Enhanced NO Inactivation and Hypertension Induced by a High-Fat, Refined-Carbohydrate Diet

Christian K. Roberts, Nosratola D. Vaziri, Xiu Q. Wang, R. James Barnard

Abstract—We have recently demonstrated that long-term consumption of a high-fat, refined-carbohydrate (HFS) diet induces hypertension (HTN) in normal rats compared with a low-fat, complex-carbohydrate (LFCC) diet. Limited evidence suggests that high-fat or high-sugar diets cause enhanced generation of reactive oxygen species (ROS). We therefore hypothesized that by inducing oxidative stress, the HFS diet may promote nitric oxide (NO) inactivation and HTN. To test this hypothesis, female Fischer rats were placed on either the HFS or the LFCC diet starting at 2 months of age. Blood pressure, urinary NO metabolites (NOx), and total renal NO synthase activity were monitored, and the tissue abundance of nitrotyrosine (NT), which is the stable “footprint” of NO oxidation by ROS, was determined. The HFS diet group exhibited a gradual rise in arterial blood pressure and were hypertensive by 18 months. This trend was accompanied by a marked accumulation of NT in all tested tissues, an initial rise and a subsequent fall in NO synthase activity, and a fall in urinary NOx excretion. The HFS diet–fed animals had a blunted blood pressure response to the NO synthase inhibitor N^6-nitro-L-arginine methyl ester (L-NAME) compared with the LFCC diet group, which showed a marked hypertensive response to L-NAME. L-NAME–induced HTN was reversible with L-arginine in the LFCC diet group; however, HTN was not corrected by L-arginine supplementation in the HFS diet group. These findings point to enhanced ROS-mediated inactivation and sequestration of NO, which may contribute to the reduction of bioactive NO and HTN in the HFS diet–fed animals. (Hypertension. 2000;36:423-429.)

Key Words: arginine ■ endothelial ■ free radicals ■ insulin resistance ■ L-NAME ■ nitric oxide

Hypertension (HTN) is the most common cardiovascular disease in the United States and is a hallmark risk factor for myocardial infarction, stroke, and congestive heart failure. It has been estimated that one quarter of all adults and one half of all individuals >65 years of age have HTN, and it is more common in men than women. The vast majority of patients with HTN have essential HTN of unknown etiology.

The vascular endothelium is responsible for the production of several vasoactive substances, 1 of the most vital of which is nitric oxide (NO), a potent vasodilator synthesized from L-arginine by the enzyme NO synthase (NOS). Several investigations have demonstrated that endothelium-dependent relaxation is impaired in patients with essential HTN. This endothelial dysfunction could be the result of a decrease in biologically active NO, ultimately leading to a depressor/pressor imbalance stemming from tonic removal of NO-mediated vasodilation.1,2

There is evidence that hyperlipidemia and high-sugar diets, high-fat diets, or both induce oxidative stress. We have recently demonstrated increased lipid peroxidation and subsequently found direct evidence of NO oxidation by reactive oxygen species (ROS) in a model of lead-induced HTN. In addition, we recently reported that female and male rats raised on a high-fat, refined-carbohydrate (HFS) diet developed HTN and endothelial dysfunction. The present study was designed to test the hypothesis that diet-induced HTN is characterized by enhanced NO oxidation by ROS. To this end, we determined tissue nitrotyrosine (NT), the “footprint” of NO-ROS interaction in rats with HFS diet–induced HTN and compared the results with those obtained in the control low-fat, complex-carbohydrate (LFCC) diet–fed animals.

Methods

Animals and Diets

All protocols were conducted in accordance with the University of California, Los Angeles, Animal Research Committee. Inbred female Fischer rats were obtained from Harlan Sprague Dawley (San Diego, Calif) at 2 months of age. We chose to study female animals because our previous studies on the diet-induced metabolic syndrome were also performed on female rats. The diets, fed ad libitum, were prepared in powder form by Purina Test Diets Inc and contained a standard vitamin and mineral mix and all essential nutrients as described previously. In the HFS diet, the fat was primarily from lard plus corn oil (~40% kcal); carbohydrate was from sucrose (~40% kcal). In the LFCC diet, 68% of kcal were from cornstarch and ~10% kcal, from corn oil. Vitamin-free casein was used for protein. Sodium intakes were lower on the HFS diet because...
the rats consumed less food to normalize energy intake. At the various time points, animals were anesthetized with chloral hydrate (250 mg/kg IP), and all of the tissues required for further analyses were excised. The rats were weighed each month.

**Blood Pressure**

Systolic blood pressure was measured by the tail-cuff method as previously described.9

1-NAME Studies

Once HTN was established in the HFS diet group, N\textsuperscript{-}-nitro-L-arginine methyl ester (1-NAME, Sigma Chemical) was added to the drinking water in a concentration of 0.5 mg/mL for 1 day,10 and subsequently the blood pressure was measured. The LFCC diet–fed rats were also given l-arginine (Sigma Chemical, 5 mg/mL in drinking water for 1 day) along with the 1-NAME, and blood pressure was subsequently measured. The HFS diet–fed rats were given only l-arginine at concentrations of 5 or 15 mg/mL for 1 day, and the blood pressure was again measured.

**Urinary NO Metabolite Excretion (Nitrate and Nitrite)**

The rats were fasted (but water was provided ad libitum) and placed into metabolic chambers for 2 days, and urine was collected and frozen until urinalyses were performed. NO metabolites were measured by first injecting the urine into a reflux flask containing 1% vanadium(IV) chloride in 2N HCl sparged with N\textsubscript{2}. At a boiling temperature of 110°C, the reflux solution reduces both nitrite and nitrate (NO\textsubscript{2}\textsuperscript{-} and NO\textsuperscript{3}\textsuperscript{-}) in the urine back to NO gas. The NO was drawn through a Dasibi model 2108 chemiluminescence NO analyzer, where it was reacted with ozone to emit photons that were detected by a photomultiplier tube. The area under the voltage peak was integrated (HP 3396 series II integrator) and converted into total NO (NO\textsubscript{2}\textsuperscript{-} and NO\textsuperscript{3}\textsuperscript{-}) by a photomultiplier tube. The area under the voltage peak was integrated (HP 3396 series II integrator) and converted into total NO\textsubscript{2}\textsuperscript{-} concentration. A second urine sample was then injected into an identical reflux system but with a reflux solution of 1.5% KI in glacial acetic acid. At a boiling temperature of 98°C, this solution will reduce only NO\textsuperscript{3}\textsuperscript{-} back to NO gas. Subtracting this value from the total NO\textsubscript{2}\textsuperscript{-} value yields the concentrations of NO\textsuperscript{3}\textsuperscript{-}.

Finally, the urine was analyzed for creatinine by using a creatinine diagnostic assay kit (Sigma Chemical). Expressing the NO-to-creatinine ratio yields the production rate of creatinine, which is produced at a constant rate; the final results are expressed as micromoles of NO per milligram of creatinine.

**NOS Activity Measurement**

Total NOS activity of both diet groups was assessed at 6 months and 2 years by measuring the conversion of [\textsuperscript{3}H]arginine to [\textsuperscript{3}H]citrulline as previously described by Kohr et al.11 Frozen renal tissues were homogenized in a solution containing 50 mmol/L Tris-HCl (pH 7.4); 1% NP-40; 0.25% sodium deoxycholate; 150 mmol/L NaCl; 1 mmol/L EGTA; aprotinin, leupeptin, and pepstatin (1 \mu mol/mL each); 1 mmol/L Na\textsubscript{2}VO\textsubscript{4}; and 1 mmol/L NaF at 0°C to 4°C in a Polytron homogenizer. Homogenates were centrifuged at 12,000g at 4°C for 5 minutes to remove tissue debris and nuclear fragments. The supernatant was processed for determination of NT abundance by Western blot analysis and for total protein concentration by a bicinchoninic acid protein assay kit (Pierce Inc). Tissue NT abundance (with 100 \mu g of protein) was determined by Western blotting as previously described\textsuperscript{8} with the use of an anti-NT monoclonal antibody (Upstate Biotechnology Inc).

**Statistical Analysis**

Data were analyzed with an ANOVA or \( t \) test. When significant F values were noted, post hoc analyses were performed with a Newman-Keuls multiple comparison test. Differences were considered statistically significant at \( P<0.05 \). Values reported are mean\( \pm \)SEM with 7 or 8 rats per group unless otherwise indicated.

**Results**

**Blood Pressure and Body Weight**

The HFS diet group exhibited a gradual rise in systolic blood pressure during the observation period (\( P<0.008 \), ANOVA), and all time points were significantly different from each other except for the difference between 6 months and 12 months. In contrast, blood pressure did not significantly change in the LFCC diet (control) group throughout the course of the study (Figure 1A). The elevation in blood pressure reached statistical significance (HFS group versus the LFCC group) at 12 and 18 months (\( n=8 \) for each group, \( P<0.01 \)). Body weight was not significantly different between the 2 groups for the first 20 weeks on the diets, after which time the HFS rats gained more weight, ending at 367±9 g versus 254±6 g for the LFCC rats.

**Response to 1-NAME and L-Arginine**

When the NOS inhibitor 1-NAME was added to the drinking water (0.5 mg/mL), blood pressure increased significantly (\( P<0.005 \)) in the LFCC diet–fed rats but was unchanged in the HFS diet–fed rats (Figure 1B). The LFCC rats were then treated with both 1-NAME and L-arginine (5 mg/mL), and blood pressure was significantly reduced, from 150.0±6.7 to 132.8±2.7 mm Hg (\( P<0.01 \)). The addition of L-arginine alone to the drinking water had no effect on the HFS rats at 5 mg/mL (151.8±4.6 mm Hg) and 15 mg/mL (161.0±7.9 mm Hg). Differences in blood pressure response could not be explained by differences in drug intake, since both groups had similar water intakes that served as the vehicle for drug delivery.

**NO Metabolite Excretion**

Urine was collected for 2 days at 6 months and 2 years from animals that were temporarily placed in metabolic chambers, and analyses were performed for both NO metabolites and creatinine. Creatinine output was not significantly different between the 2 groups at either time point. When the NO metabolites were expressed per milligram of creatinine, there was no difference in NO\textsubscript{2}\textsuperscript{-} excretion (NO\textsubscript{2}\textsuperscript{-} and NO\textsubscript{3}\textsuperscript{-} in the HFS group compared with the LFCC group at 6 months (0.38±0.06 versus 0.34±0.12 \mu mol/mg creatinine). However, at 2 years there was a marked reduction in urinary NO\textsubscript{2}\textsuperscript{-} excretion (0.54±0.17 versus 0.01±0.01 \mu mol/mg creatinine, \( P<0.05 \)).
NOS Activity
NOS activity was measured at 6 months and 2 years in renal tissue. The NOS activity was significantly elevated at 6 months in the HFS diet group compared with the LFCC diet group (42.05 ± 1.12 versus 29.74 ± 2.35 pmol/mg, *P*, <0.05). However, at 2 years the NOS activity in the HFS animals was not significantly different from that found in the LFCC group (31.77 ± 2.58 versus 29.75 ± 2.36 pmol/mg) but was significantly reduced from the 6-month value.

Nitrotyrosine
Compared with the LFCC rats, the HFS animals exhibited a marked increase in NT abundance in the aorta (Figure 2), kidney (Figure 3), heart (Figure 4), and liver (Figure 5) at 2 months and 2 years.

Discussion
We have previously reported that the HFS diet induces insulin resistance/hyperinsulinemia and endothelial dysfunction,9,10 which are prevalent in hypertensive patients.13 In the present study, the animals on the HFS diet demonstrated a significant rise in arterial blood pressure, confirming our earlier study.9 This trend was accompanied by a significant fall in urinary NOx excretion. It should be noted that the reduction in urinary NOx, was unrelated to dietary nitrate/nitrate, as the rats were fasted during urine collection. Instead,
it was most likely due to depressed total body NO production, increased NO sequestration, or both. The total renal NOS activity data indicated an initial rise (6 months) in the NO production capacity followed by a decrease at 2 years. The reduction in NOS activity, despite elevated blood pressure at 2 years, points to the partial role of depressed NO production in the chronic phase. Furthermore, increased NT abundance in all tissues studied provides evidence for enhanced inactivation and sequestration of NO in the animals on the HFS diet. It is noteworthy that enhanced NO inactivation/sequestration as NT and other nitrated products was coupled with a compensatory increase in NOS activity that prevented HTN during the early phase of the HFS diet. However, persistent ROS-mediated NO inactivation (as evidenced by increased NT abundance) in the face of decreased NOS activity and NO production resulted in depressed NO availability and HTN with long-term HFS diet consumption.

Figure 2. Effect of diet on aorta NT mass. Upper portion depicts representative Western blot of NT of 2 LFCC and 2 HFS diet–fed rats at 2 months and 2 years. Corresponding group data are illustrated below. n = 4 observations per group, *P < 0.01 vs LFCC.

Figure 3. Effect of diet on renal NT mass. Upper portion depicts representative Western blot of NT of 2 LFCC and 2 HFS diet–fed rats at 2 months and 2 years. Corresponding group data are illustrated below. n = 4 observations per group, *P < 0.01 vs LFCC.

Figure 4. Effect of diet on heart NT mass. Upper portion depicts representative Western blot of NT of 2 LFCC and 2 HFS diet–fed rats at 2 months and 2 years. Corresponding group data are illustrated below. n = 4 observations per group, *P < 0.01 vs LFCC.

Figure 5. Effect of diet on liver NT mass. Upper portion depicts representative Western blot of NT of 2 LFCC and 2 HFS diet–fed rats at 2 months and 2 years. Corresponding group data are illustrated below. n = 4 observations per group, *P < 0.01 vs LFCC.
NO-dependent vasodilation is reportedly attenuated in patients with long-standing HTN, as evidenced by a diminished response to administration of NOS inhibitors. The results of the present study with the NOS inhibitor L-NAME are consistent with the latter reports. Administration of L-NAME resulted in a marked rise in blood pressure in the LFCC diet–fed rats to a level that was comparable to the basal value in the HFS animals. In contrast, blood pressure did not change with L-NAME administration in the HFS group. These results corroborate previous evidence that N\textsuperscript{-}nitro-L-arginine markedly increases blood pressure in healthy individuals but not in hypertensive patients.\textsuperscript{2,14} Furthermore, the lack of a rise in blood pressure with L-NAME treatment in the HFS group was not due to a ceiling effect, as rat strains can exhibit blood pressures exceeding 180 mm Hg.\textsuperscript{15} Taken together, the data point to impaired NO availability for vasodilation in rats maintained on the HFS diet, which we have previously shown to cause endothelial dysfunction.\textsuperscript{10}

To investigate L-arginine depletion as a possible cause of the reduction in NO in the HFS diet group, the diet was supplemented with L-arginine. L-Arginine (5 mg/mL) supplementation abrogated the L-NAME–induced elevation of blood pressure in the LFCC group. However, L-arginine supplementation (2 different dosages) had no effect on HFS diet–induced HTN. These findings exclude substrate deficiency as a cause of the associated HTN and reduced NO availability in the HFS diet–fed animals. These data are in agreement with previous studies investigating the effects of exogenous arginine administration in HTN induced by chronic NOS inhibition.\textsuperscript{16} Normally, NO generation by NOS is not sensitive to substrate concentration because the \( \kappa_m \) of NOS (\( \approx \) 3 \( \mu \)mol/L) is well below the normal concentration of L-arginine.\textsuperscript{17} The present data suggest that L-arginine depletion was not a factor in the pathogenesis of HFS diet–induced HTN, although absorption of the arginine supplement was not determined. There is, however, no evidence of defective amino acid absorption in HTN.

Various ROS, ie, superoxide, hydrogen peroxide, and hydroxyl radical, can inactivate NO. For example, superoxide combines with NO to produce peroxynitrite (ONOO\textsuperscript{-}), a potent cytotoxic reactive nitrogen species that subsequently reacts with proteins, lipids, and DNA to induce tissue damage.\textsuperscript{18} ONOO\textsuperscript{-} reacts with tyrosine residues to produce NT, a stable footprint of NO oxidation by ROS that can be detected in tissues. In addition, ROS can react with tyrosine to produce tyrosyl radicals, which can oxidize NO to generate NT.\textsuperscript{19} Consequently, nitration of tyrosine residues can be used as a surrogate measure for ROS-mediated inactivation of NO. The elevation of NT abundance in various tissues of the HFS diet–fed group is tangible evidence for the presence of oxidative stress leading to enhanced NO inactivation and sequestration in tissues. Increased accumulation of NT in various tissues has been recently documented in lead-induced HTN,\textsuperscript{8} which is marked by increased ROS.\textsuperscript{7} This phenomenon contributes to HTN by limiting NO availability to vascular smooth muscle, causing vasoconstriction, and to renal tubular epithelial cells, causing sodium retention.

Accumulation of NT and hence, enhanced ROS-mediated inactivation of NO, was evident as early as 2 months after consumption of the HFS diet. This change was accompanied by a compensatory increase in the NOS activity that was evident at 6 months. We have recently shown a marked increase in inactivation of NO by ROS in rats with lead-induced HTN.\textsuperscript{8} This was associated with a significant fall in urinary NO\textsubscript{X} excretion and a compensatory upregulation of NOS isotype expression in these animals.\textsuperscript{20} The upregulation of NOS isotypes in the latter studies was due to ROS-mediated reduction of available NO, which has been shown to exert a negative-feedback influence on NOS protein expression.\textsuperscript{21} Despite enhanced NO inactivation and sequestration, urinary NO\textsubscript{X} excretion was normal and blood pressure was mildly elevated during the first 6 months on the HFS diet. These adaptive changes noted early in the course of feeding the HFS diet to young animals can in part be due to the observed increase in NOS activity and perhaps sufficient natural antioxidant capacity. However, with long-term HFS diet consumption, the urinary NO\textsubscript{X} excretion fell and blood pressure rose significantly, denoting a progressive decline in the animals’ capacity to overcome the effects of the HFS diet.

It should be noted that by diverting NO to reactive nitrogen species, protein nitration can contribute to an initial decline in NO\textsubscript{X} generation and its urinary excretion. However, isomerization of ONOO\textsuperscript{-} and turnover of NT and other nitrated products to nitrate and nitrite will ultimately raise NO\textsubscript{X} production and its urinary excretion to a level approximating the rate of NO generation. Thus, the fall in urinary NO\textsubscript{X} excretion observed in rats consuming the HFS diet during the chronic phase is a strong indicator of reduced NO production. This viewpoint is supported by the observation that NOS activity, which was elevated in the early phase, fell significantly in the late phase of HFS diet consumption. The reduction of NOS activity may be due to a quantitative deficiency and/or a depressed activity of the enzyme. We wish to acknowledge that there exist alternative pathways other than NOS by which citrulline may be generated in tissues.\textsuperscript{22} Although we did not measure the NOS-inhibitable fraction of citrulline generation in our assay, we have recently documented an increase in the immunodetectable levels of NOS that parallels the functional data reported here (C.K.R. et al, unpublished data, 2000). The constellation of HTN, decreased urinary NO\textsubscript{X} excretion, and increased tissue NT abundance found in the HFS diet–fed animals herein closely resembles the earlier findings in animals with lead-induced HTN.\textsuperscript{8,21} The sources of the NO recovered in the urine as NO\textsubscript{X} are not known, as NOS is present in many cell types. Nevertheless, the reduction in urinary NO\textsubscript{X} excretion in the aged, hypertensive, HFS animals is indicative of depressed NO availability in this model. This observation is consistent with earlier reports that have demonstrated a decrease in bioactive NO in hypertensive humans.\textsuperscript{23}

Böger et al\textsuperscript{24} have suggested several potential mechanisms that could explain the impaired biological activity of the NO pathway observed in HTN, including (1) defective L-arginine availability, (2) decreased expression or activity of the NOS enzymes, and (3) increased oxidative degradation of NO. The present data appear to rule out a L-arginine deficiency. However, our data suggest that the decrease in biologically active NO may be related to enhanced oxidative scavenging...
and inactivation of NO. Our previous data\textsuperscript{20,25} as well as that of others,\textsuperscript{26} suggest that NOS isotype expression paradoxically increases in some forms of HTN. Additionally, the decrease in total renal NOS activity at 2 years compared with 6 months in the HFS group may be due to NOS inhibition, possibly by an endogenous NOS inhibitor such as asymmetric dimethylarginine,\textsuperscript{27} which has been demonstrated to be elevated in hypertensive patients,\textsuperscript{28} or alternatively, due to cofactor depletion by ROS.\textsuperscript{29} These factors, as well as enzymatic inactivation due to oxidative damage, may contribute to the decrease in NOS activity coincident with the reduction in NO\textsubscript{x} excretion noted later in this model. The persistent elevation of NT abundance may appear to conflict with the declining NO production in the chronic phase of the HFS diet; however, a greater proportion of NO produced during this period appears to have been consumed by ROS-mediated inactivation and sequestration processes, which sustained the elevated NT burden while virtually exhausting the bioactive NO. The latter contention is supported by the lack of a pressor response to \textit{L}-NAME in the HFS diet group, indicating a virtual absence of NO-mediated vasodilatory tone. Accordingly, massive diversion of NO was sufficient to maintain the elevation in NT abundance despite an overall reduction in total-body NO production.

The rise in blood pressure on the HFS diet was a slow and gradual process. However, metabolic abnormalities appeared quite early. For instance, insulin resistance and hyperinsulinemia have been previously demonstrated within 2 weeks,\textsuperscript{9} and hypertriglyceridemia, which is associated with endothelial dysfunction, is observed at 2 months, well before the development of significant HTN.\textsuperscript{9} In addition to the compensatory responses noted earlier, during the early phase of the study the protective effects of estrogen as an antioxidant and a vasodilator (acting via NOS) may have helped retard HTN in the young female rats.\textsuperscript{30} The observation that significant HTN was not noted in the HFS rats until they had been on the diet for 18 months and were at a “postmenopausal” age supports this contention. A similar pattern has been observed in women in this country, in whom HTN is generally not seen until after menopause, and suggests that this dietary paradigm may be applicable to human HTN.

The precise mechanism by which the HFS diet induced oxidative stress in our animals is not clear. However recent evidence suggests that high-sugar,\textsuperscript{5,28} high-fat,\textsuperscript{6} and high-cholesterol\textsuperscript{31} diets increase free radical production. In addition, the high sucrose content of the HFS diet causes postprandial hyperglycemia, which has been recently demonstrated to increase the generation of superoxide in human endothelial cells.\textsuperscript{32} These in vitro findings are consistent with the present data that show an initial elevation in NOS activity and ROS-mediated increase in NT abundance in rats on the HFS diet. Furthermore, the role of ROS in the pathogenesis of HTN has been substantiated in a recent study by Vaziri et al.,\textsuperscript{53} who demonstrated the occurrence of severe, sustained HTN with the induction of oxidative stress by glutathione depletion in normotensive rats.

Overall, this study has demonstrated the presence of oxidative stress and inactivation of NO in rats maintained on the HFS diet, which may contribute to the development of HTN. Further work is needed to establish whether or not insulin resistance/hyperinsulinemia, obesity, or both are directly involved in the mechanism of diet-induced HTN or whether these conditions are simply another independent manifestation of the high-fat, refined-carbohydrate diet typically consumed in westernized societies. Finally, whether or not antioxidant therapy can affect HFS diet–induced HTN remains to be studied.

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