Abnormal Biopterin Metabolism Is a Major Cause of Impaired Endothelium-Dependent Relaxation Through Nitric Oxide/O_2^- Imbalance in Insulin-Resistant Rat Aorta

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To investigate underlying mechanisms responsible for the impaired nitric oxide (NO)-dependent vascular relaxation in the insulin-resistant state, we examined production of both NO and superoxide anion radical (O_2^-) and those modulating factors in aortas obtained from normal (CTR), insulin-treated (INS), or high fructose-fed (FR) rats. FR rats showed insulin resistance with endogenous hyperinsulinemia, whereas INS rats showed normal insulin sensitivity. Only FR aortic strips with endothelium elicited impaired relaxation in response to either acetylcholine or calcium ionophore A23187. Endothelial NO synthase (eNOS) activity and its mRNA levels were increased only in vessels from INS rats ($P < 0.001$), whereas eNOS activity in FR rats was decreased by 58% ($P < 0.05$) when compared with CTR rats. NO production from aortic strips stimulated with A23187 was significantly lower in FR than CTR rats. In contrast, A23187-stimulated O_2^- production was higher ($P < 0.01$) in FR than CTR rats. These differences were abolished when aortic strips were preincubated in the media including (6R)-5,6,7,8-tetrahydrobiopterin (BH_4), an active cofactor for eNOS. Furthermore, as compared with CTR rats, aortic BH_4 contents in FR rats were decreased ($P < 0.001$), whereas the levels of 7,8-dihydrobiopterin, the oxidized form of BH_4, were increased, with opposite results in INS rats. These results indicate that insulin resistance rather than hyperinsulinemia itself may be a pathogenic factor for decreased vascular relaxation through impaired eNOS activity and increased oxidative breakdown of NO due to enhanced formation of O_2^- (NO/O_2^- imbalance), which are caused by relative deficiency of BH_4 in vascular endothelial cells. *Diabetes* **48:2437–2445, 1999**

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ANOVA, analysis of variance; BH_2, dihydrobiopterin; BH_4, (6R)-5,6,7,8-tetrahydrobiopterin; ED_{50}, 50% of maximal effective dose; eNOS, endothelial nitric oxide synthase; HBSS, Hanks' balanced salt solution; HPLC; high-performance liquid chromatography; L-NAME, N^G-nitro-L-arginine methyl ester; MH_4, 6-methyl-5,6,7,8-tetrahydropterin; O_2^-, superoxide anion radical; SOD, superoxide dismutase; SSPG, steady-state plasma glucose; SSPI, steady-state plasma insulin.

Several epidemiological studies (1–3) indicate a close relationship between plasma insulin level and cardiovascular diseases, supporting the idea of hyperinsulinemia as an atherogenic factor (4,5). In 1969, Stout (5) raised the possibility that chronic hyperinsulinemia may contribute to the development of atherosclerosis by a direct effect of insulin on the artery. However, regarding the mechanisms of insulin-induced atherogenesis, considerable controversy exists, and hyperinsulinemia has been questioned as a causative factor for atherosclerotic disease (6,7). Because hyperinsulinemia is often associated with insulin resistance in both type 1 diabetic and nondiabetic subjects, the association between high plasma insulin level and atherosclerosis may be due to insulin resistance but not insulin concentration per se. We have recently shown that insulin resistance promotes the development of vasospastic angina (8) as well as obstructive coronary artery disease (9) in nondiabetic subjects. Laakso et al. (10), using the euglycemic insulin clamp technique, first demonstrated the presence of insulin resistance in patients with asymptomatic atherosclerosis (stenotic lesion) in the femoral or carotid arteries. In addition, abnormalities in endothelium-dependent arterial relaxation are also described in several insulin-resistant states, including hypertension (11) and obesity (12). These findings argue against the idea that insulin is a factor of increasing cardiovascular morbidity and suggest that endogenous and exogenous hyperinsulinemia have qualitatively different effects on atherogenic processes. However, the mechanisms responsible for the association between the insulin-resistant state and impaired endothelium-dependent vasodilatory response are poorly understood, and to date, no studies have addressed this question directly.

There are now several lines of evidence linking excess vascular oxidative stress to the impairment of nitric oxide (NO) action in patients with diabetes or hypertension (13,14). Endothelial nitric oxide synthase (eNOS) constitutively produces both NO and superoxide anion radical (O_2^-), suggesting that the effective release of NO from the vascular endothelium depends on the relative concentrations of these two species (15). Furthermore, evidence indicates that (6R)-5,6,7,8-tetrahydrobiopterin (BH_4), which is an important allosteric effector of NOS (16) through stabilization of the dimeric active form of the enzyme (17), may play a key role for the con-

trol of endothelial NO and O_2^- production in vivo. In fact, it has been demonstrated that blood vessels depleted of BH_4 produce O_2^- because of uncoupled oxygen activation (18). In contrast, BH_4 has been used to reverse vascular dysfunction induced by diabetes (19) and hypercholesterolemia (20), suggesting that BH_4 levels are compromised in these conditions. In fact, in adrenal glands, insulin stimulates BH_4 synthesis through activation of GTP cyclohydrolase I, the rate-limiting enzyme in de novo synthesis of BH_4, and BH_4 synthesis is suggested to be decreased in the insulin-deficient state (21).

Thus, we hypothesized that decreased NO-dependent vasodilation in the insulin-resistant state could be related to relative deficiency of BH_4, resulting in the aforementioned functional disturbance of eNOS and impaired endothelium-dependent relaxation. To test our hypothesis, we investigated the endothelial function, production of both NO and O_2^-, and contents of BH_4 and 7,8-dihydrobiopterin, an inactive oxidized form of BH_4, with the use of isolated thoracic aortas obtained from exogenous and endogenous hyperinsulinemic rats.

RESEARCH DESIGN AND METHODS

Materials. The insulin pellet was obtained from Linshin Canada (Ontario, Canada). Acetylcholine chloride was provided by Dai-ichi Pharmaceutical (Tokyo). Papaverine hydrochloride was obtained from Dainippon (Osaka, Japan). Concanavalin A (Con A)-sepharose was obtained from Amersham Pharmacia Biotech (Buckinghamshire, U.K.). L-[^{3}H]Arginine and [^{32}P]dCTP were purchased from New England Nuclear Research Products (Boston, MA). In vitro transcription kits (MAXIscript) and RNase protection assay kits (HybSpeed RPA) were purchased from Ambion (Austin, TX), and pCRII vector was from Invitrogen (San Diego, CA). 7,8-Dihydrobiopterin was obtained from Cayman Chemical (Ann Arbor, MI). Nitrate ion standard solution was obtained from Kanto Chemical (Tokyo). All other materials were reagent grade and purchased from Sigma (St. Louis, MO).

Animals. Male Sprague-Dawley rats (Japan SLC, Shizuoka, Japan) weighing 150 g were housed in an environmentally controlled room with a 12-h light/dark cycle and free access to a laboratory diet and water. The animals were divided into three groups and fed ad libitum on one of the following diets for 4 weeks: *1*) a normal diet (normal diet–fed), *2*) a normal diet with insulin infusion (insulin-treated), or *3*) a diet high in fructose (fructose-fed). The normal diet (Oriental Yeast, Tokyo) consisted of 58% carbohydrate (no fructose), 12% fat, and 30% protein (energy percent of diet). The high-fructose diet (Oriental Yeast) contained 67% carbohydrate (98% of which was fructose), 13% fat, and 20% protein by calorie. The high fructose–fed rats were used as an animal model for the common type of insulin resistance with endogenous hyperinsulinemia (22). For continuous delivery of insulin, an incision was made in the midscapular region, and one piece of insulin pellet (release rate 1.0 U/day for 4 weeks) was implanted in the back of the rats.

Measurement of blood pressure. Blood pressure was measured the day before the experiment, and the rats were trained to the apparatus three times before measurement. Systolic and diastolic blood pressure in the tail region were measured using an electrosphygmomanometer after the rats were prewarmed for 15 min.

Assessment of in vivo insulin action. Insulin sensitivity was measured by the steady-state plasma glucose (SSPG) method with the use of somatostatin, originally described by Harano et al. (23). Rats were administered with infusate containing somatostatin ($120\ \mu g \cdot kg^{-1} \cdot h^{-1}$), glucose ($1.0\ g \cdot kg^{-1} \cdot h^{-1}$), and insulin ($2.0\ U \cdot kg^{-1} \cdot h^{-1}$; Human Actrapid, Novo Nordisk) at a flow rate of 2.8 ml/h for 120 min. The SSPG levels and steady-state plasma insulin (SSPI) levels were measured at 120 min after the infusion. Plasma glucose and serum total cholesterol, triglyceride, and free fatty acid concentrations were measured by the standard enzymatic methods, and insulin was measured by radioimmunoassay using anti-rat insulin antibody.

Isometric tension studies. The thoracic aorta (0.6–0.8 cm outside diameter) was isolated and cut into strips with special care to preserve the endothelium. The specimens were suspended in organ chambers as previously described (24). To prevent synthesis of prostaglandins, the following experiments were performed in the presence of 10 μmol/l indomethacin. The strips were partially precontracted with L-phenylephrine ($1–3 \times 10^{-7}$ mol/l). After a plateau was attained, the strips were exposed to acetylcholine (10^{-9} to 10^{-5} mol/l), the calcium ionophore A23187 (10^{-9} to 10^{-7} mol/l), or sodium nitroprusside (10^{-11} to 10^{-6} mol/l) to construct dose-response curves. At the end of each experiment, 100 μmol/l papaverine was added to induce maximal relaxation, which was taken as 100% for relaxation induced by agonists. In some strips, the endothelium was removed by gently rubbing the intimal surface with a cotton ball. Endothelium removal was verified by abolition or marked suppression of the relaxations caused by 1 μmol/l acetylcholine.

Measurement of NO synthase activity in aortic endothelial cells. eNOS activity was measured by the conversion of L-[^{3}H]arginine to L-[^{3}H]citrulline as previously described (25). Segments of thoracic aorta (40 mm) were isolated as described above and cut longitudinally. The endothelial cells were removed with a plastic scraper and then homogenized in buffer (pH 7.4) containing 50 mmol/l Tris, 3.2 mmol/l sucrose, 1 mmol/l dithiothreitol, 10 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor, and 2 μg/ml aprotinin using a glass homogenizer. The Ca^{2+}-dependent enzyme (eNOS) activity was determined as the difference between the L-[^{3}H]citrulline generated from control samples without EGTA and those containing 3 mmol/l EGTA. Values were corrected by the amount of protein present.

Assessment of eNOS mRNA. Total RNA was prepared from thoracic aorta using the method of Chomczynski and Sacchi (26). eNOS mRNA content was determined by a RNase protection assay using an eNOS probe. A partial cDNA corresponding to nucleotides 1–189 of rat eNOS was subcloned into the pCRII vector (27). This was linealized with *Hin*dIII and labeled antisense riboprobe made with T7 RNA polymerase with [^{32}P]dCTP using the in vitro transcription kit. RNase protection assay was done with a direct protect lysate assay kit (Ambion). After autoradiography, the relative density of each band was determined using laser densitometry. The density of each band was normalized to rat β-actin mRNA content.

Measurement of NO. The concentration of NO in the aortic tissues was determined with a highly sensitive NO measurement system (FES-450; Scholar-Tec, Osaka, Japan) (28). Detection of NO with this system is based on observing chemiluminescence (660–900 nm) produced by the reaction of NO with ozone. For measurement of nitrite (NO_2^-) plus nitrate (NO_3^-) (oxidation products of NO) contents, NO_3^- was reduced to NO_2^- with a 0.2 U/ml *Aspergillus* nitrate reductase in the presence of 0.1 μmol/l flavin adenine dinucleotide and 10 μmol/l NADPH. An isolated open vascular ring was placed in the sampling tube with 2.0 ml fresh, phenol red–free Hanks' balanced salt solution (HBSS) (pH 7.4) containing (in mmol/l) NaCl 136, KCl 5.40, $CaCl_2$ 1.27, $MgSO_4$ 0.83, $Na_2HPO_4\ 7H_2O$ 0.33, $NaHCO_3$ 4.16, KH_2PO_4 0.44, $MgCl_2\ 6H_2O$ 0.49, and glucose 5.6, and incubated at 37°C for 1 h in the presence or absence of 10 μmol/l A23187. The experiment was also performed after incubation for 1 h with either 1 μmol/l BH_4 or 10 μmol/l N^G-nitro-L-arginine methyl ester (L-NAME) in HBSS. Then, the nitrite was reduced to NO by adding the 2-ml solution saturated with ascorbic acid into 1.0-ml sample solutions. The absolute concentration of NO in the sample was determined by a reference standard solution of nitrate ion.

Measurement of ex vivo aortic O_2^- production. O_2^- production in aortic segments was measured using the lucigenin-enhanced chemiluminescence method (29,30) and confirmed by the cytochrome *c* method (31). Segments of thoracic aorta (20 mm) were isolated as described above and placed in modified Krebs/HEPES buffer (pH 7.4) containing (in mmol/l) NaCl 99.01, KCl 4.69, $CaCl_2$ 1.87, $MgSO_4$ 1.20, K_2HPO_4 1.03, $NaHCO_3$ 25, Na-HEPES 20, and glucose 11.1, and allowed to equilibrate for 30 min at 37°C. After 5 min of dark adaptation, scintillation vials containing 2 ml Krebs/HEPES buffer with 50 μmol/l lucigenin were placed into a scintillation counter (TRI-CARB1500, Packard Instrument, Meriden, CT) switched to the out-of-coincidence mode. Lucigenin counts were expressed as counts per minute per milligram of dry weight of vessel. Background counts were determined by vessel-free incubations and subtracted from the readings obtained using vessels.

To confirm the validity of the lucigenin chemiluminescence method, vascular O_2^- production was also measured by superoxide dismutase inhibitable reduction of succinoylated cytochrome *c*, according to Kuthan and Ullrich (31). The rate of O_2^- production was calculated on the basis of the molar extinction coefficient of succinoylated cytochrome *c* and the portion that was inhibited by Cu,Zn-SOD (400 U/ml). The reduction of succinoylated cytochrome *c* was monitored in a spectrophotometer using the wavelength pair 550 minus 557 nm with the cuvette compartment maintained at 25°C. The calculation was based on an absorption coefficient of $E_m = 21\ mmol \cdot l^{-1} \cdot cm^{-1}$.

Biopterin content and GTP cyclohydrolase I activity in vascular tissues. Aortic tissues with or without endothelium were homogenized in 25 mmol/l triethanolamine-HCl buffer (pH 7.4), and measurements of biopterin content in aortic extract were performed by high-performance liquid chromatography (HPLC) analysis as previously described (32–34). Because the quinonoid form of dihydrobiopterin (qBH_2) is quite unstable and rapidly rearranged nonenzymatically to the more stable 7,8-BH_2 (34), the amount of BH_4 was estimated from the difference between the total (acid oxidized biopterin level = $BH_4 + BH_2$ + oxidized biopterin) and alkaline-stable biopterin (alkaline oxidized biopterin level = BH_2 + oxidized biopterin). GTP cyclohydrolase I activity was assayed using the HPLC method by measurements of neopterin, which was released from dihydroneopterin triphosphate after oxidation and phosphatase treatment (33).

Protein assay. Protein content was determined by the method of Bradford (35) with bovine serum albumin as a standard.

Statistical analysis. All values are presented as means ± SE. The dose-dependent vascular relaxation in response to either acetylcholine, A23187, or sodium

TABLE 1
Metabolic characteristics and blood pressure levels of the rats

	Normal diet–fed	Fructose-fed	Insulin-treated
Baseline data (n = 9 for each group)			
Weight (g)	320 ± 6	337 ± 6	332 ± 11
Glucose (mmol/l)	5.43 ± 0.18	5.40 ± 0.09#	4.36 ± 0.26‡
Insulin (pmol/l)	119.1 ± 7.9	253.9 ± 10.0†**	462.8 ± 73.1§¶
Total cholesterol (mmol/l)	1.70 ± 0.09	2.06 ± 0.06*‖	1.62 ± 0.11
Triglyceride (mmol/l)	1.33 ± 0.19	3.34 ± 0.39§**	0.67 ± 0.11
Free fatty acid (mmol/l)	0.15 ± 0.02	0.46 ± 0.08‡#	0.21 ± 0.03
Systolic blood pressure (mmHg)	117 ± 2	140 ± 4‡#	119 ± 3
Diastolic blood pressure (mmHg)	71 ± 3	84 ± 2‡#	73 ± 6
Insulin sensitivity test (n = 3 for each group)			
SSPG (mmol/l)	7.10 ± 0.38	10.8 ± 0.94†¶	6.71 ± 0.27
SSPI (pmol/l)	4,570 ± 631	5,044 ± 358	5,618 ± 559

Data are means ± SE. *P < 0.05, †P < 0.01, ‡P < 0.001, §P < 0.0001 vs. normal diet–fed rats. ‖P < 0.05, ¶P < 0.01, #P < 0.001, **P < 0.0001 vs. insulin-treated rats.

nitroprusside was compared among three groups using repeated-measures analysis of variance (ANOVA). Vascular responses were compared among three groups with two-way ANOVA. Comparisons for metabolic data, blood pressure, chemiluminescence, superoxide scavenging activity, and NO synthase activity were performed using ANOVA with a post hoc Scheffe's comparison. P values <0.05 were considered significant.

RESULTS

Metabolic characteristics and blood pressure of the rats. As shown in Table 1, all three treatment groups gained weight to a similar degree over the study period without any significant difference. Animals receiving insulin infusion (1.0 U/day) for 4 weeks demonstrated a significant reduction in plasma glucose levels when compared with either normal diet–fed or fructose-fed rats. Both insulin-treated and fructose-fed rats had significant increases in plasma insulin levels compared with normal diet–fed rats. Fructose-fed rats also showed a significant elevation of plasma total cholesterol, triglyceride, free fatty acid levels, and blood pressure compared with either normal diet–fed or insulin-treated rats, respectively. The fructose-fed rats exhibited a significant increase in SSPG level when compared with the other groups without difference in SSPI levels.

NO-dependent vascular relaxation in rat aorta. The addition of acetylcholine at concentrations of 10^{-9} to 10^{-5} mol/l produced a dose-dependent relaxation in aortic strips with endothelium (Fig. 1). In the aorta isolated from normal diet–fed and insulin-treated rats, the maximal relaxation to acetylcholine and 50% of maximal effective dose (ED_{50}) values did not significantly differ (Table 2). On the other hand, the maximal response was significantly reduced, and the ED_{50} values were increased in the aorta derived from fructose-fed rats as compared with those in the aortas from the other groups. The dose-response curve was also significantly shifted to the right in the fructose-fed rat aorta (Fig. 1). Similar results were also obtained for the relaxation induced by A23187, a receptor-independent endothelium-derived relaxing factors–releasing substance, among the three rat groups

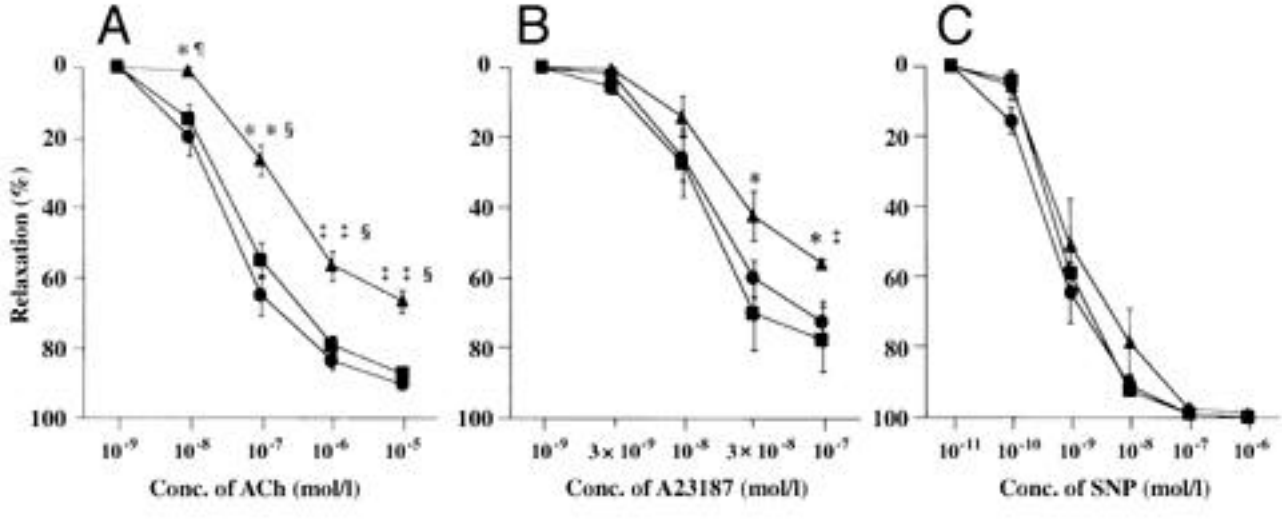

FIG. 1. Vasodilator responses to acetylcholine (ACh) (*A*), calcium ionophore A23187 (*B*), or sodium nitroprusside (SNP) (*C*) in aortic strips with endothelium from normal diet–fed (■; n = 7), fructose-fed (▲; n = 7), and insulin-treated (●; n = 7) rats. The strips were partially precontracted with L-phenylephrine. Relaxation induced by 100 µmol/l papaverine was taken as 100%. Data are expressed as means ± SE. Significantly different from the value in the strips from normal diet–fed rats: *P < 0.05, **P < 0.001, ‡‡P < 0.0001. Significantly different from the value in the strips from insulin-treated rats: ‡P < 0.05, ¶P < 0.01, §P < 0.0001. Conc., concentration of each drug.

TABLE 2
Endothelium-dependent and -independent vasorelaxation in aortic segments with endothelium from normal diet–fed, fructose-fed, and insulin-treated rats

	Normal diet–fed	Fructose-fed	Insulin-treated
Acetylcholine			
ED_{50} (nmol/l)	70.4 ± 22.3	193.8 ± 36.5†‖	51.3 ± 19.9
Maximal relaxation (%)	87.3 ± 1.5	66.6 ± 3.2‡¶	90.3 ± 1.4
A23187			
ED_{50} (nmol/l)	15.3 ± 1.6	26.1 ± 4.2*§	17.1 ± 3.0
Maximal relaxation (%)	77.8 ± 8.4	56.3 ± 1.5*§	72.7 ± 4.9
Sodium nitroprusside			
ED_{50} (nmol/l)	0.84 ± 0.16	0.81 ± 0.17	0.90 ± 0.37
Maximal relaxation (%)	100	99.0 ± 0.6	100

Data are means ± SE. n = 7 for all groups. ED_{50} is a concentration that produces 50% of the maximal response to each drug. Maximal relaxation is expressed as a percentage of relaxation induced by 10^{-4} mol/l papaverine. *P < 0.05, †P < 0.01, ‡P < 0.0001 vs. normal diet–fed rats. §P < 0.05, ‖P < 0.001, ¶P < 0.0001 vs. insulin-treated rats.

(Table 2). On the other hand, vasodilator responses to sodium nitroprusside were comparable among the three groups (Fig. 1). Treatment with 100 µmol/l L-NAME as well as removal of the endothelium abolished the acetylcholine-induced relaxation in endothelium-intact aortic strips obtained from all three groups (data not shown).

In the aortic strips with the intact endothelium obtained from normal diet–fed and insulin-treated rats that were preincubated with 200 U/ml Cu,Zn-SOD for 30 min, relaxation in response to acetylcholine and A23187 was slightly enhanced, and a greater enhancement of the response by Cu,Zn-SOD was observed in the aortas from fructose-fed rats (Fig. 2 and Table 3). In the presence of Cu,Zn-SOD, the magnitudes of relaxation to either acetylcholine or A23187 was similar among the three groups. However, vasodilator responses to sodium nitroprusside were not affected by the pretreatment of

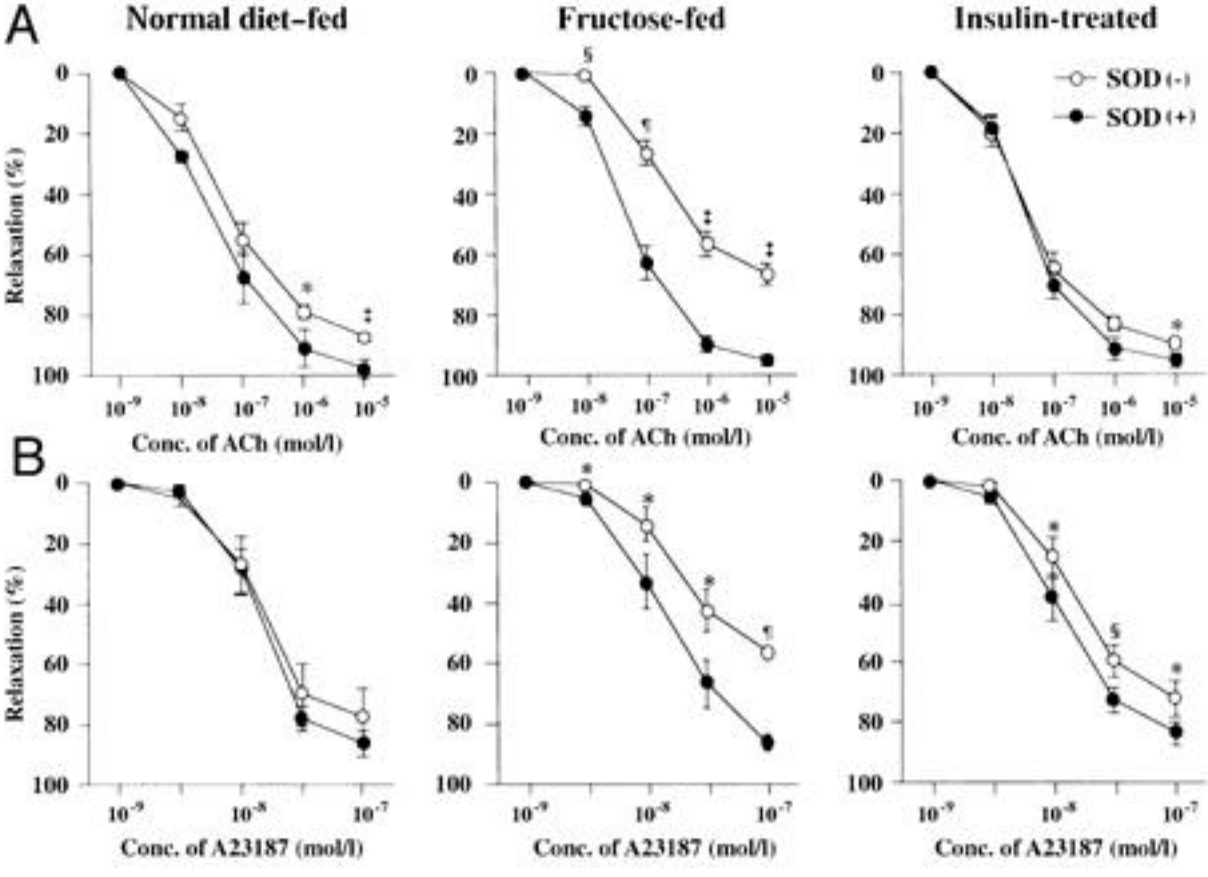

FIG. 2. Effect of SOD on relaxation induced by acetylcholine (ACh) (*A*) or calcium ionophore A23187 (*B*) in aortic strips with endothelium obtained from normal diet–fed (n = 7), fructose-fed (n = 7), and insulin-treated (n = 7) rats. The vessels from each group were pre-exposed for 30 min to bathing medium either with (●) or without (○) SOD (200 U/ml). The strips were partially precontracted with L-phenylephrine. Relaxations induced by 100 µmol/l papaverine were taken as 100%. Data are expressed as means ± SE. Significantly different from the value in the presence of SOD: *P < 0.05, §P < 0.01, ¶P < 0.001, ‡P < 0.0001. Conc., concentration of each drug.

TABLE 3
Effects of SOD on ED_{50} and maximal relaxations to acetylcholine and A23187

	Normal diet–fed		Fructose-fed		Insulin-treated	
	SOD^-	SOD^+	SOD^-	SOD^+	SOD^-	SOD^+
Acetylcholine						
ED_{50} (nmol/l)	56.1 ± 18.6	52.1 ± 18.6	192.4 ± 37.6	60.1 ± 16.7†	52.2 ± 17.3	38.5 ± 3.6
Maximal relaxation (%)	88.2 ± 1.6	98.1 ± 1.1§	65.3 ± 2.9	95.5 ± 4.5§	90.1 ± 1.6	95.4 ± 2.4*
A23187						
ED_{50} (nmol/l)	14.8 ± 1.5	11.6 ± 1.8	25.0 ± 4.7	12.9 ± 4.1†	19.2 ± 3.6	13.2 ± 3.3
Maximal relaxation (%)	73.9 ± 7.7	82.9 ± 4.5	57.1 ± 1.4	86.5 ± 2.6‡	70.8 ± 4.3	85.9 ± 2.0*
Sodium nitroprusside						
ED_{50} (nmol/l)	0.86 ± 0.15	0.84 ± 0.18	0.83 ± 0.17	0.82 ± 0.21	0.92 ± 0.34	0.88 ± 0.19
Maximal relaxation (%)	100	99.6 ± 0.5	99.1 ± 0.5	100	100	100

Data are means ± SE. $n = 6$ for all groups. ED_{50} is a concentration that produces 50% of the maximal response to each drug. Maximal relaxation is expressed as a percentage of relaxation induced by 10^{-4} mol/l papaverine. *$P < 0.05$, †$P < 0.01$, ‡$P < 0.001$, §$P < 0.0001$ vs. the corresponding vessels with endothelium incubated in buffer alone (SOD^-). In the presence of SOD, there were no significant differences in either ED_{50} or maximal relaxation among the three groups analyzed by multiple comparison test using ANOVA with a post hoc Scheffe's comparison.

Cu,Zn-SOD in any groups, and the responses in the presence of SOD were comparable among the three rat groups (Table 3).

NO synthase activity and its mRNA levels in aortic endothelium. The activity of the Ca^{2+}-dependent NO synthase from aortic endothelium in insulin-treated rats was 2.9- and 6.8-fold higher ($P < 0.0001$) than that of normal diet–fed and fructose-fed rats, respectively (Fig. 3*A*). In contrast, the enzyme activity in fructose-fed rats was significantly decreased when compared with normal diet–fed rats ($P < 0.05$). There was no significant difference in Ca^{2+}-independent NO synthase activity in endothelial homogenate among the three rat groups.

We also determined eNOS mRNA levels in the aorta using RNase protection assay (Fig. 3*B* and *C*). eNOS mRNA levels of insulin-treated rats were 2.5- and 1.9-fold higher than those of normal diet–fed and fructose-fed rats, respectively ($P < 0.01$). Interestingly, the mRNA level in fructose-fed rats was not significantly higher than that of normal diet–fed rats. However, eNOS mRNA was not detected in endothelium-denuded samples (data not shown).

NO production and its regulation through tetrahydrobiopterin, a cofactor of eNOS. The basal NO release from aorta in insulin-treated rats was 2.2- and 3.9-fold higher than that of normal diet–fed and fructose-fed rats, respectively. After stimulation with A23187, NO production in fructose-fed rats was significantly lower than that in either normal diet–fed or insulin-treated rats, respectively (Fig. 4). Neither basal nor A23187-stimulated NO release were affected after incubation with 200 U/ml Cu,Zn-SOD. However, after preincubation of the vessels with 10 µmol/l L-NAME, A23187-stimulated NO production was inhibited to the basal level and the differences among the three groups disappeared.

To determine factors that regulate NO production in the insulin-resistant state, we measured NO release in the presence of 1 µmol/l BH_4, an active cofactor for NOS (Fig. 4). In normal diet–fed rats and insulin-treated rats, exogenous BH_4 and 6-methyl-5,6,7,8-tetrahydropterin (MH_4), the lipid-soluble analog of BH_4, did not significantly stimulate NO production. However, in fructose-fed rats, addition of BH_4 and MH_4 increased NO production approximately threefold ($P < 0.01$), and there were no differences between normal diet–fed and fructose-fed rats in terms of NO production. In contrast, in insulin-treated rats, addition of BH_4 did not further increase NO production. In contrast, addition of 10 µmol/l 7,8-BH_2, an inactive oxidized form of BH_4, did not affect A23187-stimulated NO production in either normal diet–fed or fructose-fed rats, but significantly ($P < 0.01$) inhibited the NO release in insulin-treated rats (Fig. 4). Addition of 1 µmol/l vitamin C or 1 µmol/l glutathione did not affect the A23187-stimulated NO release in the three groups (Fig. 4). Furthermore, after removal of the endothelium, the rate of NO production was markedly depressed and was no longer different among the three groups.

Superoxide anion generation from aortas with or without endothelium. As shown in Table 4, basal O_2^- production by aortic segments from either insulin-treated or fructose-fed rats was significantly higher than that from normal diet–fed rats ($P < 0.05$). Endothelial removal produced 34% reduction of O_2^- levels in vessels from normal diet–fed rats, while 72% reduction of O_2^- production was found in the endothelium-denuded vessels obtained from both fructose-fed and insulin-treated rats. Thus, after removal of the endothelium, the O_2^- production rate among the three groups no longer differed. After stimulation with A23187, O_2^- production in all three groups significantly increased, whereas the magnitude of the increment in fructose-fed rats was 183% of the basal, which was significantly greater ($P < 0.01$) than that of either normal diet–fed (58% of the basal) or insulin-treated rats (52% of the basal). Incubation of intact aortic strips with Cu,Zn-SOD significantly attenuated the lucigenin signal. O_2^- production stimulated by A23187 was not affected after incubation with either L-NAME or BH_4 in both normal diet–fed and insulin-treated rats. However, in fructose-fed rats, A23187-stimulated O_2^- production was significantly decreased to its basal level after incubation with either L-NAME or BH_4.

To confirm the validity of the lucigenin method for measurement of O_2^- in our systems, we also measured vascular basal O_2^- production by the cytochrome *c* method. O_2^- production by aortic segments ($n = 4$) from insulin-treated rats (2.49 ± 0.16 nmol · min^{-1} · mg^{-1} of dry weight of vessel, $P < 0.05$) and fructose-fed rats (3.57 ± 0.32, $P < 0.01$) was significantly higher than that of normal diet–fed rats (1.48 ± 0.28).

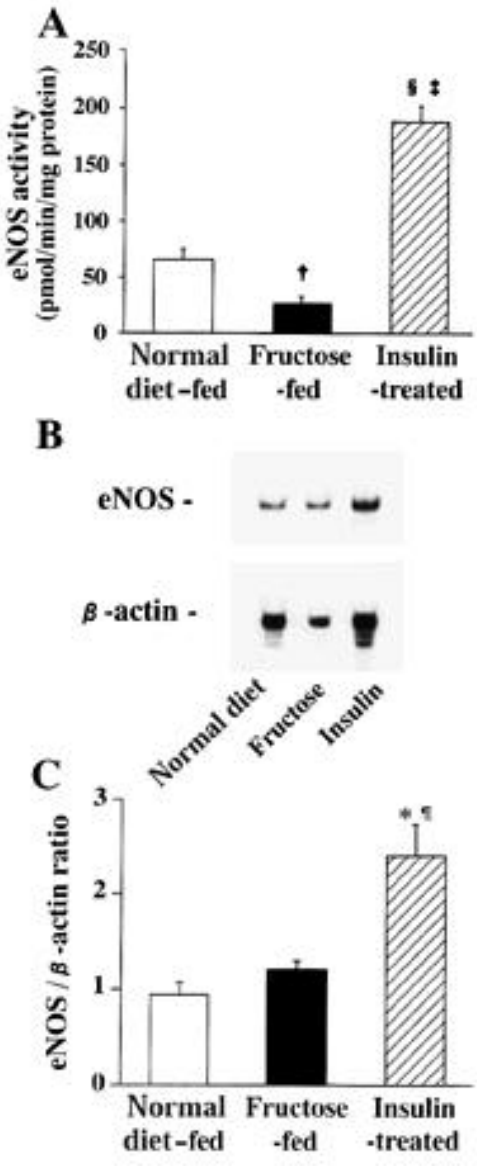

FIG. 3. *A*: **eNOS activity and mRNA expression in aortas from normal diet–fed, fructose-fed, and insulin-treated rats. The activity is expressed as picomoles per minute per milligram protein. Data are expressed as means ± SE of duplicated determinations from five different experiments.** *B*: **A set of samples from three different rats. Protected bands corresponding to eNOS and β-actin are indicated.** *C*: **The results of the RNase protection assay expressed as eNOS versus β-actin ratio: 10 µg of RNA from each aorta was analyzed by a RNase protection assay as described in RESEARCH DESIGN AND METHODS. As a control, 10 µg of rat β-actin was also analyzed. The results are expressed as means ± SE of four different experiments. †$P < 0.05$, *$P < 0.001$, §$P < 0.0001$ vs. normal diet–fed rats. ¶$P < 0.01$, ‡$P < 0.0001$ vs. fructose-fed rats.**

These results were comparable to the results obtained by the lucigenin method.

Biopterin levels in aortic tissue. In the presence of endothelium, the BH_4 level of insulin-treated rats was 1.9-fold ($P < 0.001$) higher than that of normal diet–fed rats, whereas the level in fructose-fed rats was 10% ($P < 0.001$) lower than that of normal diet–fed rats (Fig. 5*A*). In contrast to the results for BH_4, the tissue level of biopterin plus 7,8-BH_2, an oxidized form of BH_4, in fructose-fed rats was 2.4-fold ($P < 0.01$) and 7.3-fold ($P < 0.001$) higher than that of either normal diet–fed or insulin-treated rats, respectively (Fig. 5*B*). Thus, total biopterin content in normal diet–fed rats was significantly less than that of either fructose-fed or insulin-treated rats (Fig. 5*C*). The ratio of BH_4 to 7,8-BH_2 plus biopterin of intact vessels was significantly lower in fructose-fed rats than in normal diet–fed or insulin-treated rats (Fig. 5*D*). Endothelial removal produced a significant reduction of biopterin levels in each group of vessels (68–73% less than that in the intact vessel). The ratio of BH_4 to 7,8-BH_2 plus biopterin of endothelium in normal diet–fed, fructose-fed, and insulin-treated rats was calculated to be 5.83 ± 0.02, 1.56 ± 0.01, and 23.9 ± 0.02, respectively.

We measured the activity of GTP cyclohydrolase I, the rate-limiting enzyme in the de novo synthesis of BH_4, in vascular tissues. The enzyme activity of insulin-treated rats (5.48 ± 0.19 pmol/mg protein) was significantly higher than that of normal diet–fed rats (4.79 ± 0.10 pmol/mg protein, $P < 0.05$), whereas the activity in fructose-fed rats (3.31 ± 0.11 pmol/mg protein, $P < 0.001$) was significantly lower than that of normal diet–fed rats.

DISCUSSION

The central hypothesis of our laboratories is that insulin resistance but not insulin concentration per se induces endothelial dysfunction and promotes the development of atherosclerosis (8,9). In the present study, high fructose–fed rats, which are known to demonstrate impaired insulin action in both skeletal muscle and liver (22), showed a significant impairment of endothelium-dependent vasodilatation when compared with either normoinsulinemic rats or insulin-treated rats with exogenous hyperinsulinemia. However, the responsiveness of vascular smooth muscle cells to endothelium-derived NO is not impaired in high fructose–fed rats, since the vasodilatory response to sodium nitroprusside did not differ among the three groups. Consistent with our results, it has been reported that insulin receptor substrate (IRS)-1–deficient mice and Otsuka-Long-Evans Tokushima Fatty (OLETF) rats with insulin resistance also show a similar impairment of endothelium-dependent vasorelaxation (36,37). However, the underlying mechanisms responsible for the relationship between insulin-resistant state and endothelial dysfunction are not known.

Interestingly, the present study indicated that endothelium-dependent vasodilation was restored by a scavenger of O_2^- in these insulin-resistant rats. In the present study, L-NAME not only inhibited the generation of NO, but also suppressed the increased O_2^- production in fructose-fed rats. Thus, in this insulin-resistant model (endogenous hyperinsulinemia), the reduced endothelium-dependent relaxation is explained by mechanisms of a reduced NO production due to decreased NO synthase activity as well as accelerated degradation of NO by O_2^- within the vascular wall. Endothelial BH_4 levels in fructose-fed rats were significantly decreased, and BH_4 administration restored NO production as well as reduction of O_2^- production in the presence of A23187. Furthermore, despite its strong O_2^- scavenging activity, 1 µmol/l vitamin C did not affect NO production (Fig. 4). These findings suggest that exogenously added BH_4 increases NO generation as well as normalizes eNOS-dependent O_2^- production mainly through activation of eNOS in the BH_4-depleted conditions rather than by serving as a "reducing agent." During

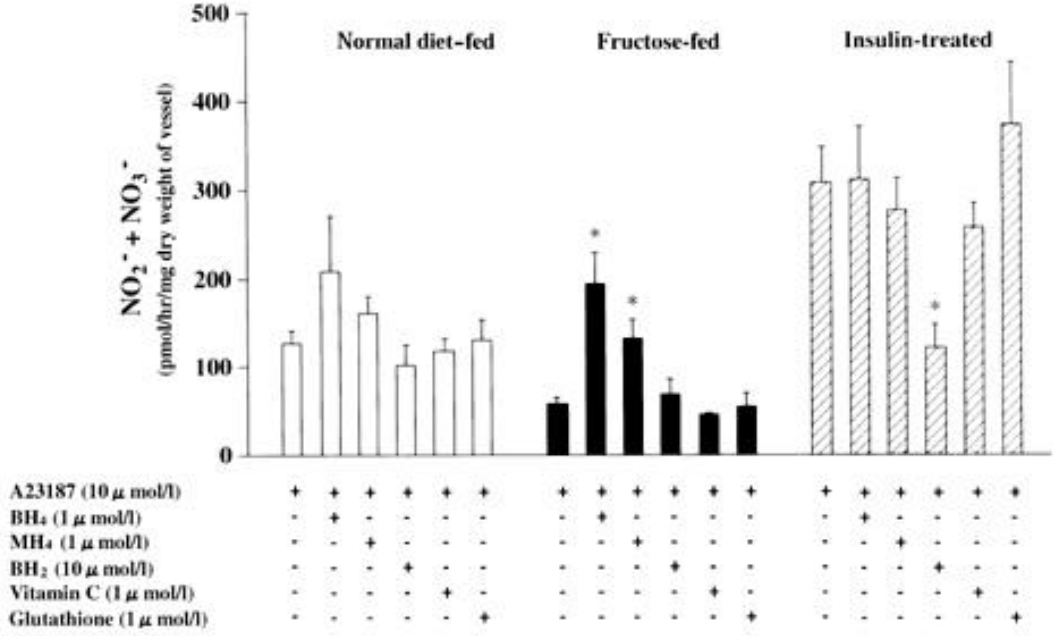

FIG. 4. Production of NO after stimulation with A23187 (10 µmol/l) in the presence of 1 µmol/l BH_4, 1 µmol/l MH_4 (a lipid-soluble analog of BH_4), 10 µmol/l BH_2, 1 µmol/l vitamin C, or 1 µmol/l glutathione. *P < 0.01 vs. corresponding values of stimulation with A23187 alone. Data are expressed as means ± SE (n = 4).

BH_4 deficiency, eNOS has been shown to produce, besides NO, a disproportionate amount of O_2^- by transferring electrons to molecular oxygen (38). This may be a reason for our findings that impaired BH_4 metabolism was closely associated with abnormalities in NO and O_2^- release in endothelium of insulin-resistant rats.

We first demonstrated that rats with chronic exogenous hyperinsulinemia had significantly increased levels of eNOS activity as well as release of NO, as suggested by the rapid stimulatory effect of insulin on NO-dependent vasodilation (11,12). Consistent with these results, we also found that eNOS mRNA content was increased in aortic endothelial cells in those rats. These results clearly contrast the results from endogenous hyperinsulinemic rats with insulin resistance.

Endothelial BH_4 content and GTP cyclohydrolase I activity were significantly decreased, whereas the levels of 7,8-BH_2 plus biopterin were markedly increased in aortas of insulin-resistant rats. In contrast to these findings, endothelial BH_4 content and GTP cyclohydrolase I activity were markedly increased, whereas the levels of 7,8-BH_2 plus biopterin were decreased in exogenous hyperinsulinemic rats. Therefore, it appears that insulin stimulates BH_4 synthesis by activation of GTP cyclohydrolase I, and this insulin effect was impaired in the insulin-resistant state (39,40). In mammalian cells, BH_4 is synthesized via two distinct pathways: a de novo synthetic pathway that uses GTP as a precursor and regeneration of BH_4 from BH_2 through a biopterin salvage pathway (41). Regardless of the decreased activity of GTP cyclohydrolase I, the

TABLE 4
Effect of Cu,Zn-SOD, L-NAME, and tetrahydrobiopterin on superoxide anion production by eNOS

Group	Endothelium	Incubation condition	A23187	Vascular superoxide production (cpm × 10^3/mg of dry weight of vessel)		
				Normal diet–fed	Fructose-fed	Insulin-treated
A	–	Buffer alone	–	21.4 ± 3.2†	20.8 ± 2.1‡	24.8 ± 1.8‡
B	+	Buffer alone	–	32.2 ± 9.7	73.4 ± 20.3#	48.4 ± 16.6
C	+	Buffer alone	+	50.8 ± 12.8*	207.6 ± 37.3* **	73.8 ± 6.8*††
D	+	+ Cu,Zn-SOD (100 U/ml)	+	28.3 ± 4.1§	47.0 ± 17.7‖	36.4 ± 8.3§¶
E	+	+ L-NAME (10 µmol/l)	+	45.7 ± 11.1	62.4 ± 14.3‖	64.8 ± 9.3
F	+	+ BH_4 (1 µmol/l)	+	60.7 ± 16.7*	70.8 ± 14.1§	71.5 ± 10.3

Data are means ± SE (n = 5). Basal concentrations were measured without stimulation of eNOS with calcium ionophore A23187 (10 µmol/l) in the absence (group A) or presence (group B) of endothelium. Vascular superoxide production was measured after stimulation with A23187 (group C) as well as in the presence of 100 U/ml Cu,Zn-SOD (group D), 10 µmol/l L-NAME (group E), or 1 µmol/l tetrahydrobiopterin (BH_4) (group F). *P < 0.05, †P < 0.01, ‡P < 0.0001 vs. corresponding vessels of group B. §P < 0.05, ‖P < 0.01 vs. corresponding vessels of group C. ¶P < 0.01 vs. insulin-treated rats of group E. #P < 0.05, **P < 0.01 vs. the corresponding values of normal diet–fed rats, using the unpaired Student's t test. ††P < 0.01 vs. the corresponding values of fructose-fed rats, using unpaired Student's t test. Statistical analysis among the three groups was analyzed by multiple comparison test using ANOVA with a post hoc Scheffe's comparison.

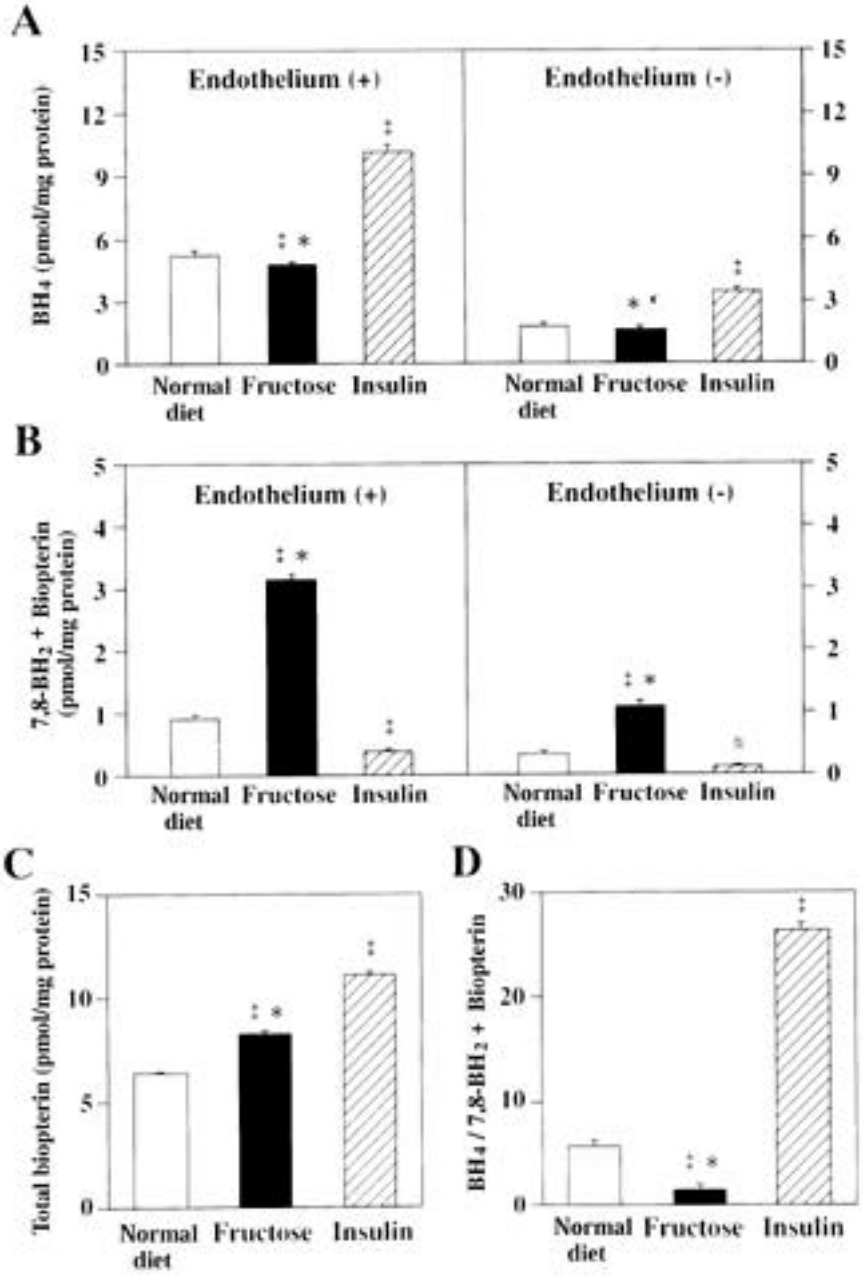

FIG. 5. Biopterin contents and GTP cyclohydrolase I activity in aortic tissue. Tissue levels of tetrahydrobiopterin (BH_4) (*A*) and 7,8-dihydrobiopterin (BH_2) + biopterin (*B*) were measured in either the presence or absence of endothelium. Total biopterin content (*C*) and the ratio of BH_4 to BH_2 plus biopterin of intact vessels are shown. ¶$P < 0.05$, §$P < 0.01$, ‡$P < 0.001$ vs. corresponding values of normal diet–fed rats. *$P < 0.001$ vs. corresponding values of insulin-treated rats. Data are expressed as means ± SE ($n = 4$).

marked increment of 7,8-BH_2 relative to BH_4 was observed in insulin-resistant rats. These results suggest that BH_4 content might be compensated through unknown mechanisms related to activation of these recycling enzymes (21). Concerning the biological significance of 7,8-BH_2, an inactive cofactor of NO synthase, it has been suggested that 7,8-BH_2 acts as a competitive antagonist of BH_4 binding (42) and inhibits GTP cyclohydrolase I and hence de novo synthesis of BH_4 (43).

In conclusion, the present study is the first to demonstrate that insulin resistance per se, by modification of biopterin levels, impairs the NO-mediated vasodilation of rat aortas. More importantly, decreased insulin action insufficiently produces BH_4, thereby allowing eNOS to become a source of O_2^-. These results indicate that impaired insulin action (insulin resistance) rather than excessive plasma insulin concentration (exogenous hyperinsulinemia) may be a pathogenic factor for abnormal regulation of vascular tones through imbalance of NO and O_2^- production, which may be associated with the development of hypertension and systemic atherosclerosis in the insulin-resistant state.

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REFERENCES

1. Pyörälä K: Relationship of glucose tolerance and plasma insulin to the incidence of coronary heart disease: relationship to the incidence of coronary heart disease: results from two population studies in Finland. *Diabetes Care* 2:131–141, 1979
2. Ducimentiere P, Eschwege E, Papoz L, Richard JL, Claude JR, Rosselin G: Relationship of plasma insulin levels to the incidence of myocardial infarction and

coronary heart disease in a middle-aged population. *Diabetologia* 19:205–210, 1980
3. Welborn TA, Wearne K: Coronary heart disease incidence and cardiovascular mortality in Busselton with reference to glucose and insulin concentration. *Diabetes Care* 2:154–160, 1979
4. Zavaroni I, Bonora E, Pagliara M, Dall'Aglio E, Luchetti L, Buonanno G, Bonati PA, Bergonzani M, Gundi L, Passeri M, Reaven GM: Risk factors for coronary artery disease in healthy persons with hyperinsulinemia and normal glucose tolerance. *N Engl J Med* 320:702–706, 1989
5. Stout RW: Insulin and atheroma: 20-yr perspective. *Diabetes Care* 13:631–654, 1990
6. Jarrett RJ: In defence of insulin: a critique of syndrome X. *Lancet* 340:469–471, 1992
7. Capron L, Jarnet J: Effects of injury and insulin on lipid synthesis from glucose by the rat thoracic aorta. *Arterioscler Thromb* 11:91–96, 1991
8. Shinozaki K, Suzuki M, Ikebuchi M, Takaki H, Hara Y, Tsushima M, Harano Y: Insulin resistance associated with compensatory hyperinsulinemia as an independent risk factor for vasospastic angina. *Circulation* 92:1749–1757, 1995
9. Shinozaki K, Suzuki M, Ikebuchi M, Hara Y, Harano Y: Demonstration of insulin resistance in coronary artery disease documented with angiography. *Diabetes Care* 19:1–7, 1996
10. Laakso M, Sarlund H, Salonen R, Suhonen M, Pyörälä K, Salonen JT, Karhapää P: Asymptomatic atherosclerosis and insulin resistance. *Arterioscler Thromb* 11:1068–1076, 1991
11. Baron AD, Brechtel-Hook G, Johnson A, Hardin D: Skeletal muscle blood flow: a possible link between insulin resistance and blood pressure. *Hypertension* 21:129–135, 1993
12. Steinberg HO, Chaker H, Leaming R, Johnson A, Brechtel G, Baron AD: Obesity/insulin resistance is associated with endothelial dysfunction: implication for the syndrome for insulin resistance. *J Clin Invest* 97:2601–2610, 1996
13. Ting HH, Timimi FK, Boles KS, Creager SJ, Ganz P, Creager MA: Vitamin C improves endothelium-dependent vasodilation in patients with non-insulin-dependent diabetes mellitus. *J Clin Invest* 97:22–28, 1996
14. Kontos HA, Kontos MC: Role of products of univalent reduction of oxygen in hypertensive vascular injury. In *Hypertension: Pathophysiology, Diagnosis, and Management*. Laragh JH, Brenner BM, Eds. New York, Raven Press, 1995, p. 685–696
15. Wever RMF, Lüscher TF, Cosentino F, Rabelink TJ: Atherosclerosis and the two faces of endothelial nitric oxide synthase. *Circulation* 97:108–112, 1998
16. Kwon NS, Nathan CF, Stuehr DJ: Reduced biopterin as cofactor in the generation of nitrogen oxides by murine macrophages. *J Biol Chem* 264:20446–20501, 1989
17. Baek KJ, Thiel BA, Lucas S, Stuehr DJ: Macrophage nitric oxide synthase subunit: purification, characterization, and role of prosthetic groups and substrate in regulating their association into a dimeric enzyme. *J Biol Chem* 268:21120–21129, 1993
18. Berka V, Chen PF, Tsai AL: Spatial relationship between L-arginine and heme binding sites of endothelial nitric-oxide synthase. *J Biol Chem* 271:33293–33000, 1996
19. Pieper GM: Acute amelioration of diabetic endothelial dysfunction with a derivative of the nitric oxide synthase cofactor, tetrahydrobiopterin. *J Cardiovasc Pharmacol* 29:8–15, 1997
20. Stroes E, Kastelin J, Cosentino F, Erkelens W, Wever R, Koomans H, Lüscher TF, Rabelink T: Tetrahydrobiopterin restores endothelial function in hypercholesterolemia. *J Clin Invest* 99:41–46, 1997
21. Hamon CG, Culter P, Blair JA: Tetrahydrobiopterin metabolism in the streptozotocin induced diabetic state in rats. *Clin Chim Acta* 181:249–254, 1989
22. Reaven GM: Insulin resistance, hyperinsulinemia, hypertriglyceridemia, and hypertension: parallels between human disease and rodent models. *Diabetes Care* 14:195–202, 1991
23. Harano Y, Ohgaku S, Kosugi K, Yasuda H, Nakano T, Kobayashi M, Hidaka H, Izumi K, Kashiwagi A, Shigeta Y: Clinical significance of altered insulin sensitivity in diabetes mellitus assessed by glucose, insulin, and somatostatin infusion. *J Clin Endocrinol Metab* 52:982–987, 1981
24. Toda N, Ayajiki K, Okamura T: Endothelial modulation of concentrations caused by oxyhemoglobin and N^G-nitro-L-arginine in isolated dog and monkey cerebral arteries. *Stroke* 24:1584–1589, 1993
25. Rees D, Ben-Ishay D, Moncada S: Nitric oxide and the regulation of blood pressure in the hypertension-prone and hypertension-resistant sabra rat. *Hypertension* 28:367–371, 1996
26. Chomczynski P, Sacchi N: Single step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 162:156–159, 1987
27. Kawai N, Bloch DB, Filippov G, Rabkina D, Suen HC, Losty PD, Janssens SP, Zapol WM, de la Monte S, Bloch KD: Constitutive endothelial nitric oxide synthase gene expression is regulated during lung development. *Am J Physiol* 268:L589–L595, 1995
28. Kita Y, Hirasawa Y, Maeda K, Nishio M, Yoshida K: Spontaneous nitric oxide release accounts for the potent pharmacological actions of FK409. *Eur J Pharmacol* 257:123–130, 1994
29. Rajagopalan S, Kurz S, Münzel T, Tarpey M, Freeman BA, Griendling KK, Harrison DG: Angiotensin II-mediated hypertension in the rat increases vascular superoxide production via membrane NADH/NADPH oxidase activation: contribution to alterations of vascular tone. *J Clin Invest* 97:1916–1923, 1996
30. Li Y, Zhu H, Kuppusamy P, Roubaud V, Zweier JL, Trush MA: Validation of lucigenin (bis-N-methylacridinium) as a chemilumigenic probe for detecting superoxide anion radical production by enzymatic and cellular systems. *J Biol Chem* 273:2005–2023, 1998
31. Kuthan H, Ullrich V: A quantitative test for superoxide radicals produced in biological systems. *Biochem J* 203:551–558, 1982
32. Masada M, Akino M, Sueoka T, Katoh S: Dyspropterin, an intermediate formed from dihydroneopterin triphosphate in the biosynthetic pathway of tetrahydrobiopterin. *Biochim Biophys Acta* 840:235–244, 1985
33. Masada M, Matsumoto J, Akino M: Biosynthetic pathways of pteridines and their association with phenotypic expression in vitro in normal and neoplastic pigment cells from goldfish. *Pigment Cell Res* 3:61–70, 1990
34. Kafman S: Metabolism of the phenylalanine hydroxylation cofactor. *J Biol Chem* 242:3934–3943, 1967
35. Bradford MM: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72:248–254, 1997
36. Abe H, Yamada N, Kamata K, Kuwaki T, Shimada M, Osuga J, Shionoiri F, Yahagi N, Kadowaki T, Tamemoto H, Ishibashi S, Yazaki Y, Makuuchi M: Hypertension, hypertriglyceridemia and impaired endothelium-dependent vascular relaxation in mice lacking insulin receptor substrate-1. *J Clin Invest* 101:1784–1788, 1998
37. Sakamoto S, Minami K, Niwa Y, Ohnaka M, Nakaya Y, Mizuno A, Kuwajima M, Shima K: Effects of exercise training and food restriction on endothelium-dependent relaxation in the Otsuka-Long-Evans Tokushima fatty rat, a model of spontaneous NIDDM. *Diabetes* 47:82–86, 1998
38. Pou S, Pou WS, Bredt DS, Snyder SH, Rosen GM: Generation of superoxide by purified brain nitric oxide synthase. *J Biol Chem* 267:24173–24176, 1992
39. Viveros OH, Lee CL, Abou-Donia MM, Nixon JC, Nichol CA: Biopterin cofactor biosynthesis: independent regulation of GTP cyclohydrolase in adrenal medulla and cortex. *Science* 213:349–350, 1981
40. Abou-Donia MM, Duch DS, Nichol CA, Viveros OH: Hormonal regulation of guanosine triphosphate cyclohydrolase activity and biopterin levels in the rat adrenal cortex. *Endocrinology* 112:2088–2094, 1983
41. Zembowicz A, Tang J, Wu KK: Transcriptional induction of endothelial nitric oxide synthase type III by lysophosphatidylcholine. *J Biol Chem* 270:17006–17010, 1995
42. Klatt P, Schmid M, Leopold E, Schmidt K, Werner ER, Mayer B: The pteridine binding site of brain nitric oxide synthase. *J Biol Chem* 269:13861–13866, 1994
43. Shen R, Alam A, Zhang Y: Inhibition of GTP cyclohydrolase I by pterins. *Biochim Biophys Acta* 965:9–15, 1988