Increased Vascular Endothelin-1 Gene Expression With Unaltered Nitric Oxide Synthase Levels in Fructose-Induced Hypertensive Rats

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The present study aimed to investigate whether altered expression levels of endothelin-1 (ET-1) and nitric oxide synthase (NOS) are related to the development of insulin-resistant hypertension. Male Sprague-Dawley rats were fed a fructose-rich diet for 5 weeks. Systolic blood pressure significantly increased in fructose-fed rats. While serum free fatty acid (FFA) and plasma nitrite/nitrate (NOx) levels did not significantly differ between the fructose-fed and control groups, plasma insulin and serum triglyceride (TG) concentrations significantly increased in the former. ET-1 mRNA expression in the aorta increased to 195% in fructose-fed rats. Neither the protein expression of constitutive NOS (cNOS) nor that of inducible NOS (iNOS) were significantly affected by fructose feeding. However, NOx levels in the aorta were significantly increased. These results indicate that an increased expression of vascular ET-1 may be causally related to the development of hypertension in fructose-fed rats. However, an altered role of the vascular nitric oxide (NO) pathway may not be primarily involved in the development of fructose-induced hypertension.

RATS THAT ARE FED a fructose-rich diet develop insulin resistance, hypertriglyceridemia, and hypertension.[1] The insulin resistance is also found in genetically hypertensive strains such as the spontaneously hypertensive rat (SHR)[2] and the Dahl salt-sensitive rat (DSR).[3] On the contrary, the amelioration of hyperinsulinemia by concomitant infusion of a somatostatin analog markedly attenuates the hypertension induced by fructose-feeding.[4] In addition, medications that decrease insulin resistance have an antihypertensive effect.[5] Collectively, hyperinsulinemia and insulin resistance may be critical factors in the pathogenesis of hypertension.

Among others, endothelial dysfunction may be an integral component of the syndrome of insulin resistance.[6] Steinberg et al[7] showed that insulin enhances the release of nitric oxide (NO), whereas Baron et al[8] observed that insulin resistance is associated with impaired endothelium-dependent vasodilation. An impaired endothelium-mediated relaxation was also noted in fructose-fed hypertensive rats.[9] Mechanisms underlying the insulin-resistant hypertension remain to be established.

Derangements of the endothelial NO system have been related to the development of hypertension. The mechanism of endothelial dysfunction differs in different models of hypertension. In the SHR, the reduced vasorelaxation response to acetylcholine was attributed to the production of cyclooxygenase-dependent constricting factors,[10] although the vascular constitutive NO synthase (cNOS) activity was increased.[11] However, in other forms of experimental hypertension, a reduced formation of NO may predominate.[12] Hayakawa and Raij[11] showed that antihypertensive therapy normalized the blood pressure and aortic cNOS activity in DSRs. No data are available on vascular NO synthesis in the fructose-fed, insulin-resistant model of hypertension.

Over the last decade, the vasoconstrictive effect of endothelin-1 (ET-1) has been known.[13] Its gene expression and release in endothelial cells are increased by insulin.[14,15] Insulin also increases ET-1 receptor expression in vascular smooth muscle cells.[16] Conversely, the blood pressure elevation is attenuated by long-term ET-1 antagonist treatment in fructose-fed rats.[17] ET-1 may be involved in the development of hypertension in a hyperinsulinemic state.

The present study aimed at determining whether altered NO synthesis is involved in the development of acquired insulin-resistant hypertension. Rats were fed a fructose-rich diet, and their vascular expression levels of ET-1 mRNA and NOS proteins were determined. The vascular NO content was also measured.

MATERIALS AND METHODS

General Protocol

Male Sprague-Dawley rats weighing about 130 g were housed in an environmentally controlled room with a 12-hour light/dark cycle. They were kept in accordance with the Institutional Guidelines of Experimental Animal Care and Use. One group was fed on a fructose diet (Harlan Teklad, Madison, WI) and the other group received normal rat chow for 5 weeks. Systolic blood pressure was measured in the conscious state by the tail-cuff method every week. On the experimental day, rats were decapitated and their trunk blood was collected. The thoracic aorta was also removed and frozen in liquid nitrogen for storage at −80°C until analysis within 3 weeks.

The blood samples were centrifuged, and either the serum or plasma was separated. The plasma glucose level was measured with a Beckman Glucose Analyzer II (Beckman-Coulter, Fullerton, CA). The serum triglyceride (TG) level was measured by an enzymatic technique (Boehringer, Mannheim, Germany). Insulin was analyzed by radioimmunoassay using a commercial kit (Linco, St. Charles, MO). Free fatty acid (FFA) levels were measured by a colorimetric method (Wako, Osaka, Japan).

Reverse Transcription–Polymerase Chain Reaction

Total RNA was extracted from the aorta as described by Chomczynski and Sacchi[18] using a commercial solution (Biotecx Laboratories, Houston, TX). Reverse transcription (RT) followed by polymerase chain reaction (PCR) was then performed. Avian myeloblastosis virus reverse transcriptase (16 U per reaction; Promega, Madison, WI) was

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used for RT, along with the reaction mixture recommended by the enzyme manufacturer, in a volume of 20 μL using 1.25 μmol/L downstream primer. The PCR was then performed with 2 μL of the resulting cDNA using the upstream and downstream primers at 1.25 μmol/L each. Each PCR mixture contained 1.5 U *Taq* polymerase (Promega) and 60 μmol/L dNTP. cDNA for ET-1 mRNA was synthesized with specific primers [upstream 5′-ATG GAT TAT TTT CCC GTG AT-3′ (1 to 20) and downstream 5′-GGG AGT GTT GAC CCA GAT GA-3′ (212 to 231)]. The PCR with these primers yielded 231 base pairs. In a preliminary study, we found that 35 PCR cycles for ET-1 were necessary to obtain a visible product on agarose gel, and the quantity of the product was in proportion to the amount of cDNA used. After an initial denaturation step at 94°C for 2 minutes, cycles of annealing at 56°C for 45 seconds, elongation at 72°C for 1.5 minutes, and denaturation at 94°C for 45 seconds were performed with cDNA (2 μL). The RT-PCR product of the gene encoding for β-actin served as a control. The upstream primer sequence 5′-GAC TAC CTC ATG AAG ATC CTG ACC-3′ used to amplify β-actin mRNA corresponds to positions 210 to 217, and the downstream primer 5′-TGA TCT TCA TGG TGC TAGG AGC C-3′ corresponds to positions 320 to 327. They yielded a 423–base pair DNA fragment. The PCR products were size-fractionated by 1.5% agarose gel electrophoresis and visualized under UV light with ethidium bromide staining. ET-1 and β-actin cDNAs were quantified by IMAGER and 1D MAIN (Bioneer, Seoul, Korea). ET-1 cDNA was normalized by comparison to β-actin cDNA.

Protein Preparation and Western Blot Analysis

For protein preparation, aortic tissues were homogenized with a Polytron homogenizer (Kinematica, Littau, Switzerland) in a solution containing 250 mmol/L sucrose, 1 mmol/L EDTA, 0.1 mmol/L phenylmethylsulfonyl fluoride, and 50 mmol/L potassium phosphate buffer at pH 7.6. Large tissue debris and nuclear fragments were removed by 2 consecutive low-speed centrifugations (3,000 × *g* for 5 minutes and 10,000 × *g* for 10 minutes). The samples were further centrifuged at 100,000 × *g* for 60 minutes. The pellet was suspended for protein blotting of cNOS, and the supernatant was used for protein blotting of inducible NOS (iNOS). The protein concentration was determined using the BCA protein assay kit (Pierce, Rockford, IL).

Proteins were separated in denaturing 7.5% sodium dodecyl sulfate polyacrylamide gel (30 μg per lane). Proteins were then blotted onto a nitrocellulose membrane. The blots were blocked overnight at 4°C with 5% nonfat dry milk in TBST (20 mmol/L Trishydrochloride, 137 mmol/L NaCl, and 0.1% Tween 20). Western blot analyses were performed with specific monoclonal antibodies against cNOS or iNOS proteins (Transduction Laboratories, Lexington, KY). Blots were incubated with the first antibody (1:2,000) for 1 hour at room temperature, and after extensive washing, they were incubated with the second antibody (horseradish peroxidase–conjugated anti-mouse immunoglobulin) at a dilution of 1:1,000 for 1 hour. Specific NOS proteins were detected by enhanced chemiluminescence (Amersham, Arlington Heights, IL). These monoclonal antibodies specifically recognize iNOS (130 kd) and cNOS (140 kd) isoforms.

Colorimetric Assay of Nitrite/Nitrate

The levels of nitrite/nitrate (NOx) in aortic tissue and plasma were measured by a colorimetric NO assay kit (Oxford, Oxford, MI). A microplate was used to perform the enzyme reaction in vitro. For spectrophotometric assay of nitrites with Griess reagent, 80 μL morpholinepropanesulfonic acid (50-mmol/L)/EDTA (1-mmol/L) buffer and 5-μL tissue samples were added to the wells. Nitrate reductase (0.01 U) and 10 μL NADH (2-mmol/L) were added to the reaction mixture, and the plate was shaken for 20 minutes at room temperature. Color reagents, sulfanilamide, and *N*-(1-naphthyl)ethylenediamine dihydrochloride were added, and absorbance values were read at 540 nm in a microtiter plate reader (Bio-Rad, Hercules, CA).

Statistical Analysis

Results are expressed as the mean ± SEM. Comparisons between control and fructose-fed rats were made by unpaired *t* test. The criterium for significance was a *P* value less than .05.

RESULTS

Systolic blood pressure significantly increased during the experimental period of fructose-feeding (Fig 1). Body weight, plasma glucose, insulin, TG, and FFA levels are shown in Table 1. There were no significant differences in body weight between the experimental and control groups at the end of the study. Plasma glucose concentrations were not significantly different between the two groups. Serum TG and plasma insulin were significantly elevated in fructose-fed rats. There were no significant differences in the serum level of FFA between the two groups.

Representative PCR products of ET-1 mRNA are shown in Fig 2. ET-1 mRNA expression increased by 195% ± 10% in fructose-fed rats compared with the control ($P < .001$).

Western blot analyses of cNOS and iNOS proteins in the aorta are presented in Fig 3. The expression of cNOS proteins was not significantly changed after 5 weeks of feeding fructose. Nor was iNOS protein expression significantly changed. However, the NOx content in the aorta increased in fructose-fed rats compared with the control group (1.34 ± 0.34 *v* 0.52 ± 0.11 nmol/mg protein, $P = .039$). The plasma concentration of NOx did not differ between the fructose-fed hypertensive and control rats (52.8 ± 2.3 and 47.0 ± 1.4 μmol/L, respectively).

DISCUSSION

There was a moderate but significant hypertension in the fructose-fed rats. Although insulin resistance and compensatory hyperinsulinemia have been associated with the hypertension,[2,3,19] the underlying mechanisms for the hypertension have to be further explained. The altered synthesis and release of endothelium-derived constricting and/or relaxing factors have been implicated in the development of hypertension.[12,20,21] An impaired endothelium-dependent vasodilation also has been

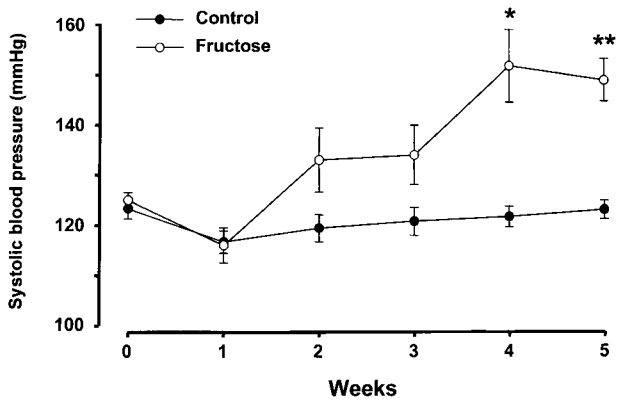

Fig 1. Systolic blood pressure in control and fructose-fed rats (n = 9 per group). *$P < .005$, **$P < .001$ *v* control.

Table 1. Metabolic Characteristics in Control and Fructose-Fed Experimental Rats After 5 Weeks of Dietary Treatment

Characteristic	Control	Fructose-Fed	*P*
Body weight (g)	296.6 ± 8.9	303.8 ± 7.6	NS
Plasma glucose (mg/dL)	66.0 ± 4.2	72.6 ± 2.3	NS
Plasma insulin (μU/mL)	18.2 ± 0.5	33.6 ± 0.8	.001
Serum TG (mg/dL)	123.3 ± 23.8	381.3 ± 37.5	.008
Serum FFA (mmol/L)	0.36 ± 0.03	0.40 ± 0.09	NS

NOTE. Data are the mean ± SEM (n = 9 per group).

observed in rats by 18 days of feeding fructose, with no significant elevations of blood pressure until day 28.[9] This finding suggests that an endothelial dysfunction precedes the development of hypertension.

In the present study, we observed that ET-1 mRNA expression in the aorta was increased 3-fold in fructose-fed rats, in line with previous findings in that ET-1 mRNA and peptide were increased in resistance arteries of fructose-fed rats.[17,22] In fact, insulin promotes the gene expression and release of ET-1 from endothelial cells.[14,15] Piatti et al[23] showed that in normal subjects, only supraphysiologic levels of insulin can increase the arterial blood ET-1 concentration. However, a physiologic increase in insulin accompanied by an elevation of serum TG could enhance arterial ET-1 to the levels found in subjects with hypertension.[23] Our rat model showed a significant elevation of serum TG with a slight elevation of the insulin level in association with hypertension, suggesting that both insulin resistance and hyperinsulinemia are necessary to increase ET-1 levels and induce hypertension in syndrome X. However,

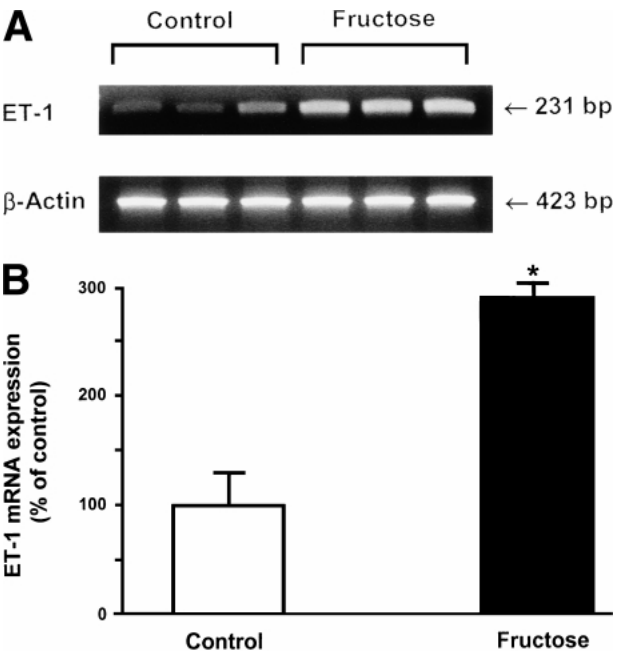

Fig 2. Representative RT-PCR products and quantification of ET-1 mRNA expression in aortic tissues (n = 6 rats each). The mean volume of amplification products in control rats was taken as 100%. *$P < .001$ *v* control.

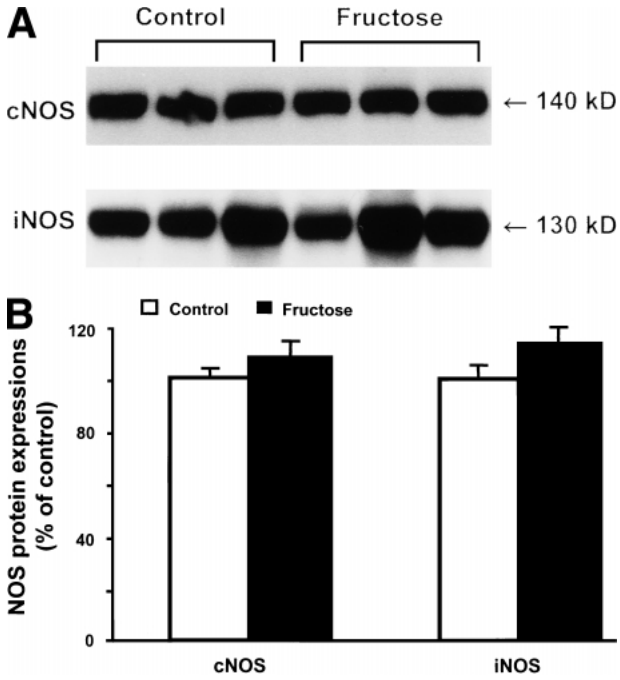

Fig 3. Representative Western blots and densitometric quantification of cNOS and iNOS protein expression in aortic tissue (n = 6 experiments each).

plasma ET-1 levels in hypertensives have shown conflicting results, being slightly increased or completely normal.[24,25] The discrepancy may be ascribed at least in part to the different characteristics of the study subjects. Although we did not measure the plasma level of ET-1, it may not be a direct measure of the endogenous production of ET-1 because of its polar secretion from endothelial cells.[26] Indeed, plasma ET-1 levels did not differ between the fructose-fed hypertensive and control rats in previous reports.[17,22] ET-1 may be causally related to the development of hypertension by acting in a paracrine manner.

One may argue that the increase in ET-1 expression is a consequence rather than a cause of hypertension. However, when infused intravenously, ET-1 causes a profound long-lasting increase in blood pressure.[27] ET-1 also has been shown to potentiate the effects of other vasoconstrictors such as norepinephrine, angiotensin II, and serotonin.[13,28] Furthermore, Verma et al[17] showed that the blood pressure elevation was prevented by long-term ET-1 antagonist treatment in fructose-fed rats, and the increase of ET-1 in the mesenteric tissue was not altered by the antagonist.

The impaired endothelium-mediated relaxation has been attributed to a decrease in NO production in many experimental hypertensive animals.[29] cNOS activity in the aorta decreased by 73% in hypertensive DSRs compared with their normotensive control rats.[11] However, in SHRs, the NO pathway was upregulated in association with an enhanced cNOS expression and hence increased NO production.[11,30,31] However, the endothelium-mediated relaxation was impaired in aortic rings from the SHR and DSR, which was normalized by indomethacin in the SHR but not in the DSR.[20] These findings suggest

a role of cyclooxygenase-derived constricting factors in the development of hypertension in SHRs. On the contrary, in hypertensive DSRs, when blood pressure was decreased by antihypertensive therapy, the endothelium-mediated relaxation and cNOS activity in the aorta were normalized as well. Taken together, changes in cNOS activity in the two strains of rats genetically prone to hypertension are the consequence of hypertension rather than the cause.

However, no studies have examined whether regulation of the vascular NO pathway is altered in fructose-fed hypertensive animals. Our study shows that the expression of NOS proteins was not significantly changed in fructose-fed hypertensive rats. When systolic blood pressure in DSRs was decreased by antihypertensive therapy from the initial value of greater than 210 mm Hg to about 146 mm Hg, similar to the level in our fructose-induced hypertension, cNOS activity in the aorta was normalized.[11] The degree of hypertension and genotypic differences among the animal models may explain the discrepancies in the activity of the NO pathway. However, NOx levels in the aorta were significantly increased in the present study. It is therefore unlikely that the fructose-induced hypertension is attributable to a decrease in the activity of the vascular NO pathway. Although we did not directly measure NOS activity in aortic tissue, the increase in NOx content along with the unchanged expression of NOS proteins suggest an increase in NOS activity. Since ET-1 has a stimulatory effect on NO production through activation of endothelial ET_B receptors,[22,32] the increase in NOx content may be explained by an enhanced activity of ET-1. Insulin may also directly stimulate the production of NO in isolated human endothelial cells.[33] Alternatively, a heightened vascular cNOS activity may be a part of the physiologic adaptation to hemodynamic forces that are increased in the hypertensive state.[6]

In summary, our results suggest that an increase in vascular ET-1 is an important contributing factor to the development of hypertension in fructose-fed rats. However, the vascular NO pathway may not be causally related to the development of fructose-induced hypertension.

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