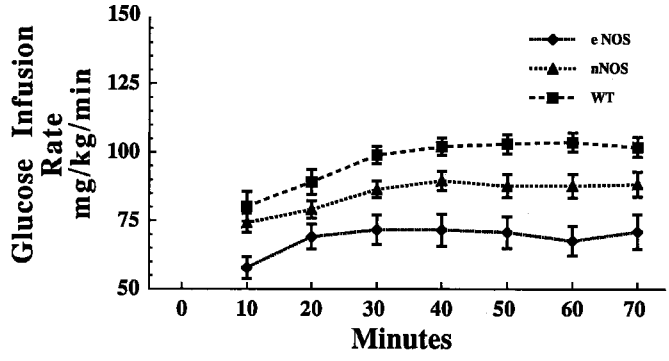

FIG. 2. GIRs during the clamp study. Steady-state infusion rates were noted by 40 min. Steady-state GIR was significantly reduced in both the eNOS and nNOS animals compared with the WT animals (P < 0.0001).

deficient in eNOS have been reported to have hypertension (22,30), enhanced blood pressure variability (31), abnormal neurotransmitter release (23), abnormal long-term potentiation in the brain (29), normal cerebral glucose utilization (40), normal coronary hemodynamics (27) but abnormal cardiac oxygen consumption (28), and exaggerated myocardial reperfusion injury (32).

Neither the tissue localization nor the previously described phenotypes in the KO mice could have predicted a role for NO in carbohydrate metabolism. However, previous studies from our laboratory and others have demonstrated a role for NO in carbohydrate metabolism, in as much as acute pharmacologic blockade of NOS activity induces insulin resistance in a rat model (17,18,41). Specifically, intravenous and intracranial administration of L-NMMA–induced hypertension and significant reduction in steady-state GIRs during euglycemic-hyperinsulinemic clamps in awake unrestrained adult male Sprague-Dawley rats. These observations prompted the current study, which was designed to evaluate insulin sensitivity in awake unrestrained mice with targeted disruption of the eNOS and nNOS genes compared with their WT counterparts.

Our data suggest that both nNOS and eNOS KO mice have insulin resistance compared with their WT counterparts. The eNOS mice are the most resistant, exhibiting resistance to the ability of insulin to suppress EGO, in addition to reduced insulin-induced glucose uptake in peripheral tissues.

Insulin levels during steady-state hyperglycemia were not statistically different in the 3 groups, and they exhibited large variability within each group. Although the steady-state insulin levels in the eNOS KO mice were somewhat lower, this is likely to be due to the overall variability (in specimen collection and assay) rather than as evidence for accelerated insulin clearance in the eNOS KO mice (33). Previously, our laboratory has demonstrated that the dose of insulin to achieve maximal rates of insulin-stimulated glucose uptake in normal mice during euglycemic clamps is 10 mU · kg^{-1} · min^{-1} (33), thus the insulin infusion rate of 20 mU · kg^{-1} · min^{-1} used in this study ensures that maximal insulin stimulation was achieved.

Most insulin-resistant animals maintain fasting normoglycemia by secreting more insulin to overcome the resistance. All 3 groups of animals had normal fasting plasma glucose, and no differences were noted in the fasting insulin levels. Although this discordance may merely reflect limitations and variability of sample collection and insulin assay, it is also possible that this reflects impaired insulin secretion. Indeed, we previously demonstrated that ICV administration of L-NMMA resulted in defects in both insulin action and secretion (18). Interestingly, in that same study, we observed an impairment in insulin's ability to suppress EGO with central NOS blockade, recapitulating our findings with the eNOS KO but not with the nNOS KO animals. If we had studied the animals at a submaximally effective insulin concentration, perhaps we might have observed resistance to EGO suppression.

This study was not designed to test the mechanism of the effect observed, and we are in the process of evaluating this important aspect. We speculate that the observed changes may be due to alterations in regional blood flow that result in impaired delivery of substrate and/or insulin to the target tissues. Alternatively or additionally, absence of NO in the target tissue may also contribute through alterations in insulin signaling to the observed insulin resistance and insulin secretory defect. Further studies will be required to sort out common and differential features between eNOS and nNOS KO mice.

In summary, we have presented genetic evidence that both eNOS and nNOS isoforms play a role in insulin action. Given the extent of evidence that NO system dysfunction coexists in many insulin-resistant states, it will be important to better understand the role of NO in insulin action and, conversely, the role of insulin in regulating the NO system. These relationships may reveal pathogenic links between insulin resistance, hypertension, and macrovascular disease.

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