Renal protection by a soy diet in obese Zucker rats is associated with restoration of nitric oxide generation

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Am J Physiol Renal Physiol 288: F108–F116, 2005. First published August 24, 2004; doi:10.1152/ajprenal.00077.2004.—The obese Zucker rat is a valuable model for studying kidney disease associated with obesity and diabetes. Previous studies have shown that substitution of animal protein with soy ameliorates the progression of renal disease. To explore the participation of nitric oxide (NO) and caveolin-1 in this protective effect, we evaluated proteinuria, creatinine clearance, renal structural lesions, nitrites and nitrates urinary excretion (UNO2\textsubscript{2}/NO3\textsubscript{3}), and mRNA and protein levels of neuronal NO synthase (nNOS), endothelial NOS (eNOS), and caveolin-1 in lean and fatty Zucker rats fed with 20% casein or soy protein diet. After 160 days of feeding with casein, fatty Zucker rats developed renal insufficiency, progressive proteinuria, and renal structural lesions; these alterations were associated with an important fall of UNO2/NO3, changes in nNOS and eNOS mRNA levels, together with increased amount of eNOS and caveolin-1 present in plasma membrane proteins of the kidney. In fatty Zucker rats fed with soy, we observed that soy diet improved renal function, UNO2/NO3, and proteinuria and reduced glomerulosclerosis, tubular dilation, interstitial fibrosis, and extracapillary proliferation. Renal protection was associated with reduction of caveolin-1 and eNOS in renal plasma membrane proteins. In conclusion, our results suggest that renal protective effect of soy protein appears to be mediated by improvement of NO generation and pointed out to caveolin-1 overexpression as a potential pathophysiological mechanism in renal disease.

plasma membrane proteins; endothelial nitric oxide synthase; nitrites and nitrates excretion

SEVERAL RISK FACTORS ASSOCIATED with an increased rate of progression in renal disease have been informed, including African-American race, male sex, hypertension, obesity, diabetes, hyperlipidemia, smoking, high-protein intake, phosphate retention, and metabolic acidosis (36). Although obesity is often associated with diabetes and hypertension, which are two of the most common risk factors for the development of end-stage renal disease (ESRD), it has been suggested that obesity per se is an independent risk factor (22). Massive obesity has been shown to produce nephrotic syndrome (56, 58), and it has been reported that proteinuria and segmental glomerulosclerosis can be present in obese patients, even in the absence of diabetes (59). In addition, a large-scale study including 6,818 renal biopsies from 1986 to 2000 revealed a 10-fold increase in renal lesion, such as glomerulomegaly and focal segmental glomerulosclerosis, which were associated with obesity. Increased frequency of obesity-related glomerulosclerosis over the studied time ran in parallel with the increased prevalence of obesity within general population (33).

Mediators involved in obesity-induced renal injury are poorly understood. Available information comes from studies performed in rats and dogs fed with high-fat diet (23, 25) and from fatty Zucker rat (17, 31, 34, 38, 41, 49), an animal model of genetic obesity that results from inactivating mutation in leptin receptor gene. Homozygous Zucker rats (fa/fa) exhibit most of the metabolic picture seen in human obesity, including hypercholesterolemia, hypertriglyceridemia, hyperinsulinemia, and proteinuria. These animals also develop glomerular hypertension, hypertrophy, and sclerosis (6, 17, 31, 49) and often die due to ESRD (31). The mechanism by which renal disease is produced in obese Zucker rats is largely unknown, but it has been shown that consumption of plant-derived proteins retards the development and progression of renal disease in humans and in several animal models of disease (9, 27, 30, 46, 52, 61). Maddox et al. (39) observed that soy protein diet reduces plasma cholesterol levels, urinary protein excretion, and the rate of progression of glomerular injury in obese Zucker rat. The mechanisms by which soy protein conferred renal protection, however, were not established, but it has been suggested that could be related to the high content of phytoestrogens in soybean products, compared with animal sources of protein (54). Isoflavones (the most common phytoestrogens in soy plant) have been shown to possess antiproliferative and antioxidant properties (51, 57) and to act as weak estrogens (40) by blocking transcription of growth factor-β1 (35) and by inhibiting activation of transcription factors such as nuclear factor-kB and activator protein-1 (62, 63).

A number of recent studies showed that nitric oxide (NO) synthesis is reduced in chronic renal disease in both humans and animals (2, 48, 55, 60). Therefore, it has been suggested that an impaired NO synthetic pathway could have a key role in mediating the complex renal hemodynamic and nonhemodynamic mechanisms associated with the progression of renal disease (for a review, see Ref. 44). In this regard, Frisbee and Stepp (16) informed that obese Zucker rats exhibit a considerable impairment of endothelium-dependent vasodilatation in

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skeletal muscle arterioles, raising the possibility that NO deficiency could contribute to progression of renal disease in this animal model. Caveolin-1, the resident integral membrane protein of caveolae, is upregulated in bovine aortic endothelial cells exposed to serum of hypercholesterolemic patients (12), and several lines of evidence demonstrate that, by interacting with endothelial NO synthase (eNOS), caveolin-1 inhibits the activity of this enzyme (19, 20, 32), thus reducing NO production. In addition, it is known that soy protein has a higher l-arginine content, the precursor of NO. Taking all this information together, we reasoned that one of the possible mechanisms by which soy diet confers renal protection in Zucker rats could be related to improvement of NO generation. Here, we show that renal protection conferred by soy protein diet in Zucker rats is associated with increase in NO metabolites excretion rate, changes in expression pattern of NO synthases, and reduction of caveolin-1 expression within the kidney.

MATERIALS AND METHODS

Twenty-four lean (Fa/Fa) and 40 obese (fa/fa) Zucker rats were obtained at 5 wk of age (Harlan, Indianapolis, IN) and randomly divided into four groups: lean and obese Zucker rats fed ad libitum obtained at 5 wk of age (Harlan, Indianapolis, IN) and randomly thereafter were anesthetized with carbon dioxide and killed by 22°C with 12:12-h light-dark cycle and free access to water recorded every day. Rats were placed in metabolic cages every 30 days at 22°C with 12:12-h light-dark cycle and free access to water and thereafter were anesthetized with carbon dioxide and killed by decapitation. Obese rats were studied at 30, 60, 90, 120, and 160 days, whereas lean rats at 90, 120, and 160 days. All procedures followed were in accordance with our institutional guidelines. Functional studies. Individual 24-h urine samples were collected by placing animals in metabolic cages. Urinary protein excretion was measured by TCA turbidimetric method (26). Serum and urine creatinine concentration were measured using an autoanalyzer (Technicon RA-1000, Bayer, Tarrytown, NY), and renal creatinine clearance was calculated by the standard formula C = U*V/P, where U is the concentration in urine, V is urine flow rate, and P is the plasma concentration. The end products of NO, nitrites, and nitrates (NO2- and NO3-) were estimated in 24-h urine samples by reducing NO2- to NO3- using nitrate reductase (Roche) and β-adenine nicotinamide (β-NADPH, Sigma), followed by nitrites quantification with the Griess reagent, as we and others previously reported (8, 43).

Serum insulin was determined by RIA with rat insulin kit (Linco Research, St. Charles, MO). The sensitivity for rat insulin assay was 0.1 ng/ml, and the intra- and interassay coefficients of variation were <5 and <5%, respectively. Immune complexes were quantified with cobra II gamma counter (Packard Instrument, Menden, CT). Serum cholesterol and tryglycerides were measured enzymatically according to the manufacturer’s instructions (Lakeside Diagnostics).

RNA isolation. Total RNA was isolated from cortices or medullas of each group following guanidine isothiocyanate-ceae chloride method (47). The integrity of isolated total RNA was examined by 1% agarose gel electrophoresis, and RNA concentration was determined by UV light absorbance at 260 nm (Beckman DU640, Brea, CA).

RT-PCR. Relative level of NOS and caveolin mRNA expression was assessed in renal cortex and medulla by semiquantitative RT-PCR, as previously described (3, 4). Briefly, all primer sequences were custom obtained from GIBCO BRL (Gaithersburg, MD). Sense nNOS primers were 5'-GGCTTTGCTCCCACAGTT-3' and antisense 5'-GCC GGA TCC TGG CCC ACAGT-3', which amplified a fragment of 308 bp, bases 692 to 999 (3); sense inducible (i)NOS primers were 5'-GGT TCT CAC CAG GAG ATG TTG-3' and antisense 5'-CTC TTC CCC ACT GAG TTT GTC-3', which amplified a fragment of 570 bp, bases 1407 to 1977 (42); sense eNOS primers were 5'-CCG GAA ATT CGA ATA CCA GCC TGA TCC ATG GAA-3' and antisense 5'-GGA TCC TCC AGG GGT TCC ACC GCA TG-3', which amplified a fragment of 614 bp, base 2456 to 3069 (1), and caveolin-1 sense primers were 5'-ATG TCT GGG GGT AAA TAC GTG-3' and antisense 5'-CCT TCT GGT TCC GAA ATC AC-3', which amplified a fragment of 230 bp, bases 1 to 230 (50). To evaluate or reduce nonspecific effects of experimental treatment and to semiquantitatively express caveolin-1, we amplified a fragment of GAPDH, using primers previously described (3). Genomic DNA contamination was checked by treating all RNA samples with DNase and by carrying samples through PCR procedure without adding reverse transcriptase.

RT was carried out using 2.5 µg of total RNA from renal cortices or medullas. Before RT reaction, total RNA was heated at 65°C for 10 min. RT was performed at 37°C for 60 min in a total volume of 20 µl using 200 U of the Moloney murine leukemia virus reverse transcriptase (GIBCO BRL), 100 pmol of random hexamers (GIBCO BRL), 0.5 mM of each dNTP (Sigma, St. Louis, MO), and 1× RT buffer (75 mM Tris-HCl, 3 mM MgCl2, 10 mM DTT, pH 8.3). Samples were heated at 95°C for 5 min to inactivate the reverse transcriptase and diluted to 40 µl with PCR grade water. One-tenth of RT individual samples of each group was used for each NOS isoform or GAPDH amplification in 20-µl final volume reactions containing 1× PCR buffer (10 mM Tris-HCl, 1.5 mM MgCl2, 50 mM KCl, pH 8.3), 0.1 mM of each dNTP, 0.2 µCi of [α32P]-dCTP (~3,000 Ci/mmol, 9.25 MBq, 250 µCi), 10 µM of each primer, and one unit of Taq DNA polymerase (GIBCO, BRL). Samples were overlaid with 30 µl of mineral oil and PCR cycles were performed in a DNA thermal cycler (M.J. Research, Watertown, MA), with the following profile: denaturation 1 min at 94°C; annealing 1 min at 55°C for nNOS, iNOS, and caveolin, 58°C for eNOS primers; and 1 min extension step at 72°C. Last cycle was followed by a final extension step of 5 min at 72°C. Control gene was coamplified simultaneously in each reaction.

Amplification kinetics were performed following our standard procedure (3–5, 11). To analyze PCR products, one-half of each reaction was electrophoresed in a 5% acrylamide gel. Bands were ethidium bromide stained and visualized under UV light, cut out, suspended in 1 ml of scintillation cocktail (Ecolomue, ICN, Aurora, OH), and counted by liquid scintillation (Beckman LS6500, Fullerton, CA). The amount of radioactivity recovered from the excised bands was plotted in a log scale against the number of cycles. To semiquantitatively express each NOS isoform, caveolin, and the control gene, all reactions were performed at least by quadruplicate.

Western blot analysis. Plasma membrane proteins from pooled renal cortices or medullas were isolated by homogenization in sucrose buffer (0.32 M sucrose, 5 mM Tris-HCl, pH 7.5, 2 mM EDTA). Homogenates were centrifuged at 3,000 g, supernatant were then centrifuged at 20,000 g, and final supernatant were centrifuged at 100,000 g. Each pellet was resuspended in a buffer containing 5 mM Tris-HCl (pH 7.5) and 2 mM EDTA. For nNOS detection, total proteins were also extracted from pooled cortices or medullas by homogenization in four volumes of lysis buffer (225 mM mannitol, 75 mM sucrose, 0.1 mM EDTA, pH 7.0; 0.5 mM MOPS, pH 7.0, containing 5 mM benzamidine and 5 mM DTT). Homogenates were centrifuged at 4,000 g for 4 min at 4°C to remove tissue debris without precipitating plasma membrane fragments. Protein concentrations were assessed in duplicate using Bio-Rad DC Protein assay (Bio-Rad, Hercules, CA). Protein samples containing 70 µg of total protein or 50 µg of plasma membrane proteins in 10 µl loading buffer (6% SDS, 15% glycerol, 150 mM Tris, 3% bromophenol blue, 2% β-mercaptoethanol, pH 7.6) were resolved by SDS-PAGE, semidried, and electrophotographed on polyvinylidene difluoride membranes (Bio-Rad). Thereafter, membranes were cut and the lower part was incubated with a rabbit caveolin-1 antibody 1:500 (Abcam, Abingdon, KT) overnight at 4°C, whereas the upper membrane was incubated with a
goat actin antibody 1:2,500 (Santa Cruz Biotechnology, Santa Barbara, CA). Additional membranes containing total proteins or plasma membrane proteins were also incubated with rabbit nNOS polyclonal antibody 1:500 (Cayman Chemical, Ann Harbor, MI) and monoclonal eNOS antibody 1:250 (Zymed Laboratories, San Francisco, CA), respectively. Thereafter, membranes were washed three times for 30 min with TBS-T. For caveolin and nNOS detection, each membrane was incubated with the secondary antibody AP-conjugated goat anti-rabbit IgG 1:2,500 (Bio-Rad) for 60 min at 37°C and washed again. For eNOS detection, membranes were incubated with a secondary antibody AP-conjugated goat anti-mouse IgG 1:3,000 (Bio-Rad) for 60 min at 37°C and washed again. Whereas for actin detection, membranes were incubated with a secondary antibody donkey anti-goat IgG-HRP (Santa Cruz Biotechnology). Proteins were detected by using an enhanced chemiluminescence kit (Bio-Rad) and autoradiography, following the manufacturer’s recommendations. Bands were scanned for densitometric analysis.

**Histological studies.** Before renal cortex and medulla separation, one-half of the left kidney was taken and fixed in formalin-phosphate buffer for light microscopy studies. After appropriate dehydration, kidney slices were embedded in paraffin, sectioned at 3 μm, and periodic acid-Schiff (PAS), eosin-hematoxilin, and trichrome stains were performed. Focal and global glomerulosclerosis as well as extracapillary proliferation were counted in at least 150 glomeruli. Tubular atrophy and dilation were evaluated in ~300 tubules. The degree of tubulointerstitial fibrosis was evaluated by morphometry as we previously reported (11). For this purpose, 10 subcortical periglomerular fields per Masson-stained section (magnification ×200) were randomly selected in kidneys from the different groups. The images were recorded, and the affected areas were delimited and semiquantified using Leica processing and analysis system (Leica Imaging System, Cambridge, UK). The proportion of fibrosis was calculated dividing the interstitial fibrosis by total area per field excluding the luminal tubular area. The histological analysis was performed without knowing the group at which each kidney belonged.

**Statistical analysis.** Comparison among the groups for continuous parameters was performed by one-way ANOVA followed by Bonferroni’s correction for multiple comparisons.

**RESULTS**

Physiological parameters of the four groups of Zucker rats at the end of the study are presented in Table 1. As expected, body weight in obese rats was significantly elevated compared with that in lean controls. At the end of the study, obese rats fed with casein protein diet gained significantly less weight than rats fed with soy probably due to their physical deterioration. Kidney weight was also higher in obese rats compared with lean rats. The obese rats fed with soy (fa/fa+soy) appeared to have less renal hypertrophy than fa/fa+cas group, given their significantly lower kidney weights. This difference remained significant when kidney/body weight ratio was calculated; the ratio in fa/fa+cas vs. fa/fa+soy was 0.42 ± 0.07 and 0.23 ± 0.01 g/100 g body wt, respectively. Obese rats developed hypercholesterolemia, hypertriglyceridemia, and hyperinsulinemia compared with lean controls. Whereas in obese soy-feeding rats, cholesterol and triglycerides serum levels were significantly lower than fa/fa+cas, without changes in insulin serum levels, confirming hypolipidemic effect of soy diet.

Figure 1 shows urinary protein excretion and creatinine clearance in lean and obese rats fed with casein or soy diet along the study. No differences in urinary protein excretion were observed in lean rats during the period of the study, which remained within normal values (Fig. 1A). In contrast, a significant progressive increase in proteinuria was observed in fa/fa+cas group throughout the study. Abnormal urinary protein excretion began at day 60, at which the average value was 70.9 ± 7.8 mg/24 h and at the end of the study proteinuria in this group was 280.5 ± 83.6 mg/24 h. Interestingly, soy protein intake was associated with a significant reduction in proteinuria levels in obese rats, because at the day 60, average value was 25.0 ± 8.0 mg/24 h and at day 160, the value was 92.8 ± 39.4 mg/24 h. These differences were statistically significant compared with fa/fa+cas group.

**Table 1. Physiological parameters in lean and obese Zucker rats at the end of the study**

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean Food Intake, g</th>
<th>Body Weight, g</th>
<th>Left Kidney Weight, g</th>
<th>Cholesterol, mg/dl</th>
<th>Triglycerides, mg/dl</th>
<th>Insulin, mg/dl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fa/Fa+cas</td>
<td>24.2±3.3</td>
<td>480±20</td>
<td>1.3±0.1</td>
<td>98±13</td>
<td>126±9</td>
<td>2.2±0.5</td>
</tr>
<tr>
<td>Fa/Fa+soy</td>
<td>24.2±3.3</td>
<td>440±16*</td>
<td>1.3±0.1</td>
<td>67±5*</td>
<td>78±5*</td>
<td>1.4±0.3</td>
</tr>
<tr>
<td>fa/fa+cas</td>
<td>22.4±1.0</td>
<td>592±48*</td>
<td>2.4±0.2*</td>
<td>575±66*</td>
<td>814±116*</td>
<td>7.5±3.1*</td>
</tr>
<tr>
<td>fa/fa+soy</td>
<td>21.2±1.3</td>
<td>708±45†</td>
<td>1.6±0.03†</td>
<td>180±31†</td>
<td>458±42†</td>
<td>6.4±0.9*</td>
</tr>
</tbody>
</table>

Values are means ± SE. *P < 0.05 vs. Fa/Fa+cas. †P < 0.05 vs. fa/fa+cas. Cas, casein.
As shown in Fig. 1B, creatinine clearance in obese rats tended to be lower than control animals, but the difference was less evident in those rats fed with soy protein. Because at 160 days obese rats fed with casein had lesser body weight than soy animals, creatinine clearance was corrected by considering body weight of each animal. Glomerular filtration rate per 100 g of body weight in Fa/Fa/cas and Fa/Fa/soy was 0.26 ± 0.01 and 0.34 ± 0.02 ml·min⁻¹·100 g⁻¹ (P = 0.06), respectively, and in fa/fa+cas and fa/fa+soy was 0.04 ± 0.02 and 0.11 ± 0.02 ml·min⁻¹·100 g⁻¹ (P = 0.03), respectively. Although creatinine clearance in fa/fa+soy was not completely normalized at the end of the study, a soy protective effect was clearly observed in this group, when it was compared with fa/fa+cas group.

Figure 2 shows the quantification of the most important lesions observed in fatty Zucker rats, and representative images are shown in Fig. 3. Fatty Zucker rats fed with casein exhibited the characteristic renal histological picture of this model that is segmental glomerulosclerosis, tubular dilation, and interstitial fibrosis. In fa/fa+cas group, percentage of glomerulosclerosis, tubular dilation, and tubulointerstitial fibrosis was 33.0 ± 11.8,
14.7 ± 9.8, and 10 ± 1.9%, respectively. All structural changes observed in obese rats were significantly reduced by soy protein diet. In *fa/ fa* soy group, mean percentage value of glomerulosclerosis, tubular dilation, and tubulointerstitial fibrosis were 8.3 ± 5.8, 2.5 ± 1.1, and 4.6 ± 0.5, respectively. Thus our results indicate that the consumption of soy diet by obese Zucker rats reduces structural and functional changes.

To characterize the soy-protective effect on NO synthesis, end NO metabolites were quantified in urine. Urinary nitrites and nitrates excretion (UNO$_3$/NO$_2$V) is shown in Fig. 4 in lean and obese Zucker rats. In *fa/ fa* soy group, mean percentage value of glomerulosclerosis, tubular dilation, and tubulointerstitial fibrosis were 8.3 ± 5.8, 2.5 ± 1.1, and 4.6 ± 0.5, respectively. Thus our results indicate that the consumption of soy diet by obese Zucker rats reduces structural and functional changes.

Fig. 4. Urinary nitrates and nitrites excretion (UNO$_3$/NO$_2$V) in lean and obese Zucker rats. Open bars represent animals fed with casein and solid bars represent rats fed with soy diet during 160 days. *$p < 0.05$ vs. all groups.

To explore if soy-induced renal protection is related to changes in intrarenal expression of NOS, neuronal (n)NOS and eNOS mRNA and protein levels were evaluated by semiquantitative RT-PCR and Western blot analysis, respectively (Figs. 5 and 6). Figure 5, left, shows that in renal cortex, soy protein diet in lean rats did not produce changes in eNOS or nNOS mRNA levels compared with rats fed with casein. In contrast, cortical nNOS mRNA level was upregulated in obese rats fed with casein, an effect that was reverted in *fa/ fa* soy group, together with a significant increase of eNOS expression in renal cortex. As shown in Fig. 5, right, eNOS mRNA in medulla was downregulated in *fa/ fa* cas rats, whereas soy protein was associated with a significant increase of eNOS in both lean and obese animals. No changes in medulla nNOS mRNA levels among all groups were observed.

Western blotting was used to assess protein expression of these enzymes. Actin was used as control protein to normalize each sample. Figure 6 shows the abundance of eNOS in plasma membrane proteins extracted from renal cortex (A) and medulla (B). Figure 6, top insets, are representative images of eNOS immunoblot. The eNOS protein level was similar in lean rats fed with casein or soy protein diet. In contrast, in *fa/ fa* cas group, there was a significant increase by threefold of eNOS in plasma membrane proteins from renal cortex; this effect was not observed in the medulla. As expected, nNOS was not detected in membrane proteins fraction (data not shown); thus total proteins were used to analyze nNOS expression. No difference in cortical nNOS expression was observed in lean and obese rats, regardless the source of protein; how-
However, an increase in medulla nNOS protein was observed in fa/fa + cas compared with Fa/Fa + cas group (1.8 ± 0.2 vs. 0.8 ± 0.1, respectively), effect that was abrogated when rats were fed with soy diet (1.0 ± 0.1; data not shown).

Because it is known that eNOS activity is modulated by caveolin-1, level of expression of this protein was evaluated in renal cortex and medulla. As shown in Fig. 7, in renal cortex Fa/Fa + cas and Fa/Fa + soy groups exhibited similar caveolin-1 mRNA levels. Caveolin-1/GAPDH ratio was 1.45 ± 0.10 and 1.25 ± 0.18, respectively. In contrast, fa/fa + cas group was associated with a significant caveolin-1 upregulation by almost twofold (2.60 ± 0.33, \( P = 0.008 \) vs. Fa/Fa + cas). Intriguingly, soy protein intake in obese rats prevented this upregulation, because caveolin-1/GAPDH ratio was 1.33 ± 0.08 (\( P = 0.004 \)). In renal medulla, similar results were observed. Casein-fed obese Zucker rats had higher caveolin-1 mRNA level than lean rats fed with casein: 2.29 ± 0.32 vs. 1.04 ± 0.04, respectively (\( P < 0.005 \)). This effect was prevented by soy protein diet, because the value in fa/fa + soy group was 1.39 ± 0.05. In addition, caveolin-1 protein levels were assayed in plasma membrane proteins obtained from rat renal cortex and medulla by using Western blot analysis (Fig. 8). Western blot analysis confirmed our findings at mRNA level, caveolin-1 protein in isolated plasma membranes from renal cortex was significantly higher in fa/fa + cas that Fa/Fa + cas rats and the increase of these protein was normalized when rats were fed with soy protein diet (fa/fa + soy group). A similar pattern was observed in renal medulla; however, the difference did not reach statistical significance.

**DISCUSSION**

In the present study, we observed that obese Zucker rats fed with casein developed a progressive renal disease characterized by proteinuria and glomerulosclerosis that were associated with hypercholesterolemia, hypertrygliceridemia, and hyperinsulinemia. Renal progressive disease, as well as the increase in cholesterol and triglycerides levels, was significantly ameliorated when the obese Zucker rats were fed with soy instead casein protein diet. These observations are consistent with a previous report (39), but little is known about the mechanism of the renal soy-protective effect. In this regard, hypertension seems not to be responsible for renal damage in this animal model, because Maddox et al. (39) observed that fatty Zucker rats exhibited only a slight increase in systolic blood pressure that cannot explain the development and progression of renal disease. Moreover, arterial pressure was not modified when rats were fed with soy. Data from this study revealed that soy diet conferring renal protection is associated with restoration of NO production.

A number of recent studies showed that NO synthesis is reduced in chronic renal disease in both humans and animals.
(2, 48, 55, 60), suggesting that availability of NO in kidney is an important factor in defining the rate of progression injury in renal disorders. To explore the role of NO in renal protection conferred by soy protein diet, we evaluated the expression pattern of nNOS and eNOS in renal cortex and medulla, as well as NO metabolites excretion. We observed that both nNOS and eNOS expression pattern in obese rats fed with casein (fa/fa + cas) was different to that observed in lean animals fed with the same diet. At mRNA levels, there was an upregulation of nNOS in cortex and a downregulation of eNOS in medulla, these changes were normalized by soy diet. At protein level, fa/fa + cas group presented a significant increase of nNOS in total proteins from renal medulla. Physiological significance of this observation is unclear because the role of nNOS in renal medulla is unknown. However, upregulation of nNOS was prevented in obese rats fed with soy diet. Interestingly, fa/fa + cas group had a greater amount of eNOS in membrane proteins fraction, an effect that was also reverted in fa/f.a + soy rats. Although eNOS expression was higher in fa/ fa + cas group, it is unlikely that eNOS in this animal model is active because most of this enzyme was located in plasma membrane, a location in which it is known that association with caveolin-1 precludes its activity. In addition to these findings, we observed an unexpected small, but significant, increase in caveolin-1 mRNA and protein expression in renal medulla from lean animals fed with soy. Because the physiological role of caveolin-1 in renal medulla is not known, interpretation of this finding is difficult. Interestingly, a recent study demonstrated that caveolin-1 knockout mouse develops hypercalcucuria and urolithiasis, suggesting that caveolin-1 is a critical determinant of urinary calcium homeostasis (7).

Supporting that changes in the NO synthetic pathway are altered in the fatty Zucker rats and that it is corrected by soy diet, we found that NO\(_2^-/NO_3^-\) urinary excretion in fa/fa + cas rats was considerably depressed and soy protein intake was associated with restoration of NO systemic production. In addition, recent studies showed that obese Zucker strain has an impairment in NO-mediated dilation of skeletal muscle arteries, suggesting a deficient NO availability (16a, 24).

NO biosynthesis is tightly regulated by a variety of mechanisms ranging from transcriptional to posttranslational level (37). Recent studies indicate that some proteins directly interact with NOS-forming complexes that regulate NOS activity or spatial distribution in the cell. For instance, eNOS is regulated by proteins residing in or recruited to plasmalemmal caveolae of endothelial cells. Caveolins, the resident scaffolding proteins of caveolae, and calmodulin undergo reciprocal Ca\(^{2+}\)-dependent association and dissociation with eNOS in the caveolar membrane that inhibits (caveolins) and activates (calmodulin) eNOS activity (10, 13, 21, 32). Thus it has been suggested that association of eNOS to the caveolae through caveolin-1 maintains eNOS in its inactive form; on the contrary, the release of eNOS from caveolae is thought to facilitate the synthesis of NO (13, 21, 32). In support to this, it has been demonstrated that the mice with targeted disruption of caveolin-1 exhibit enhanced eNOS activity (10). Given the caveolin importance in regulating NO generation, we evaluated the expression of this protein. We observed that in normal lean rats, caveolin-1 is more abundant in medulla than in cortex plasma membrane proteins and that caveolin-1 mRNA and protein levels were significantly upregulated in cortex and medulla of fatty Zucker rats fed with casein, suggesting that increased expression of caveolin-1, together with greater amount of eNOS in plasma membrane, further decreases NO production in renal tissue. An interesting observation of this study is that soy protein diet significantly reduced caveolin-1 expression and eNOS abundance in plasma membranes in the kidney of these animals, suggesting that another mechanism by which soy diet improves NO generation is by decreasing the expression of caveolin-1, a natural inhibitor of NO synthesis (10, 13, 18, 21).

Several studies in humans and animals have revealed that soy protein intake ameliorates proteinuria and progression of renal disease (9, 27, 30, 39, 46, 52, 53, 61). There are several
possible mechanisms to explain this effect of soy. 1) Isoflavones. A component of soy that is believed to be involved in this protective effect is the isoflavones, by mechanisms that are still unclear, but include the following possibilities. Isoflavones comprise the most common class of phytoestrogens present mainly in soybean products. After ingestion, isoflavones are hydrolyzed in the intestine by bacterial β-glucosidases and converted to the bioactive aglicones: genistein and daidzein. Because isoflavones possess an important hypcholesterolemic effect observed in this study (Table 1), this is one of the mechanisms that have been suggested to be involved in renal protection induced by these compounds (34, 39, 46). Renoprotection also could result from antioxidant properties of isoflavones, which not only could avoid formation of free radicals (45) but also might result in enhancing NO availability (29). Finally, preliminary evidence suggests that diazidein directly downregulates caveolin-1 protein expression in rat aorta, which in turn was associated with twofold increase in NO metabolites (61a). 2) Amino acid content in soy protein. Although both soy and casein protein sources contain the same amount of total amino acids, there are differences in the proportion of certain residues. For instance, L-arginine, precursor of NO, is more abundant in soy than in casein protein (7.6 vs. 3.7%, respectively) and glycine, another residue that has been shown to produce vasodilation (28), is also more abundant in soy protein (4.2 vs. 1.8%, respectively). Therefore, soy-fed rats received a greater proportion of two residues that could be directly involved in vasodilatory processes. 3) Soy-induced hypcholesterolemia. It is possible that caveolin-1 downregulation results from hypercholesterolemic effect of soy diet, because Fielding and Fielding (15) postulated that caveo-lae behave as sensors of free cholesterol content of the cell and that depletion of caveolar cholesterol leads to downregulation of caveolin-1 at mRNA and protein levels (14). Moreover, Feron et al. (12) observed that exposure of bovine endothelial cells to serum from hypercholesterolemic patients resulted in upregulation of caveolin-1, an effect that was associated with an impairment of basal NO release, together with an increase in formation of inhibitory caveolin-eNOS complex; thus these studies suggest that cholesterol level regulates caveolin-1 expression.

In summary, our data show that nephropathy associated with obesity in the obese Zucker rat model could be reduced by feeding animals with soy, instead casein protein diet. The protective effect of soy diet was associated with reduction in cholesterol and triglyceride levels, as well as correction of a marked reduction in NO generation, that was associated with restoration of a normal pattern of NO expression in the kidney and reduction of eNOS, together with a downregulation of caveolin-1 in plasma membrane proteins.

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