Inducible Nitric Oxide Synthase Has Divergent Effects on Vascular and Metabolic Function in Obesity

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Previous studies have suggested an involvement of inducible nitric oxide synthase (iNOS) in obesity, but the relation, if any, between this and mechanisms underlying endothelial dysfunction in obesity is unknown. We studied mice fed an obesogenic high-fat or standard diet for up to 8 weeks. Obesity was associated with elevated blood pressure; resistance to the glucoregulatory actions of insulin; resistance to the vascular actions of insulin, assessed as the reduction in phenylephrine constrictor response of aortic rings after insulin preincubation (lean −21.7 ± 11.5 vs. obese 18.2 ± 15.5%; $P < 0.05$); and evidence of reactive oxygen species (ROS)-dependent vasodilatation in response to acetylcholine in aortic rings (change in maximal relaxation to acetylcholine after exposure to catalase: lean −2.1 ± 6.0 vs. obese −15.0 ± 3.8%; $P = 0.04$). Obese mice had increased expression of iNOS in aorta, with evidence of increased vascular NO production, assessed as the increase in maximal constriction to phenylephrine after iNOS inhibition with 1400W (lean −3.5 ± 9.1 vs. obese 42.1 ± 11.2%; $P < 0.001$). To further address the role of iNOS in obesity-induced vascular and metabolic dysfunction, we studied the effect of a high-fat diet in iNOS knockout mice (iNOS KO). Obese iNOS KO mice were protected against the development of resistance to insulin's glucoregulatory and vascular effects (insulin-dependent reduction in maximal phenylephrine response: obese wild-type 11.2 ± 15.0 vs. obese iNOS KO −20.0 ± 7.7%; $P = 0.02$). However, obese iNOS KO mice remained hypertensive (124.0 ± 0.7 vs. 114.9 ± 0.5 mmHg; $P < 0.01$) and had evidence of increased vascular ROS production. Although these data support iNOS as a target to protect against the adverse effects of obesity on glucoregulation and vascular insulin resistance, iNOS inhibition does not prevent the development of raised blood pressure or oxidative stress. *Diabetes* 54: 1082–1089, 2005

The prevalence of obesity in Western societies is >30% (1). Obesity is associated with accelerated atherosclerosis (2,3), but the mechanisms underlying this association remain unclear. It has been established that a key early event in the development of atherosclerosis is endothelial dysfunction (4), a characteristic feature of which is the reduction in the bioactivity of the signaling molecule nitric oxide (NO). This can be a result of reduced production of NO or increased inactivation by reactive oxygen species (ROS). NO in small amounts is thought to be an antiatherosclerotic molecule, with properties including inhibition of vascular smooth muscle growth (5,6), leukocyte adhesion (7), and platelet adherence and aggregation (8). There is compelling evidence that obesity leads to endothelial dysfunction (9,10).

NO is synthesized by three different isoforms of NO synthase (NOS). Endothelial NOS (eNOS) and neuronal NOS (nNOS) are constitutively expressed and synthesize small amounts of NO under basal conditions and on stimulation by various stimuli, including, in the case of eNOS, shear stress and insulin (11). In contrast, inducible NOS (iNOS) is expressed when stimulated by inflammatory cytokines and can produce up to 1,000-fold more NO than eNOS (12), which, while important for the immune response, can have detrimental effects on different cell types, including vascular cells (13) and pancreatic β-cells (14). Recent studies in iNOS knockout (KO) mice have shown that iNOS-derived NO may play a role in the pathophysiology of obesity-induced metabolic dysfunction (15). Furthermore, iNOS-derived NO causes endothelial dysfunction in a number of experimental models (16–19). However, whether iNOS affects endothelial function in obesity is unknown. In recent years, the view of adipose tissue as a simple fuel store has changed. Fat tissue has emerged as a complex endocrine organ. This concept has evolved further to include a role for fat tissue in inflammatory pathways, largely because adipocytes have been shown to secrete cytokines that are generally considered to be produced by macrophages and to induce the expression of iNOS (20,21).

In this study, we used an integrated in vivo and ex vivo approach in a murine model of diet-induced obesity to explore the mechanisms of endothelial dysfunction in obesity, focusing on the possible involvement of iNOS. We report a number of novel findings: *1*) obesity is characterized by iNOS expression in the vasculature; *2*) vascular production of ROS compensates for impaired acetylcholine-mediated vasodilatation; *3*) mice lacking iNOS are protected against obesity-related resistance to the glucose-

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DHE, dihydroethidium; eNOS, endothelial NOS; HOMA, homeostatic model assessment; iNOS, inducible NOS; KO, knockout; L-NMMA, NG-monomethyl-L-arginine; nNOS, neuronal NOS; NOS, NO synthase; ROS, reactive oxygen species; SNP, sodium nitroprusside.

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lowering and vasodilatory effects of insulin but still have increased blood pressure and impaired relaxation to acetylcholine, which is compensated for by ROS production.

RESEARCH DESIGN AND METHODS

Male wild-type (WT) C57BL/6J mice were bred in our laboratories. Homozygous iNOS KO mice on a C57BL/6J background were a kind gift from Dr. A.J. Hobbs (University College London). Obesity was induced by feeding mice a high-fat diet (5,286 kcal/kg; Bioserve) from weaning. Control animals were fed a standard diet. Mice were housed in a conventional animal facility with a 12:12-h light:dark cycle. All procedures were performed in accordance with the U.K. Guidance on the Operation of the Animals (Scientific Procedures) Act (1986).

Assessment of metabolic regulation, plasma lipids, and plasma nitrite. After 4 and 8 weeks of feeding, glucose and insulin tolerance tests were performed in conscious mice by repeated blood sampling after an intraperitoneal injection of glucose (1 mg/g) or human insulin (0.75 unit/kg), as previously described (22). Blood glucose was measured using a portable device (Hemocue, Sheffield, U.K.), and insulin levels were assessed by a specific hypersensitive radioimmunoassay (Linco Research, St. Charles, MO). The homeostatic model assessment (HOMA) insulin sensitivity score was calculated as insulin × glucose ÷ 22.5. Blood samples were obtained from the lateral saphenous vein, and glucose levels were measured in whole blood. Free fatty acids and triglycerides were measured in fasting plasma using colorimetric assays (Roche, Mannheim, Germany; ThermoTrace, Victoria, Australia). Plasma nitrite was measured using the Griess reaction (14).

In vivo measurement of blood pressure. Systolic blood pressure was measured using tail-cuff plethysmography (22,23) in conscious mice prewarmed for 10 min in a thermostatically controlled restrainer (XBP1000; Kent Scientific). Three training sessions were performed during the week before measurements were taken. The mean of at least six separate recordings on three occasions was taken to calculate mean systolic blood pressure.

Ex vivo assessment of vascular function. The thoracic aorta was excised, cleaned of adherent connective tissue, and cut into rings 3 mm long. As previously described (22–24), the rings were suspended between a fixed support and a force transducer in an organ bath containing 10 ml Krebs-Henseleit solution at 37°C, bubbled with 95% O_2/5% CO_2. After a 45-min equilibration at a resting tension of 3 g, a level that we have found to be optimal, the maximal contractile response to 40 mmol/l KCl was assessed. After a wash out and reequilibration, a cumulative dosage-response curve to phenylephrine was performed. Rings were then preconstricted to 70% of the maximal phenylephrine-induced tension; relaxation responses to the cumulative addition of acetylcholine (1 nmol/l to 10 μmol/l) or sodium nitroprusside (SNP; 0.1 nmol/l to 1 μmol/l) were then determined. In some rings, the acetylcholine response was repeated in the presence of catalase (1,250 unit/ml), which degrades H_2O_2. Basal NO bioavailability was assessed by measuring the constriction to phenylephrine in the absence and presence of the NOS inhibitor NG-monomethyl-L-arginine (L-NMMA; 0.1 mmol/l; 30 min). The role of iNOS-derived NO was assessed in a similar fashion by measuring the effect of the iNOS-specific inhibitor 1400W (10 μmol; Alexis Biochemicals, San Diego, CA) (25) on phenylephrine constriction.

The NO-mediated vascular response to insulin was assessed by constructing phenylephrine dosage-response curves before and after a 2-h exposure to insulin (100 mU/ml). We have previously demonstrated in healthy mice that insulin causes an endothelium-dependent blunting of the vasoconstrictor response to phenylephrine and that this effect is abolished by L-NMMA; that is, it is NO-mediated (23).

In situ measurement of vascular production of reactive oxygen species. ROS generation within the aorta in situ was measured using dihydroethidium (DHE) fluorescence, as previously reported (24,26). To explore the possibility that vascular ROS production was stimulated by acetylcholine, DHE fluorescence was assessed in aortic rings that had been exposed to acetylcholine or saline. Fluorescence intensity at the endothelial surface was quantified microscopically from at least five random fields (1,024–1,022 pixels; 269.7 × 269.2 μm) per slide and three slides per experimental condition, using a computerized image analysis system (Improvision).

Expression of NOS mRNA in aorta. Total RNA was extracted from aortas (Mini Fibrous Kit; Quiagen), and equal amounts were reverse-transcribed using Superscript II RT (Invitrogen) and random decamer oligonucleotides. Real-time RT-PCR analyses for eNOS, nNOS, iNOS, and β-actin mRNA expression were performed in duplicate using the ABI Prism 7000 sequence detection system (23). Primers and FAM-labeled probes specific for these genes were used (Assays-on-Demand; Applied Biosystems). The cDNA was amplified at the following conditions: 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. The standard curve method (User Bulletin No 2; ABI Systems) was used for quantification, and results were normalized for the expression of β-actin.

TABLE 1
Metabolic and blood pressure data in WT and iNOS knockout mice fed a standard or high-fat diet at 4 and 8 weeks of feeding

	4 weeks	8 weeks
Body mass (g)		
WT, standard	24.1 ± 0.3	29.1 ± 0.4
WT, high-fat	27.3 ± 0.4*	38.4 ± 0.4*
iNOS KO, standard	22.0 ± 0.8	27.2 ± 1.1
iNOS KO, high-fat	27.9 ± 0.5	36.6 ± 1.3
Epididymal fat pad (mg)		
WT, standard	11.7 ± 1.1	12.0 ± 0.3
WT, high-fat	24.4 ± 1.5*	33.6 ± 0.9*
iNOS KO, standard	11.3 ± 0.5	13.2 ± 0.5
iNOS KO, high-fat	23.4 ± 0.8	32.1 ± 0.7
Fasting glucose (mmol/l)		
WT, standard	6.4 ± 0.6	6.5 ± 0.4
WT, high-fat	12.0 ± 0.3*	11.2 ± 0.6*
iNOS KO, standard	7.0 ± 0.2	6.1 ± 0.2
iNOS KO, high-fat	8.5 ± 0.5	7.7 ± 0.2†
Fasting insulin (pg/ml)		
WT, standard	0.5 ± 0.1	0.3 ± 0.1
WT, high-fat	1.0 ± 0.2*	3.5 ± 0.5*
iNOS KO, standard	0.5 ± 0.1	0.4 ± 0.1
iNOS KO, high-fat	0.6 ± 0.1	0.5 ± 0.1†
HOMA (unit)		
WT, standard	0.15 ± 0.01	0.15 ± 0.2
WT, high-fat	0.55 ± 0.1*	2.03 ± 0.2*
iNOS KO, standard	0.14 ± 0.01	0.13 ± 0.01
iNOS KO, high-fat	0.18 ± 0.01	0.22 ± 0.02†
Free fatty acids (mmol/l)		
WT, standard	—	0.42 ± 0.1
WT, high-fat	—	1.41 ± 0.1*
iNOS KO, standard	—	0.35 ± 0.1
iNOS KO, high-fat	—	0.56 ± 0.1†
Decline in glucose in response to insulin (%)		
WT, standard	70.2 ± 1.9	65.6 ± 1.2
WT, high-fat	54.9 ± 0.6*	48.5 ± 1.7*
iNOS KO, standard	68.1 ± 2.6	66.2 ± 1.7
iNOS KO, high-fat	68.0 ± 1.3	68.5 ± 1.2
Systolic blood pressure (mmHg)		
WT, standard	117.6 ± 0.3	118.6 ± 0.5
WT, high-fat	122.7 ± 0.3*	124.8 ± 0.7*
iNOS KO, standard	113.9 ± 0.4	114.9 ± 0.5
iNOS KO, high-fat	122.5 ± 0.5	124.0 ± 0.7

Data are means ± SE. *$P < 0.05$ for standard vs. high-fat diet; †$P < 0.05$ for WT vs. iNOS KO mice on a high-fat diet.

Statistical analysis. Data are means ± SE. Comparisons were made using an unpaired *t* test, one-way ANOVA, or repeated-measures ANOVA, where appropriate. $P < 0.05$ was considered significant.

RESULTS

Progression of obesity. Within 4 weeks of commencing the high-fat diet, mice were significantly heavier than littermates fed the standard diet (Table 1). This weight gain was accompanied by a significantly greater epididymal fat pad mass. The obese mice had higher systolic blood pressures after 4 and 8 weeks of feeding. They were also insulin resistant, as manifested by significantly higher fasting glucose and insulin levels, a higher HOMA score, and a smaller decrement in glucose levels in insulin tolerance tests than standard diet−fed controls. Obese

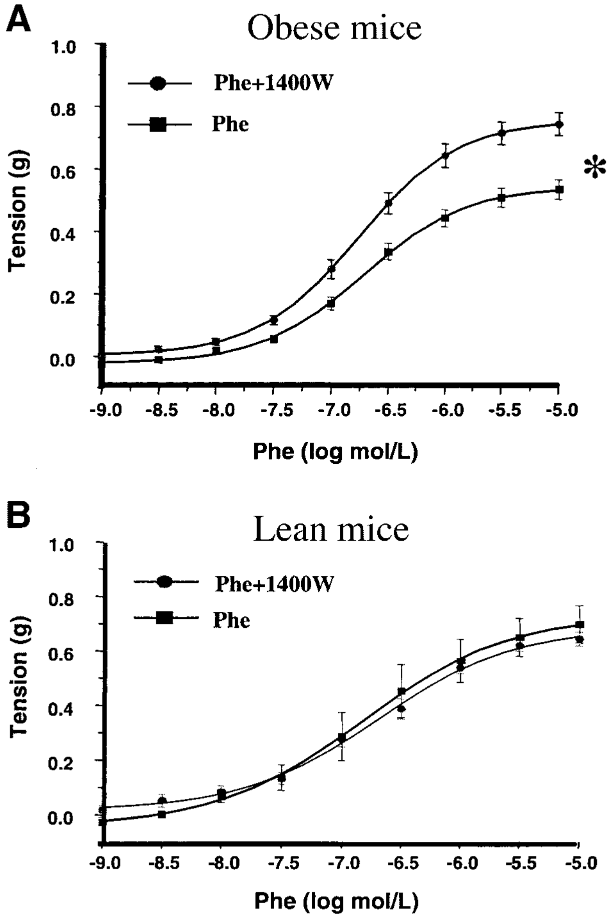

FIG. 1. Constrictor response to the specific iNOS inhibitor 1400W in mice fed a high-fat diet for 8 weeks (*A*) and lean mice fed a standard diet (*B*). *$P < 0.01$ by ANOVA ($n = 8$–16). Phe, phenylephrine.

mice also had significantly higher fasting free fatty acid levels.

Basal aortic nitric oxide bioavailability in obese and lean mice. Basal aortic NO bioavailability was assessed from the effect of treatment with the nonselective NOS inhibitor L-NMMA on vasoconstrictor responses to phenylephrine. After 4 or 8 weeks of feeding, there was a greater constrictor response with L-NMMA in obese than in lean mice: at 4 weeks, the change in tension in response to L-NMMA in obese versus lean mice was 86.4 ± 21.0 vs. 35.4 ± 12.9% ($P < 0.001$); at 8 weeks, it was 135.1 ± 13.2 vs. 57.7 ± 14.1% ($P < 0.001$). (Data for 8 weeks of feeding are shown in Fig. 6*C*.) To explore the source of this NO, we used the selective iNOS inhibitor 1400W. In aortas from animals that had been fed an obesogenic diet for 8 weeks, the constrictor response to 1400W was significantly greater than in lean mice (Fig. 1*A* and *B*); indeed, 1400W had no effect in lean mice.

Plasma nitrite levels. Consistent with increased production of NO in obese mice, plasma nitrite was significantly higher in obese than in lean mice (16.9 ± 1.45 vs. 8.66 ± 1.23 μmol/l; $n = 8$–16 per group; $P < 0.01$) (Fig. 2*A*).

NOS isoform expression in the aorta. We performed real-time RT-PCR on aortic ring segments from obese and lean mice ($n = 8$ per group). There was no difference in mRNA levels of eNOS and nNOS in obese and lean mice (Fig. 2*B* and *C*). However, consistent with the results of experiments with 1400W in aortic rings, iNOS mRNA was significantly higher (Fig. 2*D*) in aortas of obese mice compared with lean mice (δCT 0.98 ± 0.09 vs. 0.72 ± 0.07 units; $P = 0.03$), demonstrating a twofold increase in iNOS mRNA.

ROS-dependent vasorelaxation in obese mice. Vasorelaxation to acetylcholine was similar in obese and lean mice after 4 and 8 weeks of feeding (8-week data shown in Fig. 3*A* and *B*). Previous studies in humans and mice have shown that in situations where NO-mediated vasorelaxation is impaired (e.g., hypertension, hyperlipidemia), this impairment may be compensated for by increased generation of the ROS H_2O_2, which may act as an endothelial-derived hyperpolarizing factor that compensates for NO. We therefore studied the effect of catalase treatment (to degrade H_2O_2) on the vasodilator response to acetylcholine. Although catalase had no effect on acetylcholine responses in lean mice (8-week data shown in Fig. 3*A* and *B*), there was a significant blunting (by ~20%) of acetylcholine-induced vasodilatation in obese mice. Endothelial-independent relaxation to SNP was similar in lean and obese mice (Fig. 3*C*).

Dihydroethidium fluorescence. To more directly assess ROS production, we examined in situ ROS production with or without acetylcholine using DHE fluorescence in frozen aortic sections. In the basal state, there was minimal ROS, but in response to acetylcholine there was a significant rise in ROS that was greater in obese mice (Fig. 3*D*).

Insulin-mediated vasorelaxation. To investigate whether the obesity-related resistance to the glucoregulatory actions of insulin was paralleled by resistance to the vascular actions of insulin, we performed phenylephrine dosage-response curves before and after incubation with insulin. After 4 weeks of a high-fat diet, both obese and lean mice had preserved vascular insulin responses, as evidenced by blunting of the vasoconstrictor response to phenylephrine in the presence of insulin (Fig. 4*A* and *B*). After 8 weeks on a high-fat diet, however, this response

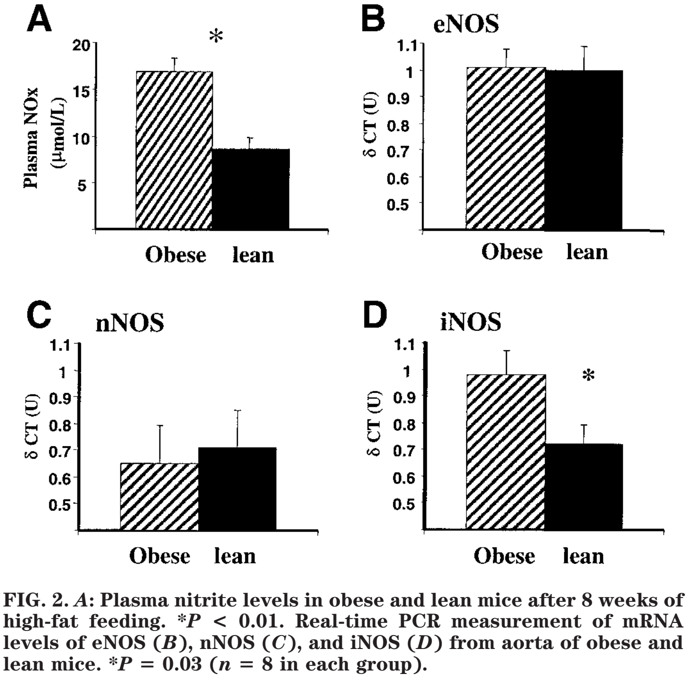

FIG. 2. *A*: Plasma nitrite levels in obese and lean mice after 8 weeks of high-fat feeding. *$P < 0.01$. Real-time PCR measurement of mRNA levels of eNOS (*B*), nNOS (*C*), and iNOS (*D*) from aorta of obese and lean mice. *$P = 0.03$ ($n = 8$ in each group).

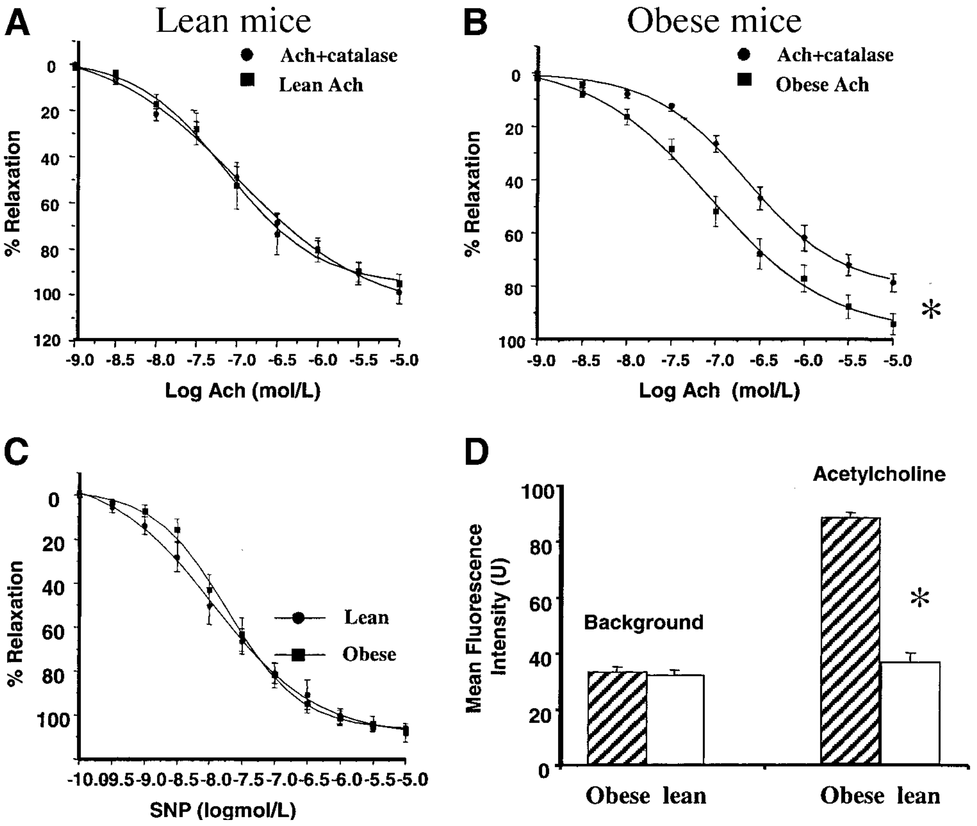

FIG. 3. *A*: Relaxation curves to acetylcholine (Ach) in lean mice at 8 weeks before and after catalase (1,250 units/l) was given. *B*: Relaxation curves to acetylcholine in obese mice before and after catalase was given after 8 weeks of high-fat feeding. $^*P < 0.01$ by ANOVA. *C*: Relaxation curves to SNP in lean and obese mice at 8 weeks. There was no difference between groups (n = 8–16 in each group). *D*: Mean DHE fluorescence intensity in lean and obese mice. $^*P < 0.0001$.

was lost in obese mice, whereas it was still present in lean mice (Fig. 4*C* and *D*). These results seem to indicate that resistance to the vascular actions of insulin occurs later than resistance to its glucoregulatory effects in this model of diet-induced obesity, given that glucoregulation was already impaired after 4 weeks on a high-fat diet (Table 1), whereas vascular insulin responsiveness was preserved.

Influence of iNOS on metabolic and vascular function in obese mice. iNOS KO mice were fed a high-fat or standard diet for up to 8 weeks and compared with WT mice in terms of their metabolic and vascular outcomes. The weight gain in iNOS KO mice fed the high-fat diet was similar to that of WT mice on the same diet (Table 1). As reported previously, iNOS KO mice were protected against

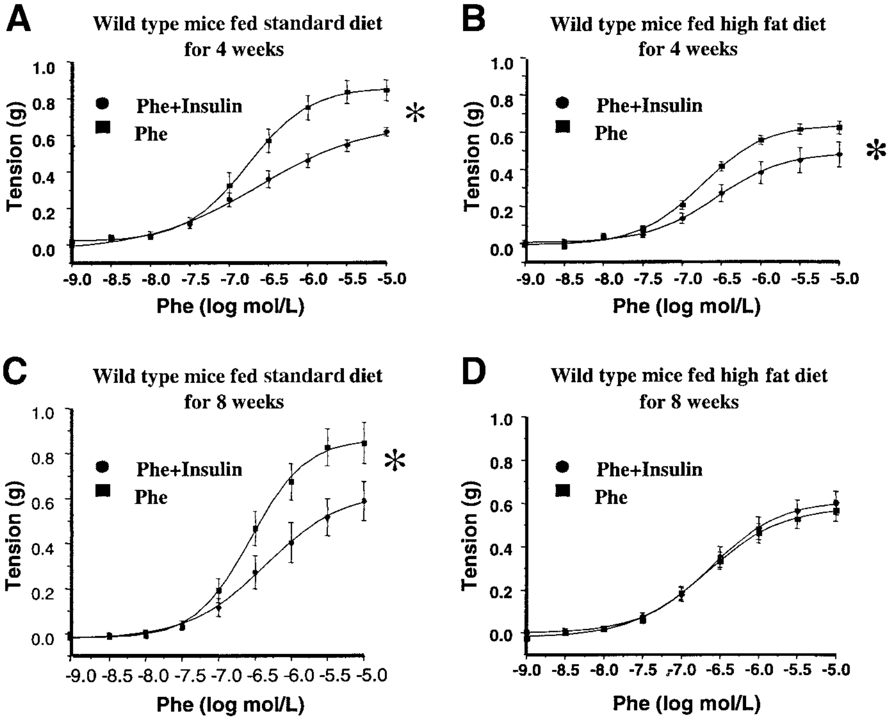

FIG. 4. Phenylephrine (Phe)-dependent vasoconstriction before and after exposure of rings to insulin in WT mice fed a standard (*A*) or high-fat (*B*) diet for 4 weeks or fed a standard (*C*) or high-fat (*D*) diet for 8 weeks. $^*P < 0.01$.

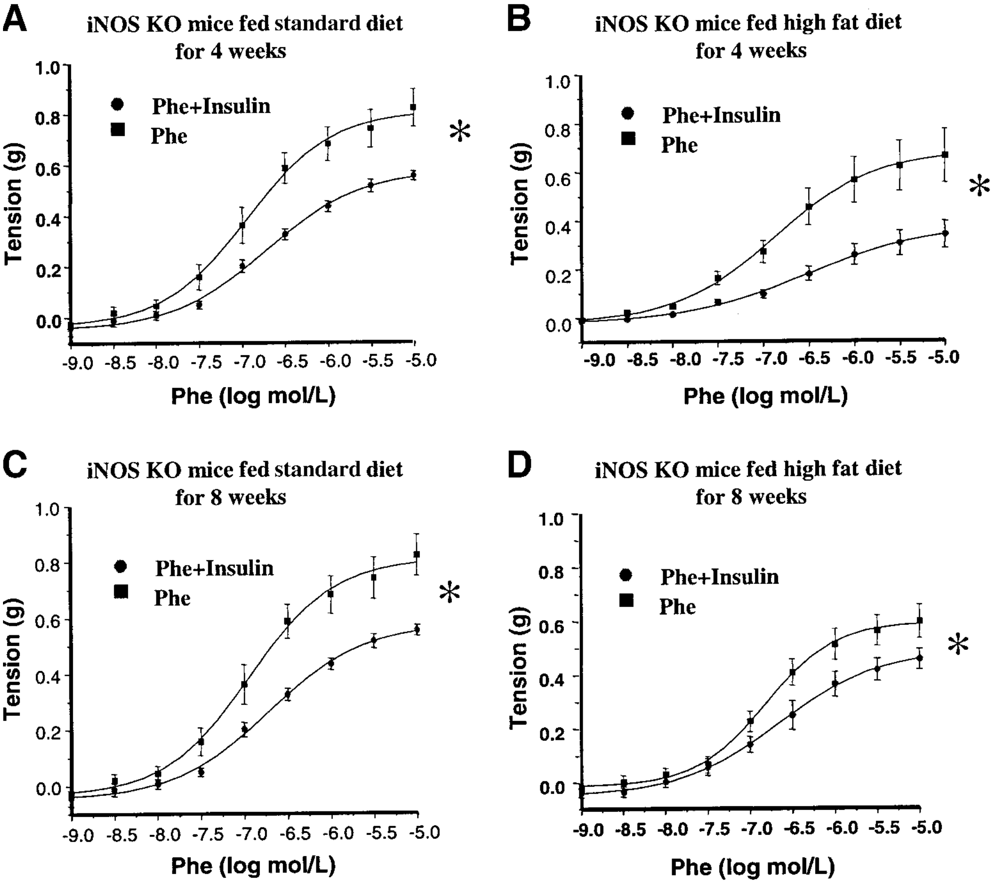

FIG. 5. Phenylephrine (Phe)-dependent vasoconstriction before and after exposure of rings to insulin in iNOS KO mice fed a standard (*A*) or high-fat (*B*) diet for 4 weeks or fed a standard (*C*) or high-fat (*D*) diet for 8 weeks. $^{*}P < 0.01$ by ANOVA (n = 8–16).

the development of resistance to insulin's glucoregulatory effects, manifested as lower fasting glucose and insulin levels, a lower HOMA index, and a greater insulin-induced decrement in blood glucose than WT mice fed the high-fat diet (Table 1).

Basal aortic NO production was assessed by measuring the vasoconstrictor response to L-NMMA. iNOS KO mice fed the high-fat diet had a similar constrictor response compared with WT or iNOS KO mice fed the standard diet (Fig. 6*C*). Consistent with a reduction in total NO production, iNOS KO mice fed the high-fat diet had less plasma nitrite than WT mice on the same diet (12.0 ± 0.3 vs. 16.9 ± 1.45 μmol/l; P = 0.02). iNOS KO mice fed the high-fat diet were also protected against loss of insulin-mediated vasodilation (Fig. 5*B* and *D*); however, they still developed elevated blood pressure (Table 1). Furthermore, iNOS KO mice on the obesogenic diet had evidence of ROS-mediated vasodilation in aortas in the same fashion as did WT mice (Fig. 6*B*), as well as increased ROS production in the DHE fluorescence assay (Fig. 6*A*).

DISCUSSION

Obesity is a major health care problem in the Western world and is set to reach epidemic proportions in the next 20 years. A major consequence of obesity is premature vascular disease. Understanding the mechanisms linking obesity and endothelial dysfunction is an important aim. The present study has demonstrated a number of findings important to our understanding of the interrelations among obesity, resistance to insulin's glucoregulatory and vascular actions, and endothelial-dependent vasodilator function as well as the role of iNOS-derived NO in these different facets of obesity-associated metabolic and vascular perturbations.

In this study, we found that insulin-mediated glucose uptake was impaired at an earlier stage than the blunting of insulin-mediated NO release from the vascular endothelium, indicating a divergence between these abnormalities. We also demonstrated that an early feature of obesity is a (covert) blunting of acetylcholine-mediated vasodilatation of aortas that was compensated for by ROS-mediated vasodilatation. In addition, as obesity progressed, there was an increase in basal vascular NO bioavailability, at least part of which was derived from iNOS. Using iNOS KO mice, we confirmed the findings of Perreault and Marette (15) that these animals are protected against obesity-induced metabolic insulin resistance despite a similar weight gain as WT mice. In addition, we showed that iNOS KO mice fed a high-fat diet were also protected against impairment of insulin-mediated NO release from aortas. However, obese iNOS KO mice still developed elevated blood pressures and showed evidence of impaired acetylcholine-induced vasodilatation, which was compensated for by ROS-dependent vasodilation. These data demonstrate a divergence in the mechanisms by which obesity leads to impairment of insulin's vascular effects and the classical endothelium-dependent vasodilator function induced by agents such as acetylcholine.

ROS-dependent vasodilation in early obesity. In vivo and in vitro studies have demonstrated that in humans, relaxant factors other than NO compensate to maintain endothelium-dependent vascular function in disease states.

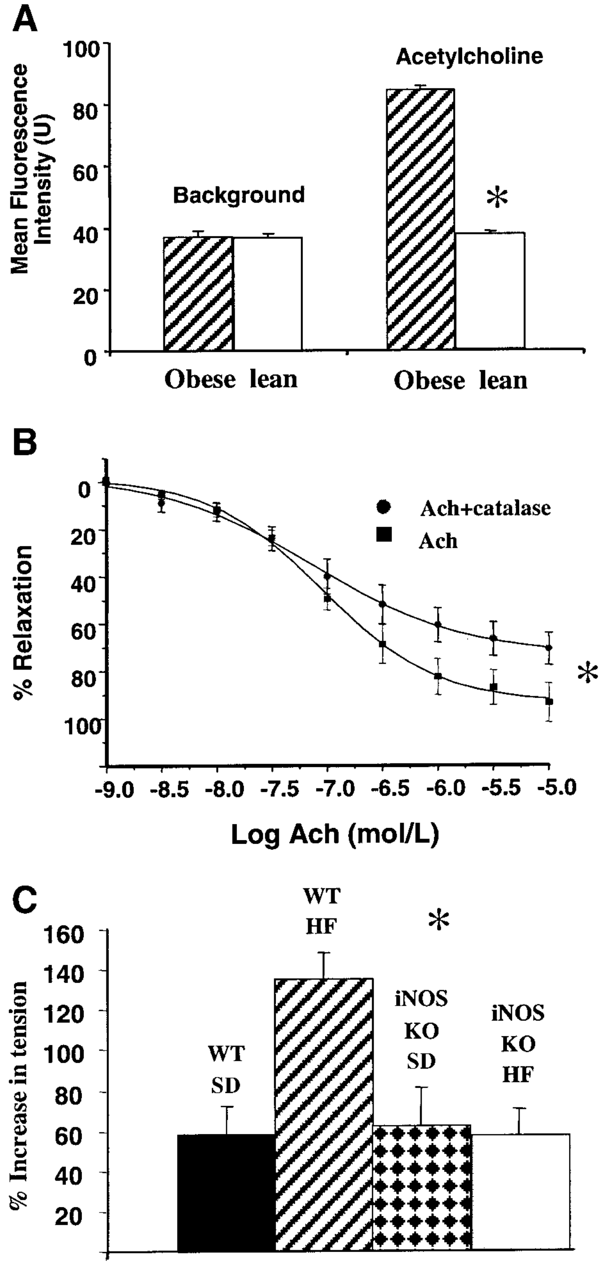

FIG. 6. ROS production and effect of catalase in iNOS KO mice. *A*: In situ DHE detection of ROS production in obese and lean iNOS KO mouse aortic sections (n = 18 sections from six mice in each group). A similar pattern was seen to that observed in WT mice, with no difference in basal ROS between mice fed a standard diet and those fed a high-fat diet, but a significant increase in ROS in response to acetylcholine was seen in the high-fat–fed iNOS KO mice. $^*P < 0.001$. *B*: Relaxation curve to acetylcholine (Ach) before and after exposure to catalase in iNOS KO mice fed a high-fat diet for 8 weeks. $^*P < 0.01$. *C*: Constrictor response to L-NMMA in lean mice fed a standard diet (SD), obese mice fed a high-fat (HF) diet, iNOS KO mice fed a standard diet, and iNOS KO mice fed a high-fat diet. $^*P < 0.01$ by ANOVA (n = 8–16). The constrictor response to L-NMMA in WT mice fed a high-fat diet was significantly greater than that of each of the other mice.

One of these relaxant factors is H_2O_2, which may relax smooth muscle by hyperpolarization or cyclic guanosine monophosphate−dependent mechanisms. Matoba et al. (27) showed that catalase, which degrades H_2O_2, inhibits vasodilatation to acetylcholine in murine mesenteric arteries. In a subsequent study, Ellis et al. (28) found no effect of catalase on acetylcholine-induced vasodilatation in aortas of normal mice. Consonant with that study, we found no effect of catalase on acetylcholine-induced vasodilatation in aorta of lean mice, whereas in obese mice, ~20% of acetylcholine-induced vasodilatation was accounted for by ROS. The mice in the present study were obese, insulin resistant, and mildly hypertensive. In a recent study (29), ROS-dependent vasodilation was documented in the aortas of deoxycorticosterone acetate−salt hypertensive rats, and was postulated to compensate for impaired endothelium-dependent vasodilation, as in the present study. There are several potential sources of ROS, such as NAD(P)H oxidase, xanthine oxidase, mitochondria, and uncoupled eNOS, that may account for the increased oxidative stress demonstrated in the present study (rev. in 30). The sources of ROS production in the current diet-induced obesity model require further study.

Increasing evidence has emphasized an important role for ROS and oxidative stress in vascular disease. ROS may play a critical role in modulating redox-sensitive signaling pathways and are implicated in vascular smooth muscle cell growth and hypertrophy (31). Thus, although the ROS-dependent vasodilation observed in the present study may be regarded as potentially beneficial, in the long term, increased ROS production within the vasculature may be detrimental by promoting maladaptive processes such as cell proliferation (32). Moreover, and of particular relevance to the present study, the simultaneous production of O_2^- and NO leads to near diffusion-limited production of the reactive species, peroxynitrite ($ONOO^-$). Peroxynitrite is thought to play a role in the development of the vasculopathy associated with obesity and diabetes via a number of mechanisms, including protein nitrosylation (33). In a study by Marfella et al. (34), iNOS KO mice rendered diabetic by streptozotocin were protected against the formation of tissue nitrotyrosine after myocardial infarction. The effect of iNOS KO on obesity-induced peroxynitrite in vascular tissue warrants further study.

Mechanisms underlying development of hypertension in obesity. The mechanisms responsible for the development of hypertension in obesity may include abnormalities at the level of the resistance vasculature, central changes that affect autonomic neural output, and/or alterations at the level of the kidneys, including activation of the renin-angiotensin system (35). The precise mechanisms underlying the blood pressure elevation observed after high-fat feeding in the present study remain to be defined. However, the current results provide some interesting insights. First, we have demonstrated a clear dissociation between metabolic/vascular insulin resistance (which was prevented in obese iNOS KO mice) and hypertension (which was not). Therefore, hypertension is apparently not the direct result of insulin resistance. Second, we demonstrated evidence of significant ROS production in the vasculature of obese mice, independent of metabolic dysfunction. Although increased ROS was found to contribute to better endothelium-dependent vasorelaxation in the aorta in the present study, there are several mechanisms by which ROS could be involved in hypertension. Increased ROS at the level of the resistance vasculature (as opposed to conduit vessels such as the aorta) may augment vascular tone either directly or by inactivation of NO (30–32). ROS contribute to vascular smooth muscle hypertrophy and remodelling and have been shown to be implicated in hypertension through this mechanism (31,32). Recent studies have also implicated ROS production in the central nervous system (36) and kidneys (37) in the development of hypertension. Finally, it is possible that non−ROS-dependent pathways, such as the activation of the renin-angiotensin system, leptin, and other adipocyte-derived peptides, may also be involved (rev. in 10).

Role of iNOS in obesity and relation to inflammation. There is compelling evidence from large epidemiological studies, detailed characterization of human vascular lesions, and experimental investigations that inflammation plays a key role in the development and progression of atherosclerosis (4). It is also well established that obesity is associated with a chronic inflammatory response characterized by abnormal cytokine production, increased acute phase reactants, and activation of inflammatory signaling pathways (20,21). Recent studies (38,39) have demonstrated that murine models of obesity similar to the one used in the present study and more severe models of obesity are associated with infiltration of adipose tissue by macrophages and activation of a portfolio of inflammatory genes. The expression of iNOS may be regarded as one aspect of such inflammatory activation. iNOS expression is primarily regulated at a transcriptional level and, once expressed, the enzyme may generate large amounts of NO over long periods of time. In other models of increased iNOS-derived NO (e.g., sepsis), this excessive NO may contribute to increased basal bioactive NO and blunt, classic, calcium-dependent vasodilatation (e.g., in response to acetylcholine) (16–19). In other systems, excessive NO has been shown to reduce insulin-mediated glucose uptake (40) and induce cellular stress (41) and pancreatic β-cell death (14). Thus, iNOS expression could potentially contribute to several of the obesity-associated abnormalities reported in this study.

In the present study, we were able to discern the likely contribution of iNOS to the abnormalities identified during the progression of obesity. In early obesity, we found evidence of a significant increase rather than a reduction in basal NO using several different approaches. We demonstrated an increased vasoconstrictor response to the nonselective NOS inhibitor L-NMMA, an effect of obesity that was lost in iNOS KO mice. A role for iNOS was further supported by the effect of the specific iNOS inhibitor 1400W to increase tone in the aorta of obese mice, whereas no effect was seen in lean mice. Evidence for an increase in whole-body NO production was seen in the elevated plasma nitrite levels in obese mice, which were blunted in iNOS KO mice. Finally, iNOS expression was found to be significantly increased in the aorta of obese mice.

Previous studies in vessels exposed to inflammatory cytokines, mice rendered septic using injection of lipopolysaccharide (17) and iNOS gene transfer studies have all supported a role for iNOS-derived NO in causing vascular dysfunction (18,19). If one considers obesity to be an inflammatory condition analogous to low-grade sepsis, iNOS KO mice might be expected to be protected against classical endothelial dysfunction, as has been demonstrated to be the case in sepsis (17). However, this was not observed in the present study, where iNOS KO mice rendered obese still had evidence of ROS-mediated vasodilation masking covert endothelial dysfunction. In one intriguing finding, we observed that despite preserved glucocompetence, obese iNOS KO mice developed elevated blood pressures just as obese WT mice did.

Potential implications of the present study. Although the current study was conducted in mice, it could have clinically relevant implications in humans. If similar mechanisms are at play in human diet−induced obesity, iNOS-derived NO may be a reasonable target for slowing the progression of resistance to the metabolic and vascular actions of insulin. However, based on the current study, such an approach may not have favorable effects on blood pressure and endothelial dysfunction in obesity. Protecting the vasculature in obesity and insulin-resistant states is therefore likely to require an integrated approach, with total fat probably being a key factor.

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REFERENCES

1. Flegal KM, Carroll MD, Ogden CL, Johnson CL: Prevalence and trends in obesity among US adults, 1999–2000. *JAMA* 288:1723–1727, 2002
2. Hubert HB, Feinleib M, McNamara PM, Castelli WP: Obesity as an independent risk factor for cardiovascular disease: a 26-year follow-up of participants in the Framingham Heart Study. *Circulation* 67:968–977, 1983
3. Manson JE, Colditz GA, Stampfer MJ, Willett WC, Rosner B, Monson RR, Speizer FE, Hennekens CH: A prospective study of obesity and risk of coronary heart disease in women. *N Engl J Med* 322:882–889, 1990
4. Ross R. Atherosclerosis is an inflammatory disease. *N Engl J Med* 340: 115–126, 1999
5. Garg UC, Hassid A: Nitric oxide generating vasodilators and 8-bromo-cyclic guanosine monophosphate inhibit mitogenesis and proliferation of cultured rat vascular smooth muscle cells. *J Clin Invest* 83:1774–1777, 1989
6. Tanner FC, Meier P, Greutert H, Champion C, Nabel EG, Luscher TF: Nitric oxide modulates expression of cell cycle regulatory proteins: a cytostatic strategy for inhibition of human vascular smooth muscle cell proliferation. *Circulation* 101:1982–1989, 2000
7. Kubes P, Suzuki M, Granger DN: Nitric oxide: an endogenous modulator of leukocyte adhesion. *Proc Natl Acad Sci U S A* 88:4651–4655, 1991
8. Cooke JP, Dzau VJ: Nitric oxide synthase: role in the genesis of vascular disease. *Annu Rev Med* 48:489–509, 1997
9. 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
10. Williams IL, Wheatcroft SB, Shah AM, Kearney MT: Obesity, atherosclerosis and the vascular endothelium: mechanisms of reduced nitric oxide bioavailability in obese humans. *Int J Obes Relat Metab Disord* 26:754–764, 2002
11. Michel T, Feron O: Nitric oxide synthases: which, where, how, and why? *J Clin Invest* 100:2146–2152, 1997
12. Nathan C: Inducible nitric oxide synthase: what difference does it make? *J Clin Invest* 100:2417–2423, 1997
13. Iwashina M, Shichiri M, Marumo F, Hirata Y: Transfection of inducible nitric oxide synthase gene causes apoptosis in vascular smooth muscle cells. *Circulation* 98:1212–1218, 1998
14. Shimabukuro M, Ohneda M, Lee Y, Unger R: Role of nitric oxide in obesity-induced β cell disease. *J Clin Invest* 100:290–295, 1997
15. Perreault M, Marette A: Targeted disruption of inducible nitric oxide synthase protects against obesity-linked insulin resistance in skeletal muscle. *Nat Med* 7:1138–1143, 2001
16. Kessler P, Bauersachs J, Busse R, Schini-Kerth VB: Inhibition of inducible nitric oxide synthase restores endothelium-dependent relaxations in proinflammatory mediator-induced blood vessels. *Arterioscler Thromb Vasc Biol* 17:1746–1755, 1997
17. Chauhan SD, Seggare G, Vo PA, Macallister RJ, Hobbs AJ, Ahluwalia A:

Protection against lipopolysaccharide-induced endothelial dysfunction in resistance and conduit vasculature of iNOS knockout mice. *FASEB J* 17:773–775, 2003

18. Gunnett CA, Lund DD, Chu Y, Brooks RM II, Faraci FM, Heistad DD: Nitric oxide dependent vasorelaxation is impaired after gene transfer of inducible NO synthase. *Arterioscler Thromb Vasc Biol* 21:1281–1287, 2001
19. Gunnett, Heistad DD, Faraci FM: Gene targeted mice reveal a critical role for inducible nitric oxide synthase in vascular dysfunction during diabetes. *Stroke* 34:2970–2974, 2003
20. Hotamisligil GS, Arner P, Caro JF, Atkinson RL, Spiegelman BM: Increased adipose tissue expression of tumor necrosis factor-α in human obesity and insulin resistance. *J Clin Invest* 95:2409–2415, 1995
21. Mazurek T, Zhang L, Zalewski A, Mannion JD, Diehl JT, Arafat H, Sarov-Blat L, O'Brien S, Keiper EA, Johnson AG, Martin J, Goldstein BJ, Shi Y: Human epicardial adipose tissue is a source of inflammatory mediators. *Circulation* 108:2460–2466, 2003
22. Wheatcroft SB, Kearney MT, Shah AM, Grieve DJ, Williams IL, Miell JP, Crossey PA: Endothelial function and blood pressure homeostasis in mice overexpressing IGF binding protein-1. *Diabetes* 52:2075–2082, 2003
23. Wheatcroft SB, Shah AM, Li JM, Crossey PA, Noronah BT, Duncan E, Kearney MT: Preserved glucoregulation but loss of the vascular actions of insulin in mice heterozygous for a knockout for the insulin receptor. *Diabetes* 53:2645–2652, 2004
24. Li JM, Wheatcroft SB, Fan LM, Kearney MT, Shah AM: Opposing roles of $p47^{phox}$ in basal versus angiotensin II-stimulated alterations in vascular O_2^- production, vascular tone and MAPK activation. *Circulation* 109: 1307–1313, 2004
25. Garvey EP, Oplinger JA, Furfine ES, Kiff RJ, Laszlo F, Whittle BJ, Knowles RG: 1400W is a slow, tight binding, and highly selective inhibitor of inducible nitric-oxide synthase in vitro and in vivo. *J Biol Chem* 272:4959–4963, 1997
26. Li JM, Mullen AM, Yun S, Wientjes F, Brouns GY, Thrasher AJ, Shah AM: Essential role of the NADPH oxidase subunit p47 (phox) in endothelial cell superoxide production in response to phorbol ester and tumor necrosis factor-α. *Circ Res* 90:143–150, 2002
27. Matoba T, Shimokawa H, Nakashima M, Hirakawa Y, Mukai Y, Hirano K, Kanaide H, Takeshita A: Hydrogen peroxide is an endothelium-derived hyperpolarizing factor in mice. *J Clin Invest* 106:1521–1530, 2000
28. Ellis A, Pannirselvam M, Anderson TJ, Triggle CR: Catalase has negligible inhibitory effects on endothelium-dependent relaxations in mouse isolated aorta and small mesenteric artery. *Br J Pharmacol* 140:1193–1200, 2003
29. Landmesser U, Dikalov S, Price SR, McCann L, Fukai T, Holland SM, Mitch WE, Harrison DG: Oxidation of tetrahydrobiopterin leads to uncoupling of endothelial cell nitric oxide synthase in hypertension. *J Clin Invest* 111: 1201–1209, 2003
30. Li JM, Shah AM: Endothelial cell superoxide generation: regulation and relevance for cardiovascular pathophysiology. *Am J Physiol* 287:R1014–R1030, 2004
31. Griendling KK, Harrison DG: Dual role of reactive oxygen species in vascular growth. *Circ Res* 85:562–563, 1999
32. Ushio-Fukai M, Zafari AM, Fukui T, Ishizaka N, Griendling KK: p22phox is a critical component of the superoxide-generating NADH/NADPH oxidase system and regulates angiotensin II induced hypertrophy in vascular smooth muscle cells. *J Biol Chem* 271:23317–23321, 1996
33. Brodsky SV, Gealekman O, Chen J, Zhang F, Togashi N, Crabtree M, Gross SS, Nasjletti A, Goligorsky MS: Prevention of premature endothelial cell senescence and vasculopathy in obesity-induced diabetes by ebselen. *Circ Res* 94:377–384, 2004
34. Marfella R, Di Filippo C, Esposito K, Nappo F, Piegari E, Cuzzocrea S, Berrino L, Rossi F, Giugliano D, D'Amico M: Absence of inducible nitric oxide synthase reduces myocardial damage during ischemia reperfusion in streptozotocin-induced hyperglycemic mice. *Diabetes* 53:454–462, 2004
35. Boustany CM, Bharadwaj K, Daugherty A, Brown DR, Randall DC, Cassis LA: Activation of the systemic and adipose renin-angiotensin system in rats with diet-induced obesity and hypertension. *Am J Physiol* 287:R943–R949, 2004
36. Zimmerman MC, Lazartigues E, Lang JA, Sinnayah P, Ahmad IM, Spitz DR, Davisson RL: Superoxide mediates the actions of angiotensin II in the central nervous system. *Circ Res* 91:1038–1045, 2002
37. Wilcox CS: Reactive oxygen species: roles in blood pressure and kidney function. *Curr Hypertens Rep* 4:160–166, 2002
38. Xu H, Barnes GT, Yang Q, Tan G, Yang D, Chou CJ, Sole J, Nichols A, Ross JS, Tartaglia LA, Chen H: Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. *J Clin Invest* 112:1821–1830, 2003
39. Weisberg SP, McCann D, Desai M, Rosenbaum M, Leibel RL, Ferrante AW Jr: Obesity is associated with macrophage accumulation in adipose tissue. *J Clin Invest* 112:1796–1808, 2003
40. Bedard S, Marcotte B, Marette A: Cytokines modulate glucose transport in skeletal muscle by inducing the expression of inducible nitric oxide synthase. *Biochem J* 325:487–493, 1997
41. Hofseth LJ, Saito S, Hussain SP, Espey MG, Miranda KM, Araki Y, Jhappan C, Higashimoto Y, He P, Linke SP, Quezado MM, Zurer I, Rotter V, Wink DA, Appella E, Harris CC: Nitric oxide-induced cellular stress and p53 activation in chronic inflammation. *Proc Natl Acad Sci U S A* 100:143–148, 2003