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Oxidative stress and dysregulation of NAD(P)H oxidase and antioxidant enzymes in diet-induced metabolic syndrome

Christian K. Roberts[a], R. James Barnard[a], Ram K. Sindhu[b], Michael Jurczak[c,d], Ashkan Ehdaie[b], Nosratola D. Vaziri[c,d,*]

[a]Department of Physiological Science, University of California, Los Angeles, CA 90095, USA
[b]Division of Endocrinology, Metabolism and Molecular Medicine, Charles R. Drew University, Los Angeles, CA 90059, USA
[c]Division of Nephrology and Hypertension, Department of Medicine, University of California, Irvine, CA 92697, USA
[d]Department of Physiology and Biophysics, University of California, Irvine, CA 92697, USA

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Abstract

Previously, we have demonstrated that chronic consumption of a high-fat, high-refined sugar (HFS) diet results in metabolic syndrome which is marked by obesity, insulin resistance, hyperlipidemia, and hypertension in Fischer rats. Metabolic syndrome in this model is associated with oxidative stress, avid nitric oxide (NO) inactivation by reactive oxygen species (ROS), diminished NO bioavailability, and dysregulation of NO synthase isotypes. Although occurrence of oxidative stress and its impact on NO metabolism are well established, the molecular source(s) of ROS in this model is unknown. In an attempt to explore this issue, we measured protein expressions of the key ROS-producing enzyme, NAD(P)H oxidase, and the main antioxidant enzymes, superoxide dismutase (CuZn SOD and Mn SOD), catalase, glutathione peroxidase (GPX), and heme oxygenase-2 (HO-2), in the kidney and aorta of Fischer rats fed an HFS or low-fat, complex-carbohydrate diet for 7 months. In addition, plasma lipid peroxidation product (malondialdehyde) as well as endothelium-dependent and -independent vasorelaxation (aorta rings) was determined. The results showed a significant upregulation of $gp91^{phox}$ subunit of NAD(P)H oxidase and downregulations of SOD isoforms, GPX, and HO-2 in the kidney and aorta of the HFS-fed animals. This was associated with increased plasma malondialdehyde concentration and impaired vasodilatory response to acetylcholine, but not the NO donor, Na nitroprusside. The latter findings confirm the presence of oxidative stress and endothelial dysfunction in the HFS-fed rats. Oxidative stress and endothelial dysfunction in the diet-induced metabolic syndrome are accompanied by upregulation of NAD(P)H oxidase, pointing to increased ROS production capacity, and downregulation of SOD isoforms, GPX, and HO-2, the key enzymes in the antioxidant defense system.

1. Introduction

Metabolic syndrome, otherwise known as syndrome X or insulin resistance syndrome, is defined by the presence of insulin resistance, hyperinsulinemia, and some combination of obesity, dyslipidemia, inflammation, endothelial dysfunction, and hypertension [1-9]. The incidence of metabolic syndrome has reached epidemic proportion worldwide due primarily to prevailing sedentary lifestyle and unhealthy dietary habits. Consequently, metabolic syndrome has emerged as a major cause of diabetes, cardiovascular, and kidney diseases in the industrialized societies [10,11].

In a series of earlier studies, we demonstrated that chronic consumption of a high-fat, high-refined sugar (HFS) diet induces obesity, insulin resistance, dyslipidemia, and hypertension in genetically normal rats [12,13]. Hypertension in this model is associated with oxidative stress [14], avid nitric oxide (NO) inactivation, depressed NO bioavailability [15], and downregulations of NO synthase (NOS) isoforms, AKT (eNOS activator), and soluble guanylate cyclase (downstream target of NO) [14]. It is of note that diminished NO production and NOS expression found in rats with diet-induced metabolic syndrome are also seen in the obese Zucker rats with hereditary metabolic syndrome [16]. The reduced NO production capacity in the obese Zucker rats is associated with inflammation and activation of inflammatory pathways [17].

Oxidative stress plays a major role in the pathogenesis of endothelial dysfunction, hypertension, inflammation, and

* Corresponding author. Division of Nephrology and Hypertension, Department of Medicine, University of California, Irvine, CA 92668, USA. Tel.: +1 714 456 5142; fax: +1 310 456 6034.
E-mail address: ndvaziri@uci.edu (N.D. Vaziri).

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Table 1
Low-fat, complex-carbohydrate and high-fat, high-refined sugar diet composition

Diet	LFCC	HFS
% Energy as carbohydrate	59.81	40.12
% Energy as fat	12.14	39.09
% Energy as protein	28.05	20.72
Fiber (g/100 g)	5.3	1.6
Sucrose (g/100 g)	3.68	45.10
% Cholesterol	0.0200	0.0175
% Saturated fat	1.50	7.91
% Monounsaturated fat	1.58	8.22
% Polyunsaturated fat	0.71	3.04
Physiological energy (kJ/g)	13.97	19.63

atherosclerotic cardiovascular disease. Although the presence of oxidative stress and its impact on NO metabolism in rats with diet-induced metabolic syndrome are well established [14,15], the molecular sources of the reactive oxygen species (ROS) in this model are uncertain. Oxidative stress can result from either excess ROS production and/or deficient antioxidant capacity. The present study was designed to test the hypothesis that oxidative stress in rats with diet-induced metabolic syndrome may be due to upregulation of NAD(P)H oxidase (a major source of ROS in the kidney and cardiovascular tissues) and downregulation of the main antioxidant enzymes, superoxide dismutases, glutathione peroxidase (GPX), catalase (CAT), and heme oxygenase.

2. Methods

2.1. Animals and diets

All protocols were approved and conducted in accordance with the University of California, Los Angeles, Animal Research Committee. Two-month-old female Fischer 344 rats were obtained from Harlan Sprague Dawley (San Diego, Calif). We have used this rat model in previous studies, as the female Fischer rat normally shows little weight gain after the maturation phase [12,18]. The animals (6 per group) were housed in a light-controlled (12-hour light cycle starting at 7 AM) and temperature-regulated (24°C) space. They were allowed to acclimatize to their environment for 1 week, consuming standard rat chow (Purina 5001), before the dietary intervention was initiated. The animals were randomly assigned to either the LFCC or HFS diet with food and water ad libitum. The diets were prepared in powder form by Purina Test Diets (Richmond, Ind) and contained a standard complement of vitamin and mineral mix and all essential nutrients. The LFCC diet (Purina 5001) is low in saturated fat and contains mostly complex carbohydrate, whereas the HFS diet is high in saturated and monounsaturated fat (primarily from lard plus a small amount of corn oil) and high in refined sugar (sucrose). The diet components are listed in Table 1. Blood pressure was measured by tail cuff plethysmography (Harvard Apparatus, Holliston, Mass) in conscious rats placed on a heated pad in a quiet room as previously described [15]. Four consecutive measurements were obtained and the average of the 4 values was used.

At the conclusion of the study period, the animals were anesthetized and exsanguinated by cardiac puncture and plasma was stored at −80°C until processed. The tissues were flash frozen in liquid nitrogen and stored at −80°C until processed.

Lipoperoxides were determined in plasma samples by measuring malondialdehyde (MDA) via high-performance liquid chromatography as described previously [19].

2.2. Measurement of endothelial function

The animals were anesthetized by intraperitoneal injection of ketamine (100 mg/kg) and xylazine (5 mg/kg). After adequate anesthesia, a thoracotomy was performed and the descending thoracic aorta was carefully removed and placed in chilled Krebs-Henseleit physiological solution containing (in millimoles per liter) 131.5 NaCl, 5.0 KCl, 1.2 $MgCl_2 \cdot 6H_2O$, 2.5 $CaCl_2 \cdot 2H_2O$, 1.2 $NaH_2PO_4 \cdot H_2O$, 11.2 glucose, and 20.8 $NaHCO_3$. All remaining fat and connective tissue were gently removed, and the aorta was sectioned into 5-mm segments. The segments were mounted on standard tungsten wire triangles (A-M Systems, Everett, Wash), attached to isometric force displacement transducers (FTO3C, Grass Instrument, Quincy, Mass), and placed into tissue baths. The transducer output was amplified and recorded continuously on a portable computer with digital analysis software (Femto Tek, Mt Laurel, NJ). The tissue baths were temperature-controlled via a heated water jacket at 37°C. A 95% oxygen, 5% carbon dioxide gas mixture was bubbled

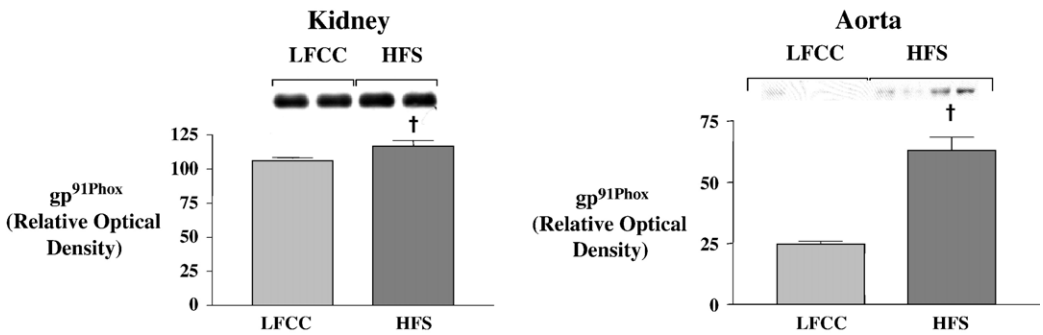

Fig. 1. Representative Western blots and group data depicting protein abundance of $gp91^{phox}$ subunit of NAD(P)H oxidase in the kidney and thoracic aorta of rats fed an HFS or an LFCC diet for 7 months. n = 6 in each group, $P < .05$.

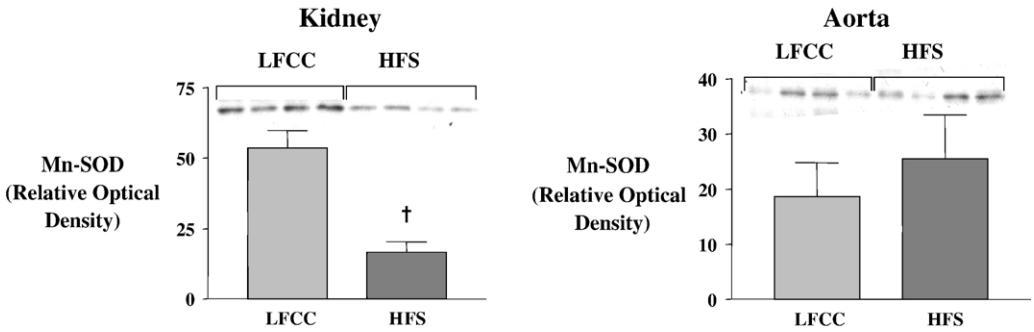

Fig. 2. Representative Western blots and group data depicting protein abundance of Mn SOD in the kidney and thoracic aorta of rats fed an HFS or an LFCC diet for 7 months. n = 6 in each group, $P < .05$.

into the tissue baths. Preload (2 g) was applied to the arterial rings and the vessels were allowed to equilibrate for 45 minutes. Thereafter, the arteries were constricted using a high-potassium Kreb's solution (18 mmol/L KCl) and allowed to reequilibrate. This represented the maximal contractile force for the artery (F_{max}). The baths were then emptied and rinsed 3 times with Kreb's solution and again allowed to equilibrate. The arteries were then constricted to 75% of F_{max} with phenylephrine (10^{-5} mol/L, Sigma, St Louis, Mo). Acetylcholine (Sigma) was then added in incremental log concentrations from 10^{-8} to 10^{-4} mol/L for determination of endothelium-dependent relaxation. Endothelium-independent relaxation was measured in a similar fashion using sodium nitroprusside (Sigma) in incremental log concentrations from 10^{-9} to 10^{-5} mol/L. Baths were rinsed and arterial segments were brought to 75% F_{max} with phenylephrine between each reagent. Tension was measured in grams, and contraction and relaxation were recorded as percentages of 75% F_{max} for each incremental dose of reagent.

2.3. Immunoblot analyses

Immunoblotting was performed in the kidney and thoracic aorta to quantify NADPH oxidase gp91^{phox}, Mn SOD, CuZn SOD, GPX, CAT, and heme oxygenase-2 (HO-2) protein levels. Homogenates (25% wt/vol) of kidney and thoracic aorta were prepared in 10 mmol/L *N*-[2-hydroxyethyl]-piperazine-*N*′-2-ethanesulfonic acid buffer, pH 7.4, 1 mmol/L ethylenediaminetetraacetic acid, 1 mmol/L dithiothreitol, 10 μg/mL leupeptin, 2 μg/mL aprotinin, and 1 mmol/L phenylmethylsulfonyl fluoride at 0°C to 4°C with a polytron tissuemizer (Kinematica AG, Luzernerstrasse, Switzerland). Homogenates were centrifuged at 9000 × *g* for 10 minutes at 4°C to remove nuclear fragments and tissue debris without precipitating plasma membrane fragments. A portion of the supernatant was used for the determination of total protein concentration by using a Bio-Rad kit (Bio-Rad, Hercules, Calif). Western blot analyses for the above proteins were performed as described in our previous studies [20,21] using the primary antibodies against NADPH oxidase gp91^{phox} (Transduction Labs, Franklin Lakes, NJ), Mn SOD (Calbiochem, San Diego, Calif), CuZn SOD and CAT (Calbiochem), GPX (Cortex Biochem, San Leandro, Calif), and HO-2 (rabbit polyclonal, Stressgen, Victoria, DC, Canada).

2.4. Statistical analysis

Data were analyzed using paired Student *t* tests. Vascular ring experiments were analyzed using analysis of variance, and post hoc analyses were performed when significant differences were noted using a Newman-Keuls multiple comparison test. Differences were considered statistically significant at $P < .05$. Values reported are means ± SE with 6 rats per group unless otherwise indicated.

3. Results

3.1. Blood pressure and MDA

Tail arterial blood pressure at the conclusion of the 7-month study period was slightly higher in the HFS group

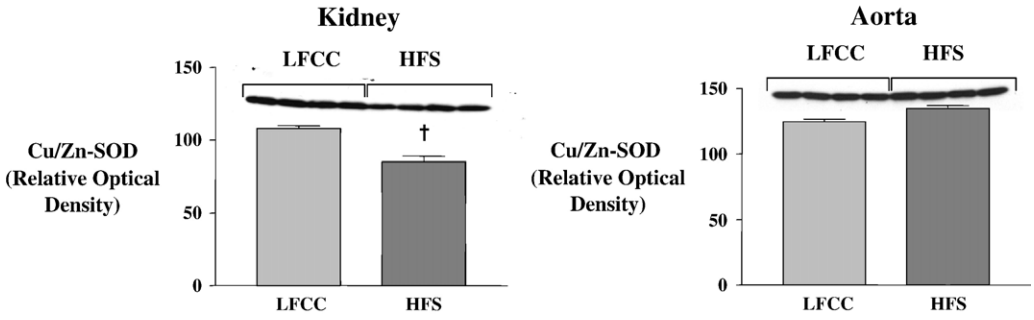

Fig. 3. Representative Western blots and group data depicting protein abundance of CuZn SOD in the kidney and thoracic aorta of rats fed an HFS or an LFCC diet for 7 months. n = 6 in each group, $P < .05$.

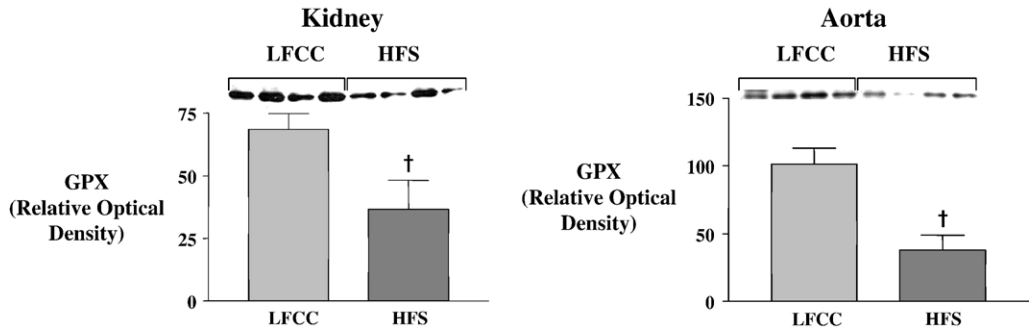

Fig. 4. Representative Western blots and group data depicting protein abundance of GPX in the kidney and thoracic aorta of rats fed an HFS or an LFCC diet for 7 months. n = 6 in each group, $P < .05$.

(131 ± 6 mm Hg) than in the LFCC group (120 ± 2 mm Hg, $P > .05$). Likewise, plasma MDA level in the HFS group (1.91 ± 0.08 μmol/L) was significantly greater than in the LFCC group (1.37 ± 0.06 μmol/L, $P < .05$) denoting the presence of oxidative stress in the HFS rats.

3.2. NAD(P)H oxidase

Data are illustrated in Fig. 1. The $gp91^{phox}$ protein abundance in the thoracic aorta was significantly greater in the HFS-fed group than that found in the LFCC-fed group. Likewise, the $gp91^{phox}$ protein abundance was increased in the kidneys of the HFS-fed group when compared to that in LFCC-fed group.

3.3. Superoxide dismutase data

Data are shown in Figs. 2 and 3. Manganese SOD abundance was significantly lower in the kidneys of the HFS group compared to the LFCC group. Likewise, CuZn SOD abundance was significantly reduced in the kidney and aorta of the HFS group.

3.4. Glutathione peroxidase and CAT

Results are depicted in Figs. 4 and 5. Glutathione peroxidase abundance was significantly reduced in the kidney and aorta of the HFS group when compared with that in the LFCC group. However, CAT abundance in the kidney and aorta was similar among the HFS and LFCC groups.

3.5. Heme oxygenase-2

Results are given in Fig. 6. Heme oxygenase-2 abundance was significantly lower in the kidney and aorta of the HFS group compared to the LFCC group.

3.6. Endothelium-dependent vasorelaxation

Data are shown in Fig. 7. Vasorelaxation response to acetylcholine in phenylephrin-precontracted aorta rings was significantly reduced in the HFS group as compared to that found in the LFCC group. In contrast, the vasodilatory response to the NO donor nitroprusside was comparable among the LFCC and the HFS groups. These observations point to impaired endothelium-dependent vasorelaxation response in the HFS-fed animals.

4. Discussion

Several studies have shown that diets high in fat and refined sugars (sucrose or fructose) cause endothelial dysfunction and ultimately hypertension [15,22-25]. Endothelial dysfunction and hypertension in these and other

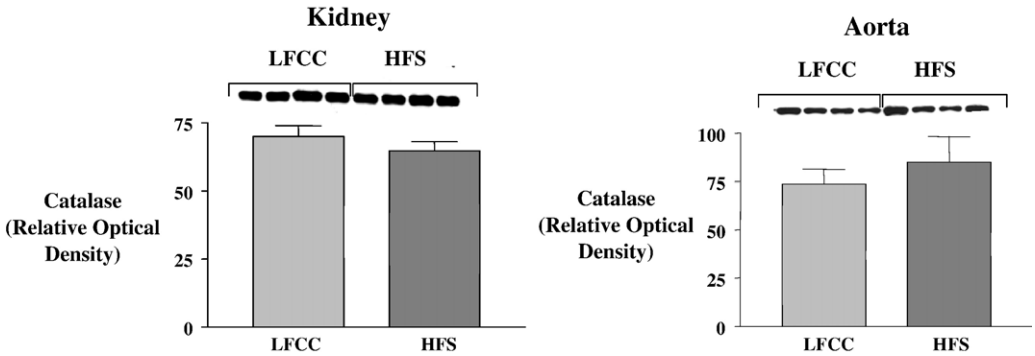

Fig. 5. Representative Western blots and group data depicting protein abundance of catalase in the kidney and thoracic aorta of rats fed an HFS or an LFCC diet for 7 months. n = 6 in each group.

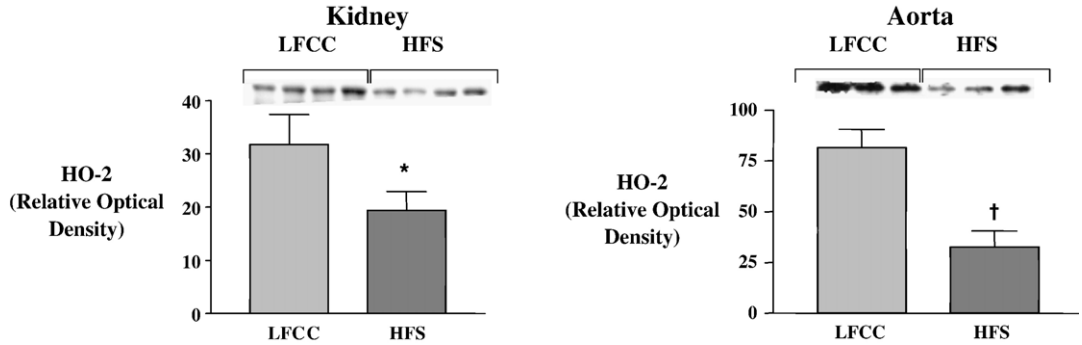

Fig. 6. Representative Western blots and group data depicting protein abundance of HO-2 in the kidney and thoracic aorta of rats fed an HFS or an LFCC diet for 7 months. n = 6 in each group, $P < .05$.

models are associated with and, at least in part, due to oxidative stress [26,27]. Oxidative stress in the kidney and vascular tissues can promote endothelial dysfunction by several mechanisms. For instance, oxidative stress limits the bioavailability of NO via ROS-mediated inactivation of preformed NO [28-30]. In addition, oxidative stress results in depletion of the NOS cofactor tetrahydrobiopterin and in uncoupling of the NO syntheses which normally exists in a homodimeric state. The latter events, in turn, reduce NO production and promote superoxide generation by NO syntheses [31]. In addition, as reviewed by Rodriguez-Itrube et al [26], oxidative stress adversely affects renal and cardiovascular function and structure by promoting inflammation (NFκB activation), endothelial injury, vascular smooth muscle cell/fibroblast/mesangial proliferation, matrix accumulation, and generation of isoprostanes from nonenzymatic oxidation of arachidonic acid.

Superoxide ($O_2^{\bullet}$) avidly reacts with NO producing peroxynitrite ($O_2^{\bullet} + NO \rightarrow ONOO^-$), a highly reactive and cytotoxic radical that attacks and denatures lipids, nucleic acids, and proteins [30]. For instance, peroxynitrite reacts with tyrosine residues in protein molecules producing nitrotyrosine which is frequently used as a stable marker of ROS-NO interaction [28-30]. In an earlier study, we found significant accumulation of nitrotyrosine in the kidney and cardiovascular tissues denoting increased NO inactivation by ROS in rats fed an HFS diet for 2 months [15]. Although these and other observations illustrate the presence of oxidative stress and its impact on NO metabolism in HFS-fed animals, to our knowledge, the possible origin of oxidative stress in this model has not been investigated.

NAD(P)H oxidase is a major source of ROS in the kidney and cardiovascular tissues [27,32-35]. In fact, oxidative stress is associated with upregulation of this enzyme in the kidney and vascular tissues in several animal models of acquired and genetic hypertension [20,21,32,34,36-39]. The enzyme catalyzes the transfer of a single electron to molecular oxygen to produce superoxide ($O_2 + e^- \rightarrow O_2^{\bullet -}$). The $gp91^{phox}$ membrane subunit of NAD(P)H oxidase was significantly elevated in the kidneys and aortas of our HFS-fed animals. Upregulation of NAD(P)H oxidase can raise ROS production and thereby contribute to the pathogenesis of oxidative stress and NO inactivation in the HFS-fed rats [15].

Normally, superoxide is converted to hydrogen peroxide by superoxide dismutases ($O_2^{\bullet -} + O_2^{\bullet -} + 2H^+ \rightarrow H_2O_2 + O_2$). Hydrogen peroxide is, in turn, inactivated by either CAT ($2H_2O_2 \rightarrow 2H_2O + O_2$) or GPX ($H_2O_2 + 2GSH \rightarrow GSSG + 2H_2O$). Heme oxygenases catalyze the conversion of heme (which is a pro-oxidant factor) to carbon monoxide

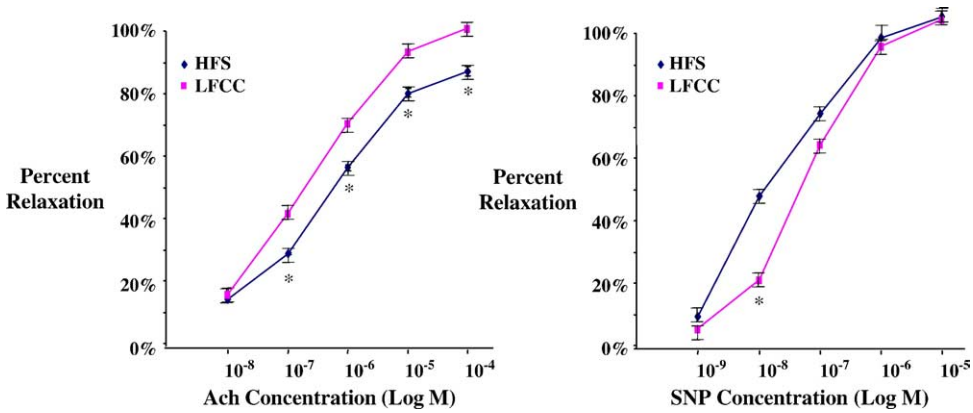

Fig. 7. Vasorelaxation response to different concentrations of acetylcholine (endothelium-dependent) and NO-donor nitroprusside (endothelium-independent) in aorta rings obtained from rats fed an HFS or LFCC diet for 7 months. n = 6 observations per group, $*P < .05$.

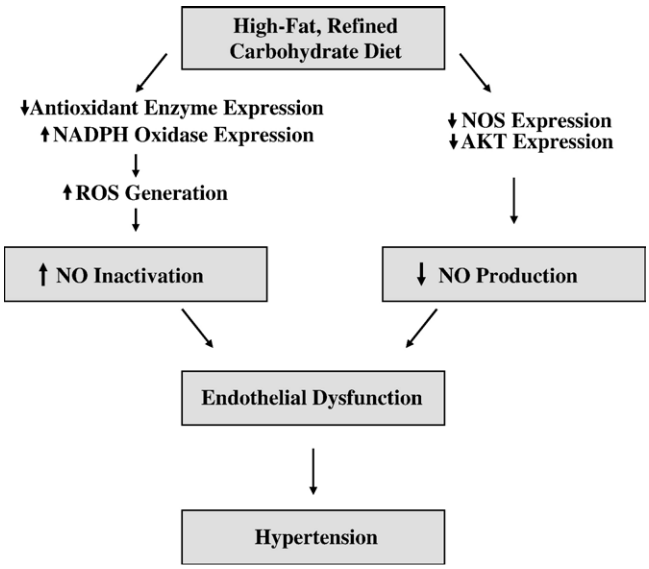

Fig. 8. Proposed pathways involved in the pathogenesis of endothelial dysfunction and hypertension caused by chronic consumption of an HFS diet.

and biliverdine/bilirubin which are potent antioxidants [40]. Thus, SODs, CAT, GPX, and heme oxygenase represent important components of the antioxidant defense system. Consequently, deficiencies of one or more of these enzymes can contribute to oxidative stress.

The HFS-fed animals exhibited significant reductions of mitochondrial SOD (Mn SOD), cytoplasmic SOD (CuZn SOD), GPX, and HO-2 in the kidney. In addition, GPX, CuZn SOD, and HO-2 were reduced in the aorta. Thus, upregulation of ROS-generating enzyme, NAD(P)H oxidase, in HFS-fed animals is compounded by downregulations of several key antioxidant enzymes, that is, SOD isoforms, GPX, and heme oxygenase. These events can work in concert to promote oxidative stress, endothelial dysfunction, hypertension, and cardiovascular disease in animals maintained on the HFS diet. It is of note that the HFS diet used in our studies was formulated to resemble diets commonly consumed in westernized societies. As noted above, oxidative stress is a common cause of endothelial dysfunction. The acetylcholine-mediated vasorelaxation response in the aorta rings obtained from the HFS-fed rats was significantly impaired when compared to that found in the LFCC-fed animals. In contrast, the vasodilatory response to the NO donor, sodium nitroprusside, was similar in the 2 groups. These observations confirm the presence of endothelial dysfunction in HFS-fed animals shown in our earlier studies [41].

In conclusion, consumption of an HFS diet results in upregulation of the ROS-generating enzyme, NAD(P)H oxidase, and in downregulation of several key antioxidant enzymes in the kidney and vascular tissues. These events can, in part, account for oxidative stress and endothelial dysfunction in this model. In addition, these findings extend our previous findings that an HFS diet increases NO inactivation by ROS and decreases NO production. Fig. 8 depicts a proposed model of the events induced by an HFS diet, leading to endothelial dysfunction and subsequent hypertension.

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