Decrease in Renal Medullary Endothelial Nitric Oxide Synthase of Fructose-Fed, Salt-Sensitive Hypertensive Rats

Yasuhiro Nishimoto, Takahito Tomida, Hideo Matsui, Takayuki Ito, Kenji Okumura

Abstract—We investigated the expression of endothelial NO synthase (eNOS) in the kidneys of fructose-fed insulin-resistant rats (FFR) with a low- or high-sodium diet. Male Sprague-Dawley rats were fed a control (C) or high-fructose (40% fructose; F) diet, with each coming in low-sodium (0.024% NaCl; LS-C or LS-F) or high-sodium (3% NaCl; HS-C or HS-F) varieties, for 2 weeks. Half of the FFR were orally administered pioglitazone (10 mg·kg⁻¹·day⁻¹), an insulin-sensitizing agent (LS-FP or HS-FP). The systolic blood pressure was significantly higher in the HS-F rats than in the LS-F rats or the HS-C rats (HS-F rats, 129±4 mm Hg, versus LS-F rats, 115±3 mm Hg, P<0.05; or versus HS-C rats, 116±5 mm Hg, P<0.05), which indicated the salt dependence of hypertension in FFR. The protein expression of eNOS in the renal medulla of FFR was significantly lower than that in control rats during a high sodium load. The administration of pioglitazone prevented the hypertension (HS-F rats, 129±4 mm Hg, versus HS-FP rats, 113±3 mm Hg, P<0.05) and the reduction of medullary eNOS protein expression in HS-F rats. There was no significant difference in eNOS expression in the renal cortex or aorta between FFR and control rats, regardless of sodium load. These results suggest that the decrease in renal medullary NO production by eNOS during a high sodium load may play a role in fructose-fed, salt-sensitive hypertension. (Hypertension. 2002;40:190-194.)

Key Words: insulin resistance • fructose • hypertension, sodium-dependent • nitric oxide synthase • kidney

Previous studies have shown an association between insulin resistance and systemic hypertension.1,2 Compen-satory hyperinsulinemia was thought to cause hypertension because it led to sodium retention, sympathetic nerve activation, and vascular smooth muscle cell proliferation.2 However, Bursztyn et al3 demonstrated that chronic exogenous hyperinsulinemia itself did not elevate blood pressure; only when in combination with a defective NO system, might hyperinsulinemia cause hypertension. Some investigators revealed a correlation between insulin resistance and defects of the NO system,4,5 and others have shown that a defective NO system in the kidney of insulin-resistant subjects, they may develop salt-sensitive hypertension. Indeed, several investigators indicate that patients or animals11,12 with insulin resistance tend to develop salt-sensitive hypertension. If the defect of the NO system is seen in the kidney of insulin-resistant subjects, they may develop salt-sensitive hypertension. Indeed, several investigators indicate that patients11,12 or animals13,14 with insulin resistance tend to develop salt-sensitive hypertension. Although the mechanisms underlying the coexistence of insulin resistance and hypertension remain unclear, a defect of the NO system in the kidney is thought to be a cause of hypertension in insulin-resistant subjects.13 NO is produced from the conversion of L-arginine to L-citrulline by a family of enzymes known as NO synthase (NOS).15 NOS exists in 3 isoforms: neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS).16 Recent studies indicate that all 3 isoforms of NOS are constitutively expressed in the kidney of normal rats.8 Among the 3 isoforms, eNOS is known to be important for sodium excretion,17,18 but little is known about changes in eNOS levels in the kidneys of insulin-resistant subjects. In the present study, we investigated the expression of eNOS in the kidney by use of a dietary model of insulin resistance with a low- or high-sodium diet. We also evaluated the effect of pioglitazone, an insulin-sensitizing agent, on blood pressure and eNOS expression in this model.

Methods

Animals

Seven-week-old male Sprague-Dawley rats (Japan SLC Inc, Hamamatsu, Japan) were divided first into 4 groups according to diet: standard rat food (Clea Japan) containing 0.024% NaCl (low-sodium control diet; LS-C) or 3% NaCl (high-sodium control diet; HS-C), and a high-fructose diet (Clea Japan) containing 40% fructose and 7% lard as a percentage of total calories with 0.024% NaCl (low-sodium high-fructose diet [LS-F]) or 3% NaCl (high-sodium high-fructose diet [HS-F]). The content of other minerals, protein, fat, and vitamins in the control and high-fructose diets was matched. A previous study in our laboratory demonstrated that this high-fructose diet obviously produced insulin-resistant states in the animals over 2 weeks of feeding.18 The high-fructose diet groups were divided into rats that were administered vehicle only (LS-F and...
HS-F) or rats that were administered pioglitazone (Takeda Chemical Industries; ie, a low-sodium high-fructose diet plus pioglitazone group [LS-FP] and a high-sodium high-fructose diet plus pioglitazone group [HS-FP]) for 2 weeks from the beginning of the administration of the diets. Ultimately, then, the rats were divided into 6 groups. Six animals were studied for each group. Pioglitazone was suspended in 0.5% methylcellulose solution and administered at a dose of 10 mg kg\(^{-1}\) day\(^{-1}\) orally by gastric gavage in the evening. Both the low- and high-sodium diet control rats were also administered vehicle, as were the high-fructose diet rats. The same amount of methylcellulose solution was administered orally as the vehicle. The rats were housed in an environmentally controlled room with a 12-hour/12-hour light/dark cycle and were allowed free access to the assigned diet and water. Daily food consumption was recorded. All procedures were approved by the animal ethics committee of Nagoya University.

**Blood Pressure Measurements**

Two weeks after the experiment began, systolic blood pressure and heart rate were measured using the tail-cuff method (BP98A, Softron) after the rats were externally prewarmed for 15 minutes at 39°C.

**Urine Collection and Assays**

After the measurements of blood pressure, the rats were placed in metabolic cages without food preparation for 24-hour urine collection. Urinary protein excretion was determined by improved Lowry assay (DC protein assay; Bio-Rad Laboratories). Urinary nitrite and nitrogen and nitrate (N\(_\text{OX}\)) excretion was determined using the Greiss reaction with an automated NO detector high-performance liquid chromatography system (ENO-10, Eicom), as previously reported.\(^9\) Urine sodium concentration was determined using ion-sensitive electrodes.

**Blood and Tissue Sampling**

After the 24-hour fast in the metabolic cages, rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (50 mg/kg), and blood samples were obtained from the inferior vena cava. Each kidney was immediately removed and divided into cortex and medulla, and the thoracic aorta was also excised rapidly. The renal cortex, medulla, and aorta were immediately frozen in liquid nitrogen and stored at −80°C. The plasma glucose level was measured by the glucose oxidase method, whereas the plasma insulin level was measured by radioimmunoassay. Plasma sodium concentration was determined using ion-sensitive electrodes.

**Western Blotting**

Tissues were homogenized in 25 mmol/L of Tris-HCl buffer (pH 6.8) with (in mmol/L) sodium dodecylsulfate (SDS) 100, ethylenediaminetetraacetic acid 5, dithiothreitol 56, and phenylmethylsulfonyl fluoride 1. Homogenates were centrifuged at 14 000g for 120 minutes at 4°C, and the supernatant was boiled for 10 minutes. Tissue lysates (80 μg for each sample) were separated on 7.5% SDS-polyacrylamide gels, and transferred to a polyvinyl difluoride membrane (Millipore Corp.). The membranes were blocked with 10% skim milk in Tris-buffered saline pH 7.4, containing 0.1% Tween 20 for 60 hours at room temperature, and incubated overnight at 4°C with a 1:500 dilution of mouse monoclonal antibody to eNOS (Transduction Laboratories). The membranes were incubated for 1 hour with 1:1000 dilution of antimouse secondary antibody conjugated to horseradish peroxidase (Amersham). Bound antibody was detected by enhanced chemiluminescence (ECL kit, Amersham) on XAR film (Kodak). β-actin (monoclonal anti-β-actin antibody, Sigma) was used for all membranes as an internal control, and signals on Western blots were quantified by densitometry and normalized relative to the β-actin signal by use of an image analysis software program (Pharmacia Biotech).

**Reverse Transcription–Polymerase Chain Reaction**

Reverse transcription–polymerase chain reaction (RT-PCR) was performed for semiquantification of tumor necrosis factor-α (TNF-α) mRNA in the renal cortex and medulla of these rats. The total cellular RNA was extracted from the renal cortex and medulla by use of a total RNA extraction kit (ISOGEN, Nippon Gene) according to the manufacturer’s instructions. The total RNA (5 μg) was subjected to RT using a first-strand cDNA synthesis kit (Pharmacia Biotech) with oligo(dt)\(12–18\) primer (GIBCO BRL). By use of the primer pairs for TNF-α and GAPDH,\(^{11}\) PCR was performed for 33 cycles. The PCR product was electrophoresed on a 2% agarose gel, stained with ethidium bromide and photographed on an UV transilluminator. The intensity of bands was measured by densitometry. There was a linear increase in each PCR product at least 35 cycles in preliminary experiments using total RNA obtained from these rats.

**Statistical Analysis**

Data are expressed as mean±SE. Comparisons among the groups were performed with ANOVA followed by the Student-Newman-Keuls post hoc test. A value of \(P<0.05\) was considered statistically significant.

**Results**

**General Features of Animals**

As shown in the Table, there was no difference in final body weight among the 6 groups at 2 weeks after beginning the
to the data in the medulla of high-sodium diet control rats (A and B) and in the aorta of high-sodium diet control rats (C). Values are mean±SE. *P<0.05 vs low-sodium diet rats; †P<0.05 vs control-diet rats; and ‡P<0.05 vs high-fructose diet rats.

Expressions of eNOS protein in the Kidney and Aorta

As shown in the Figure, the expression of cortical eNOS was lower than that of medullary eNOS (eg, the cortical eNOS/β-actin values were 23±7% of medullary eNOS/β-actin values in HS-C rats). There was no significant difference in eNOS protein expression in the renal cortex. In low-sodium diet groups, the expression of medullary eNOS protein in the high-fructose diet rats was not significantly lower than in the control diet rats, whereas the administration of pioglitazone significantly increased the medullary eNOS protein mass compared with that of the high-fructose diet rats. In the high-sodium diet groups, the expression of medullary eNOS protein in the high-fructose diet rats was significantly lower than in the control diet rats. The administration of pioglitazone significantly increased the medullary eNOS protein expression compared with that in the LS-FP, HS-C, or HS-F rats. As shown in the Figure, panel C, there was no significant difference in eNOS protein expression in the aorta. Although we attempted to investigate the expressions of nNOS and iNOS proteins using monoclonal antibodies for them (Transduction Laboratories), neither nNOS nor iNOS proteins were detected distinctly in the kidney and the aorta of these rats.

Expression of TNF-α mRNA in the Renal Cortex and Medulla

TNF-α mRNA existed in the renal cortex and medulla in all of the rats in all 6 groups. However, there was no difference in the ratio of TNF-α mRNA/GAPDH mRNA by RT-PCR among the groups (data not shown).

Discussion

Other investigators have reported that aortic4-22 or renal23 eNOS activity in fructose-fed insulin-resistant rats (FFR) was decreased. There are a few studies4,24 on aortic eNOS expression in FFR, and no studies have examined renal eNOS expression in FFR. Our study is considered to be the first report on renal eNOS protein expression in FFR.

Our present data showing that systolic blood pressure increased significantly only in the HS-F rats suggested the salt dependence of hypertension in this high fructose-fed insulin-resistant model, and it is consistent with a previous report. As in the report by Iyer et al,25 there was no significant difference in urinary sodium excretion between the control rats and the FFR in the present study. A decreased slope of the pressure-natriuretic curve existed in the subjects with salt-sensitive hypertension,26 so these results indicate that as a consequence of the sustained increase in blood pressure, FFR were able to excrete plenty of sodium and maintain fluid balance during a long-term increase in sodium intake. Renal abnormalities that cause increased proximal, distal, and collecting tubule reabsorption; a decreased glomerular filtration coefficient; or loss of nephrons also cause a decreased slope of pressure natriuresis.26 There are some mediators between increased renal perfusion pressure and decreasing tubular reabsorption, such as atrial natriuretic peptides, prostaglandin, renal interstitial hydrostatic pressure, renal medullary blood flow, or renin-angiotensin-aldosterone system. Among these mediators, the NO system participated in the regulation of renal interstitial hydrostatic pressure27 and renal medullary blood flow,28 and the derangement of the NO system in the kidney became the cause of abnormal pressure-natriuresis29 and salt-sensitive hypertension.30 In particular, NO in the renal medulla is important for regulating sodium and water reabsorption.6,12 All 3 isoforms of NOS in the renal medulla are reported to increase for adaptation to increased sodium intake in normal rats.28 In the present study, HS-C rats tended to increase (P=0.06) and HS-FP rats significantly

special diets. Averaged food consumptions for 2 weeks were not different between control and fructose-fed groups. The heart rate was significantly higher in each high-sodium group than in each low-sodium group except in the HS-FP groups. The systolic blood pressure was significantly higher in the HS-F rats than in the LS-F rats or the HS-C rats. The administration of pioglitazone prevented the increase in blood pressure in the HS-F rats. Systolic blood pressure in the LS-F rats was not significantly higher than in the LS-C rats (Table). There were no differences in urine volume, urinary protein, or urinary NOx. The values for urinary sodium excretion in the present study were very low because they reflected the excretion of sodium in rats denied food for 24 hours. Nevertheless, urinary sodium excretion was significantly increased in each high-sodium group, and the values were not different between the control rats and the fructose-fed rats (data not shown). No significant differences were found in plasma glucose, insulin, or sodium among any of the groups (Table).
increased ($P<0.05$) in medullary eNOS expression compared with that of LS-C rats and LS-FP rats, respectively, whereas HS-F rats did not. It is suggested that the defect of increase in medullary eNOS expression on a high sodium intake in FFR may inhibit the adaptive increase in medullary NO production, then may become the cause of salt-sensitive hypertension in this model. Although urinary NOx excretion of the FFR was not significantly lower than that of the control rats in the present study, this finding does not disprove this hypothesis because urinary NOx level reflected not only renal medullary NO metabolic products but also renal cortical or other circulating NO metabolic products. Renal cortical and aortic eNOS protein expressions were not significantly different between FFR and control rats in the present study, which may attenuate the power of the statistical difference in urinary NOx excretion between FFR and control rats. Previous studies of eNOS in the renal medulla of various hypertensive models have demonstrated an increase,\textsuperscript{29–31} a decrease,\textsuperscript{32–35} or no change.\textsuperscript{36} The importance of eNOS expression and protein in the renal medulla for hypertension is therefore not clear. However, a decrease in renal medullary eNOS is considered to be a cause of salt-sensitive hypertension.\textsuperscript{32,33} The smaller expression of cortical eNOS in the present study, which was consistent with others,\textsuperscript{28} and/or the smaller effect of cortical eNOS on long-term control of blood pressure\textsuperscript{6} may explain the reasons why the changes in eNOS were isolated in the medulla of the kidney of these rats. Because nNOS and iNOS were not clearly detected by immunoblotting in the present study, we could not confirm that the alteration of NOS in the renal medulla was selective for eNOS. In the renal medulla, however, eNOS is reported to be most important for prevention of hypertension.\textsuperscript{17,29}

Several studies revealed that insulin caused an upregulation of eNOS expression and NO production both in vitro\textsuperscript{37} and in vivo.\textsuperscript{38} Kuboki et al reported that insulin could regulate the expression of eNOS gene, mediated by the activation of phosphatidylinositol-3 kinase (PI-3 kinase), in endothelial cells and microvessels.\textsuperscript{5} So, there is partly a common pathway through the insulin receptor followed by PI-3 kinase between eNOS production and the glucose uptake by insulin, and renal medullary eNOS production might also be regulated by insulin through PI-3 kinase activation, because renal medulla has a high number of microvessels. The effects of insulin, fructose, high salt, and pioglitazone on eNOS expression remain unclear in the present study, but there are some possibilities about the impairment of renal eNOS upregulation in FFR during a high salt load. Kim et al\textsuperscript{39} reported that fluid share stress increased PI-3 kinase activity; therefore, eNOS upregulation mediated by sodium-induced fluid overload or shear stress in the renal medullary collecting duct cells\textsuperscript{40} may depend on PI-3 kinase activation.\textsuperscript{5} Because PI-3 kinase activity in the muscle and liver of FFR was reported to be reduced,\textsuperscript{41} renal PI-3 kinase activity in FFR is expected to be reduced in the same way, and eNOS upregulation by the sodium-induced fluid overload might be blunted. TNF-$\alpha$ was shown to decrease PI-3 kinase activity\textsuperscript{42} and to degrade eNOS mRNA directly.\textsuperscript{43} We could not show the changes of renal TNF-$\alpha$ mRNA in FFR by RT-PCR, but skeletal muscle TNF-$\alpha$ was reported to increase in FFR,\textsuperscript{44} and if circulating TNF-$\alpha$ also increased in FFR, it might decrease in medullary eNOS expression. Pioglitazone may interrupt these impaired pathways for eNOS upregulation by increasing PI-3 kinase activity\textsuperscript{45} and/or by decreasing TNF-$\alpha$ activity.\textsuperscript{46}

In the present study, the expression of aortic eNOS protein was not significantly different between the control rats and FFR, and this finding is consistent with other reports.\textsuperscript{1,24} It is speculated that there may be different underlying mechanisms to enhance the production of eNOS in the aorta and kidney. Because the systolic blood pressure of FFR was slightly higher than that of control rats, even on a low-sodium diet, the hypertension seen in FFR was thought to be not only salt sensitive but also salt insensitive. Salt-insensitive hypertension was induced by increasing preglobular resistance.\textsuperscript{26} Abnormal function of the aorta in FFR, such as decreased vascular relaxation,\textsuperscript{4} can also cause salt-insensitive hypertension. It is suggested that the defect of increase in renal medullary eNOS on a high sodium intake coexists with many other factors that have been previously reported,\textsuperscript{4,14,22–25,44} and induces hypertension in this animal model. In conclusion, renal medullary eNOS protein expression of FFR was significantly lower than that of control rats during a high sodium load. These results suggest that attenuated responses of increasing medullary NO production by eNOS to the enhanced sodium load may play a role for the salt-sensitive hypertension in FFR. Pioglitazone enhances renal medullary eNOS expression and may ameliorate hypertension in FFR.

**Perspectives**

When elaborating the strategy for the treatment of hypertension based on pressure natriuresis, it is important to restore the blunted slope and right-shift of the pressure natriuresis curve in insulin-resistant subjects. For the former, the agents that can enhance medullary NO production, such as pioglitazone, may be adequate, and for the latter, other current available antihypertensive agents may be useful. Pioglitazone is an activator of peroxisome proliferator-activated receptor-$\gamma$ (PPAR$\gamma$) shown to function in adipogenesis.\textsuperscript{47} PPAR$\gamma$ mRNA was detected only in the inner medulla but not in the cortex in the kidney of Sprague-Dawley rats.\textsuperscript{48} Although the role of PPAR$\gamma$ in the kidney is unknown, it is interesting that PPAR$\gamma$ exists only in the medulla and that changes in eNOS were also seen only in the medulla of FFR in the present study. There is a report that pioglitazone improved insulin resistance by an independent mechanism of adipogenic activity of PPAR$\gamma$,\textsuperscript{45} which indicates that PPAR$\gamma$ agonists can act even on a kidney that has few adipocytes. We would like to clarify the interaction between renal NO and PPAR$\gamma$ for the salt-sensitive hypertension in insulin resistant subjects.

**Acknowledgment**

We thank Takeda Chemical Industries, Osaka, Japan, for providing pioglitazone.

**References**

194 Hypertension August 2002


Decrease in Renal Medullary Endothelial Nitric Oxide Synthase of Fructose-Fed, Salt-Sensitive Hypertensive Rats
Yasuhiro Nishimoto, Takahito Tomida, Hideo Matsui, Takayuki Ito and Kenji Okumura

Hypertension. 2002;40:190-194; originally published online June 17, 2002; doi: 10.1161/01.HYP.0000024267.71656.0D
Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2002 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/40/2/190

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Hypertension can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Hypertension is online at:
http://hyper.ahajournals.org//subscriptions/