Nephropathy in Zucker Diabetic Fat Rat Is Associated with Oxidative and Nitrosative Stress: Prevention by Chronic Therapy with a Peroxynitrite Scavenger Ebselen

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Abstract. Zucker diabetic fat (ZDF) rats with the metabolic syndrome and hyperlipidemia develop focal and segmental sclerosis. The role of oxidative and nitrosative stress in the nephropathy in ZDF was studied. Renal histology, function, and immunohistologic and biochemical parameters of oxidative and nitrosative stress were evaluated at 8 and 22 wk of age in ZDF and Zucker lean (ZL) rats and after chronic treatment with ebselen, an antioxidant and peroxinitrite scavenger. At 8 wk, ZDF rats showed hyperglycemia, no proteinuria or nephropathy, but higher levels of dihydrobiopterin and 3-nitrotyrosine (3-NT)–modified proteins compared with age-matched ZL rats. At 22 wk, ZDF rats developed focal and segmental sclerosis, proteinuria, decreased creatinine clearance, and renal tissue levels of glutathione and tetrahydrobiopterin with further elevation in dihydrobiopterin and 3-NT–modified proteins, in contrast to age-matched ZL rats. Renal immunohistologic expression of lipid peroxidation products and 3-NT–modified proteins also increased in 22-wk-old ZDF but not in ZL rats. Chronic ebselen treatment of ZDF rats restored renal tissue levels of glutathione and tetrahydrobiopterin; prevented significant accumulation of dihydrobiopterin, lipid peroxidation products, and 3-NT–modified proteins; and ameliorated focal and segmental sclerosis, proteinuria, and fall in creatinine clearance without affecting mean BP, body weight, and blood glucose, compared with the untreated ZDF rats. Chronic ebselen therapy also ameliorated vasculopathy with lipid deposits and tubulointerstitial scarring, inflammation, and upregulated α-smooth muscle actin expression. These findings suggest that ZDF rats develop a progressive nephropathy with glomerular, vascular, and tubulointerstitial pathology. Oxidative and nitrosative stress predates the nephropathy, which is improved by peroxinitrite scavenger ebselen, and thus considered its cause and not consequence.

The growing population of patients with the metabolic syndrome and with obesity-associated type 2 diabetes has resulted in a dramatic increase in the number of patients who have ESRD and require dialytic life support (1,2). This challenge is being met by interdisciplinary efforts to elucidate the characteristics and mechanisms of nephropathy and to develop animal models and therapeutic tools. The Zucker diabetic fat (ZDF) rat represents a well-characterized model of the metabolic syndrome. Developed more than three decades ago (3), the pathophysiologic mechanism of this model has recently been attributed to the missense mutation of leptin receptor (4). The evolution of the metabolic syndrome in the ZDF rat is associated with the development and progression of nephropathy, together with obesity, insulin resistance, hyperglycemia, and hyperlipidemia (5–10). Focal and segmental sclerosis (FSGS) develops at the age of 18 wk, although severe hyperlipidemia, hyperglycemia, and obesity predate it (8).

Previous studies suggested the role of oxidative stress and endothelial dysfunction in the pathophysiology of systemic vasculopathy in ZDF rats (11–13). Indeed, increased generation of superoxide radicals has been demonstrated directly in the islets of Langerhans from young prediabetic ZDF rats (14). Whether these mechanisms are operant in the kidney and whether they play a pathophysiologic role in the progression of nephropathy in the ZDF rat remains a compelling but an unexplored possibility.

We attempted to address this possibility by morphologic, biochemical, and functional testing of ZDF rats during the evolution of nephropathy. In addition, animals received chronic therapy with a seleno-organic compound, ebselen, a bona fide peroxynitrite scavenger and an antioxidant (15,16). In our previous studies, ebselen ameliorated oxidative and nitrosative stress associated with acute renal ischemia (17) and prevented macrovascular disease in ZDF rats (13), and it was found to be well tolerated by rats at the doses used. To examine

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the efficacy of ebselen in preventing nephropathy in ZDF rats, we initiated chronic therapy at 8 wk, the age when nephropathy is undetectable, and continued until the age of 22 wk, when nephropathy advances to chronic renal insufficiency.

Materials and Methods

Materials

The following antibodies were used: anti-α-smooth muscle actin (α-SMA; DakoCytomation, Carpinteria, CA), anti–CD-68 (Serotec Ltd, Oxford, UK), 4-hydroxy-2-nonenal (HNE; gift of Dr. M. Tanaka, Nagoya Univ., Japan), and anti–3-nitrotyrosine (3-NT; Upstate Biotechnology, Lake Placid, NY). Ebselen was purchased from Alexis Biochemicals (San Diego, CA) or provided by Daiichi Pharmaceutical Co. (Tokyo, Japan).

Experimental Animals

Studies were carried out in ZDF and lean control (ZL) rats (Charles River Laboratories, Wilmington, MA) aged 8 and 22 wk. At least five animals were used in each group. The animals were housed in animal quarters kept at 20 to 22°C with a 12-h light/dark cycle and were allowed free access to rat diet and water throughout the study. ZDF rats were randomly divided into two groups. The first group received daily ebselen administered by gavage in two doses, 5 mg/kg body wt each, dissolved in 5% CM-Cellulose (Sigma, St. Louis, MO) starting at the age of 8 wk and continuing until the rats were killed at 22 wk. The control (vehicle) groups of ZDF and ZL rats were treated with 5% CM-Cellulose.

Before the rats were killed, they were anesthetized by intraperitoneal injection of Ketamin-Xylazin (60 and 7.7 mg/kg body wt, respectively). A mid-laparotomy was performed, the abdominal aorta was cannulated with a P-50 catheter, and mean BP (MBP) was measured using a pressure monitor BP-1 (WPI). Subsequently, blood was collected, and the right kidney was removed, cross-sectioned, fixed, and processed for paraffin embedding or cryopreservation and cryosectioning. Glucose concentration in the blood was measured using the modified Trinder color reaction according to the manufacturer’s protocol (Raichem, San Diego, CA). The animal study protocol was approved by the institutional Animal Care and Use Committee.

Histologic and Immunohistologic Evaluation

Midcoronal cross-sections of kidneys from all animals were paraffin embedded, cut at 2- to 3-μ thickness, and stained with hematoxylin and eosin, periodic acid-Schiff, and Trichrome stains. Glomeruli were evaluated for size and mesangial expansion. On Trichrome stain, 100 consecutive glomeruli from one end of the section from each animal were counted for the presence of lesions of FSGS. Foci of tubulointerstitial scars and of interstitial inflammation were also counted in the same area that contained these 100 glomeruli in each section. Tubulointerstitial scarring index was evaluated by counting the total number of atrophic or atrophying tubular profiles in these foci of scarring per 100 glomeruli. Each of the 100 glomeruli counted were scored from 0 to 3+ on the basis of the glomerular tuft surface area obliterated with sclerosis (0 = none, 1 = <25%, 2 = up to 50%, and 3 = >50%), and glomerulosclerosis index was calculated by adding all of the scores and dividing by 100. A 2- to 4-μ cyrosection from each kidney was evaluated with Oil-Red-O staining. Tubular staining was evaluated from 0 to 3+ on the basis of the degree and distribution of the positive droplets.

For immunohistology, 2- to 3-μ-thick paraffin sections were stained by using specific antibodies to α-SMA (1 μg/ml), CD-68 (50 μg/ml), HNE (25 μg/ml), and 3-NT (5 μg/ml). Immunoperoxidase staining was performed by using LSAB+ system (DakoCytomation) according to the manufacturer’s protocol. AEC peroxidase substrate kit (Vector Laboratories, Burlingame, CA) was used to visualize the staining. For α-SMA, the number of glomeruli with positive mesangial staining was counted among 100 consecutive glomeruli. Bowman’s capsules and tubular profiles surrounded with positive staining were also counted as indicators of interstitial myofibroblastic activation per 100 glomeruli. Immunostaining for HNE and 3-NT was graded semi-quantitatively from 0 to 3+ in glomeruli; cortical, outer, and inner medullary tubules; and blood vessels. For further evaluation of the 3-NT immunoexpression in glomeruli, 10 of the most intensely stained glomeruli from sections of each animal were captured by digital imaging and submitted for image analysis by using Adobe Photoshop 7.0 software. The image complexity was reduced using “Curves” and “Replace Color” tools until all but specific staining remained. The specific staining was then replaced by a primary color. Single color area was measured with the Histogram tool. All numbers are given as a percentage of specific staining in glomerular tuft area.

Pterin analysis by CoulArray Electrochemical Detection

Tissues (kidney) were rinsed of blood using ice cold PBS solution (pH 7.2 to 7.4) and then homogenized in 10 ml/g wet wt tissue in a buffer that consisted of 50 mM Tris, 150 mM NaCl, 0.1 mM EDTA, and 20 mM CHAPS (pH 7.4). Ice-cold acid precipitation buffer (0.1 M phosphoric acid, 0.23 M TCA) was added to a 100-μl portion of the sample (3:1 vol/vol) and then centrifuged for 1 min at 12,000 × g at 4°C. Two aliquots of supernatant (120 μl) were removed into HPLC vials for the analysis of total biopterin, tetrahydrobiopterin (BH$_4$), dihydrobiopterin (q-BH$_2$), and 7,8-BH$_2$ (18).

To the first vial, 1 μl of sodium bisulfite (final concentration 3 mM) was added immediately followed by 1 μl of dithioerythritol (DTE; 6 mM final). To the second, DTE (6 mM final) only was added. Samples were then injected onto an isocratic HPLC system with a multichannel electrochemical CoulArray (ESA Inc., Chelmsford, MA) detection with a 100-mm C-18 column (Microsorb-MV; Varian, Palo Alto, CA) running mobile phase, comprising 50 mM sodium acetate, 5 mM citric acid, 48 mM EDTA, and 0.3 mM DTE (pH 5.2). The flow rate was set at 0.75 ml/min, and the temperature was set at 30°C. The optimum potential for detection of BH$_4$ was determined to be 125 mV. Two other sequential electrodes were set at –350 mV and 700 mV to reduce the BH$_4$ and oxidize all of the pterin within the sample, respectively, allowing for confirmation of BH$_4$ presence and detection of all pterins, irrespective of their redox state. Quantitation of q-BH$_2$ was done by subtracting value sample 1 from sample 2, and 7,8-BH$_2$ is quantified by fluorescence detection linked to the fourth channel of the electrochemical detector. Quantitation of BH$_4$ and 7,8-BH$_2$ was done by comparison with external standards after normalizing for sample protein content.

Glutathione Analysis in the Renal Tissue

A microtiter plate enzymatic recycling assay was adapted (19,20), in which cellular glutathione (GSH) is oxidized by 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) and reduced by NADPH in the presence of glutathione reductase. A buffer that contained sodium phosphate (125 mM) and EDTA (1 mM) and a reaction solution (2.8 ml of 1 mM DTNB, 3.75 ml of 1 mM NADPH, 5.85 ml of buffer, and 20 U of GSH reductase) were freshly prepared. GSH standards and kidney homogenate (50 μl) were loaded onto a microtiter plate. Immediately, 100 μl of the reaction mixture was added and the rate of 2-nitro-5-
thiobenzoic acid formation was quantified at 405 nm over a 2-min period.

**Protein-Incorporated 3-NT Assay of Renal Tissue**

All chemicals, unless otherwise stated, were purchased from Sigma Chemical Co. The water used for the HPLC mobile phase and sample preparation was from a MilliQ water purification system (Millipore, MA) and >18 MΩ resistance.

Kidneys were rinsed of blood using ice-cold PBS solution (pH 7.2 to 7.4), minced with scissors, and then homogenized in 10 ml/g wet wt tissue in a buffer that consisted of 50 mM Tris, 150 mM NaCl, 0.1 mM EDTA, and 20 mM CHAPS (pH 7.4). Ice-cold acid precipitation buffer (0.1 M phosphoric acid and 0.23 M TCA) was added to a 100-μl portion of the sample (3:1 vol/vol), allowed to set for 5 min at room temperature, and then centrifuged for 15 min at 12,000 × g at 4°C. The supernatant was removed and placed in HPLC vials for the injection of the sample.

A second 100-μl aliquot of the tissue homogenate was subjected to proteolytic digestion before the extent of protein incorporated 3-NT was quantified. Proteinase K (1 U/10 mg protein) was added to the tissue homogenate and incubated for 8 h at 55°C. Samples were allowed to come to room temperature before the addition of the precipitation buffer and further preparation as above.

An isocratic HPLC system with multichannel electrochemical Coul-Array detection was used to effectively resolve 3-NT from background species with a 100-mm C-18 column (Microsorb-MV) running the mobile phase, 90 mM sodium acetate, 35 mM citric acid, 130 mM EDTA, and 460 μM sodium octane sulfonate (pH 4.35) (21,22). The flow rate was set at 0.75 ml/min, and the temperature was set at 30°C. The optimum potential for detection of 3-NT was found to be 800 mV. For maximum selectivity of the system for 3-NT, two other electrodes were set at either side of the optimum potential for 3-NT, 700mV and 900 mV, respectively. Further confirmation of the elution of 3-NT was established by addition of 10 mM sodium hydrosulfite to nitrotyrosine. This treatment chemically reduces 3-nitro- to 3-amino-tyrosine, silencing the electrochemical signal.

**Statistical Analyses**

The data were expressed as mean ± SEM. The means of two populations were compared by a t test. For multiple comparisons, one-way ANOVA was used, followed by Tukey posttest. Differences were considered significant at P < 0.05.

**Results**

**General Characterization of the Model**

At 8 wk of age, ZDF rats were obese (332 ± 7 versus 214 ± 9 g in age-matched ZL rats; P < 0.05); developed mild hyperglycemia (198 ± 11 versus 144 ± 16 mg/dl in ZL rats; P < 0.05); but showed no hypertension (98 ± 12 versus 105 ± 4 mmHg in ZL rats), decline in creatinine clearance (Ccr; 2.6 ± 0.2 versus 2.8 ± 0.2 ml/min in ZL rats), or proteinuria (32.9 ± 1.2 versus 16.2 ± 0.6 mg/dl in ZL rats). By the age of 22 wk, the glucose level in ZDF rats averaged 400 ± 33 mg/dl (181 ± 17 mg/dl in age-matched ZL rats; P < 0.01), Ccr decreased to 1.9 ± 0.4 ml/min (3.2 ± 0.2 ml/min in ZL rats; P < 0.05), and proteinuria increased to 197.7 ± 15.7 mg/dl (29.2 ± 2.7 in ZL rats; P < 0.001). MBP remained within normal range in both ZDF and ZL animals (108 ± 5 versus 103 ± 3 mmHg). Ebselen treatment affected neither BP (101 ± 6 mmHg) nor the level of hyperglycemia (393 ± 18 mg/dl) or body weight (410 ± 28 g) in ZDF rats (Figure 1).

**Morphologic Characterization of the Model**

Consonant with the lack of proteinuria and normal renal function, 8-wk-old ZDF animals showed neither glomerulosclerosis nor evidence of tubulointerstitial scarring or inflammation. Compared with 8-wk-old ZL rats, glomeruli in ZDF rats seemed slightly hypertrophied with further increment at 22 wk of age along with mild to moderate mesangial expansion (Figure 2, B versus A and D versus C in age-matched ZL rats).

FSGS was noted in 9.5 ± 1.8% glomeruli (versus 1.2 ± 2.7 in 22-wk-old ZL rats; P < 0.001) with focally swollen and vacuolated podocytes that contained protein resorption droplets overlying such areas and frequent segmental adhesions to the Bowman’s capsules (Figure 2D). Glomerulosclerosis index was scored at 0.34 ± 0.04 in 22-wk-old ZDF (versus 0.02 ± 0.02 in 22-wk-old ZL rats; P < 0.001). Oil-Red-O staining revealed lipid deposits in the tubular epithelium, focal in 8-wk-old and widespread in 22 wk-old ZDF rats (Figure 3B). At this age, lipid deposits also accumulated focally in podocytes and glomerular capillary tufts (Figure 3A) with or without overt FSGS and in the endothelial cells of peritubular capillaries, vasa rectae (Figure 3D), and arteries of all sizes including perihilar and interlobar arteries. Lipid deposits were extra- and intracellular in the medial layer of these larger (Figure 3C) and a few smaller arteries (Figure 4B) with thickened vessel walls, focally swollen endothelium, and occasionally subintimal leukocytes (Figure 4, B versus A) compared with age-matched ZL rats despite comparable MBP, suggesting a lipid-induced vasculopathy. Trichrome staining revealed patchy areas of tubulointerstitial scars in 22-wk-old ZDF rats (16 ± 5 versus 1.2 ± 2.7/100 glomeruli in 22-wk-old ZL rats; P < 0.001; Figure 5, B versus A) and aggregates of mononuclear leukocytes. Similar aggregates were seen in other areas without overt scars (15 ± 2 versus 1.8 ± 2 22-wk-old ZL rats; P < 0.001). From 35 to 50% of the leukocytes stained positive for monocyte/macrophage marker CD-68. A significantly greater number of atrophic/atrophying tubular profiles, representing the total tubulointerstitial scarring index, were quantified in 22-wk-old ZDF rats (93.25 ± 0.12 versus 6.40 ± 6.40/100 glomeruli in 22-wk-old ZL rats; P < 0.001). ZDF rats at 8 wk of age and ZL rats at 8 and 22 wk age showed no significant scars or inflammation.

**Effects of Chronic Ebselen Treatment on Renal Function, Morphology, Tubulointerstitial Scarring, and α-SMA Immunoexpression**

Ebselen-treated 22-wk-old ZDF rats showed partial amelioration in proteinuria (123.5 ± 11.2 versus 197.7 ± 15.7 mg/24 h; P < 0.001 versus untreated 22-wk-old ZDF rats), plasma creatinine and Ccr (0.8 ± 0.1 versus 1.6 ± 0.3 mg/dl and 1.9 ± 0.4 versus 2.5 ± 0.6 ml/min, respectively; P < 0.05 versus untreated 22-wk-old ZDF rats) despite the absence of any notable changes in the body weight, MBP, or blood glucose. In parallel with the improved functional parameters, morphologic indices of renal damage were also downgraded compared with
the untreated ZDF rats. These included glomeruli with FSGS (5.3 ± 1 versus 9.5 ± 1.8/100 glomeruli in the untreated 22-wk-old ZDF rats; P < 0.001; Figure 2, D versus F), glomerulosclerosis index (0.15 ± 0.04 versus 0.34 ± 0.04 in the untreated 22-wk-old ZDF rats; P < 0.001; Figure 2E), tubulointerstitial scarring index (40.25 ± 15.34 versus 93.25 ± 12.11/100 glomeruli in the untreated 22-wk-old ZDF rats; P < 0.001; Figure 5D), and foci of tubulointerstitial scars (Figure 5,
C versus B) and inflammation to 9 ± 3.2 and 8 ± 1/100 glomeruli, respectively (versus 16 ± 5 and 15 ± 2%, respectively in the untreated 22-wk-old ZDF rats; P < 0.01). Ebselen had no effect on Oil-Red-O–positive lipid deposits in the tubules and the glomeruli; however, vessels, including endothelial cells, revealed considerably less lipid deposits compared with the untreated ZDF rats.

Immunostaining for α-SMA was examined to evaluate the relationship of mesangial activation with FSGS and of interstitial myofibroblasts with tubulointerstitial scarring (Table 1). Only rare glomeruli in ZL and ZDF rats showed α-SMA immunoreactivity in mesangial cells at 8 wk of age with significant increase only in ZDF rats at 22 wk (P < 0.05 versus 8-wk-old ZDF rats). Similarly, rare interstitial cells around the Bowman capsules or tubular profiles were α-SMA positive at age 8 wk in either ZL or ZDF rats. Consonant with increased tubulointerstitial scarring, marked upregulation of α-SMA immunoreactivity occurred in the same areas, only in 22-wk-old ZDF rats, around the Bowman’s capsule (P < 0.05 versus 8-wk-old ZDF rats; P < 0.01 versus 22-wk-old ZL rats) and tubular profiles (P < 0.05 versus 8-wk-old ZDF rats; P < 0.05 versus 22-wk-old ZL rats; Figure 6, B versus A).

Ebselen-treated 22-wk-old ZDF rats showed markedly fewer α-SMA immunoreactive cells in the mesangium (P < 0.01), around the Bowman’s capsules (P < 0.05), and in the tubular profiles (P < 0.05; Figure 6, C versus B) compared with untreated 22-wk-old ZDF rats.

Effects of Chronic Ebselen Therapy on Parameters of Oxidative and Nitrosative Stress in ZDF Rats

Lipid peroxides, immunodetectable with antibody to HNE, were localized predominantly in the renal tubules of all animals (Table 2). The distribution pattern in 8-wk-old ZL rats consisted of outer and inner medullary tubules; cortical distal tubules; and only rarely proximal tubules, glomeruli, and blood vessels. Age-matched ZDF rats revealed a slightly stronger expression in all compartments, but it was significantly different only in the inner medullary tubules (P < 0.05 versus 8-wk-old ZL rats). In glomeruli, HNE immunoeexpression was focal, often in the vicinity of tubulointerstitial scarring, and mostly in the podocytes and the parietal epithelium. Vascular staining was also focal and usually affected the arterial media and rarely the endothelium of the peritubular capillaries and vasa rectae. ZDF at 22 wk of age showed enhanced HNE immunoeexpression in nearly all of the compartments but were different from age-matched ZL rats in the distal tubules in the cortex (P < 0.05) and outer and inner medullary tubules (P < 0.001 and P < 0.05, respectively; Figure 7, B versus A). ZDF
rats that were on chronic ebselen therapy displayed significantly less HNE immunoexpression only in the inner medullary tubules \((P < 0.05)\) versus 22-wk-old untreated ZDF rats; Figure 7, C versus B).

Oxidative stress was confirmed by the renal tissue measurements of glutathione (Figure 7D) and the pterins (Figure 7E). Tissue levels of glutathione were comparable in 8-wk-old ZL and ZDF rats and in 22-wk-old ZL rats but were markedly less in 22-wk-old ZDF rats \((P < 0.05)\). These levels were restored to those comparable to 22-wk-old ZL rats in the rats that received chronic ebselen treatment \((P < 0.01)\) versus untreated 22-wk-old ZDF rats. Similarly, renal tissue levels of BH₄ were comparable in ZL and ZDF rats at 8 wk of age, remained stable in 22-wk-old ZL rats, but dropped considerably in age-matched ZDF rats \((P < 0.05)\), suggesting marked consumption as a result of persistent oxidative stress. In contrast, renal tissue levels of BH₂, the oxidative product of BH₄, were significantly elevated in ZDF rats at 8 \((P < 0.05)\) versus 8-wk-old ZL rats) and 22 wk of age \((P < 0.01)\) versus 8- and 22-wk-old ZL rats, and \(P < 0.05\) versus 8-wk-old ZDF rats) but were restored to levels comparable to 22-wk-old ZL rats in ZDF rats that received chronic treatment with ebselen \((P < 0.05)\) versus 22-wk-old untreated ZDF rats). The lower levels of BH₄ in the renal tissue were also similarly restored in ebselen-treated 22-wk-old ZDF rats.

Immunodetectable 3-NT was used as a marker of nitrosylated products, a fingerprint of peroxynitrite formation. At 8 wk of age, greater immunostaining for 3-NT was noted in glomeruli and the outer medullary tubules in ZDF rats \((P < 0.05)\) versus ZL rats, which showed only minimal expression without further increment at 22 wk of age (Table 3).

### Table 1. Quantitative analysis of α-SMA staining

<table>
<thead>
<tr>
<th>Positive Stain/100 Glomeruli</th>
<th>8-Week-Old ZL</th>
<th>8-Week-Old ZDF</th>
<th>22-Week-Old ZL</th>
<th>22-Week-Old ZDF</th>
<th>22-Week-Old ZDF + E</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mesangium</td>
<td>0.50 ± 0.50</td>
<td>0.75 ± 0.48</td>
<td>2.25 ± 0.85</td>
<td>2.75 ± 0.63</td>
<td>0.06 ± 0.06</td>
</tr>
<tr>
<td>Bowman’s capsule</td>
<td>0.50 ± 0.50</td>
<td>1.00 ± 1.00</td>
<td>1.00 ± 0.41</td>
<td>7.50 ± 1.94</td>
<td>2.75 ± 1.31</td>
</tr>
<tr>
<td>Tubular profile</td>
<td>0</td>
<td>1.00 ± 0.58</td>
<td>1.25 ± 0.95</td>
<td>43.25 ± 17.09</td>
<td>6.00 ± 2.68</td>
</tr>
</tbody>
</table>

*α-SMA, α-smooth muscle actin; E, ebselen treated. Among 100 consecutive glomeruli in each section, those with positive mesangial α-SMA staining were counted. Bowman’s capsules and tubular profiles surrounded by positive α-SMA staining were also quantified in the areas next to the same consecutive 100 glomeruli.

\(\overset{b}{*}P < 0.05\) versus 8-wk-old ZDF rats.

\(\overset{c}{*}P < 0.01\) versus 22-wk-old ZDF rats.

\(\overset{d}{*}P < 0.01\) versus 22-wk-old ZL rats.

\(\overset{e}{*}P < 0.05\) versus 22-wk-old ZDF rats.

\(\overset{f}{*}P < 0.05\) versus 22-wk-old ZL rats.
in ZDF rats at 22 wk in glomeruli and the outer medullary tubules (P < 0.01 versus 8-wk-old ZDF rats), and the staining was greater in glomeruli (P < 0.001 in 22-wk-old ZL rats; Figure 8, B versus A), cortical proximal (P < 0.01), outer medullary (P < 0.001; Figure 9A), and inner medullary (P < 0.01) tubules versus age-matched ZL rats. ZDF rats at this age also displayed marked and frequent 3-NT products in the peritubular capillaries and vasa rectae (Figure 9B) and focal deposits in the lumen, media, and/or endothelium of arteries (Figure 9, C and D) and venules in two of four rats examined. Immunoreactive products in glomeruli (Figure 8B) and peritubular capillaries (Figure 8E) and veins seemed to be confined to the endothelial lining. No vascular staining was observed in ZL rats at any age (Figure 8D). Ebselen-treated ZDF rats revealed significantly less staining in glomeruli (P < 0.01; Figure 8, C versus B), cortical proximal tubules (P < 0.01), and outer (P < 0.01) and inner medullary tubules (P < 0.05) versus untreated 22-wk-old ZDF rats. None of the arteries contained 3-NT products, and only scattered, albeit weaker, staining of peritubular capillaries was evident in ebselen-treated 22-wk-old ZDF rats (Figure 8, F versus E).

Glomerular immunostaining for 3-NT products evaluated by computer image analysis gave similar results, i.e., markedly enhanced expression in 22-wk-old ZDF rats (2.052 ± 0.582 versus 0.338 ± 0.135% of positively stained area/glomerulus; P < 0.05 in age-matched ZL rats), and restored level in ebselen-treated 22-wk-old ZDF rats (0.517 ± 0.097% of positively stained area/glomerulus; P < 0.05 versus untreated 22-wk-old ZDF rats; Figure 10A). Concordant with the above morphologic findings, 3-NT–modified proteins measured by HPLC in the renal parenchyma (Figure 10B) showed higher levels in ZDF at 8 wk of age (P < 0.05) compared with age-matched ZL rats. At age 22 wk, marked accumulation occurred in ZDF rats (P < 0.001 versus 8-wk-old ZL and ZDF and 22-wk-old ZL rats), but the levels were unchanged in ZL rats. Chronic ebselen treatment resulted in significant prevention of the accumulation of renal tissue 3-NT–modified proteins in the 22-wk-old ZDF rats (P < 0.01 versus untreated ZDF rats).

Collectively, these findings are indicative of oxidative and nitrosative stress in ZDF rats, operant at 8 wk, not only in the tubules but also in the glomeruli and a variety of vessels, particularly involving the endothelial lining. Chronic administration of *bona fide* peroxynitrite scavenger, ebselen, significantly prevented the accumulation of 3-NT–modified proteins, detectable both chemically and immunohistologically; partially

### Table 2. Semiquantitative analysis of HNE staining

<table>
<thead>
<tr>
<th></th>
<th>8-Week-Old ZL</th>
<th>8-Week-Old ZDF</th>
<th>22-Week-Old ZL</th>
<th>22-Week-Old ZDF</th>
<th>22-Week-Old ZDF + E</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glomeruli</td>
<td>0.31 ± 0.06</td>
<td>0.38 ± 0.22</td>
<td>0.63 ± 0.24</td>
<td>0.88 ± 0.13</td>
<td>0.81 ± 0.28</td>
</tr>
<tr>
<td>Peritubular capillaries</td>
<td>0.38 ± 0.07</td>
<td>0.75 ± 0.44</td>
<td>0.63 ± 0.13</td>
<td>1.13 ± 0.13</td>
<td>1.13 ± 0.13</td>
</tr>
<tr>
<td>Arteries</td>
<td>0.44 ± 0.06</td>
<td>0.38 ± 0.07</td>
<td>0.63 ± 0.13</td>
<td>1.13 ± 0.24</td>
<td>0.88 ± 0.24</td>
</tr>
<tr>
<td>Cortical distal tubules</td>
<td>0.88 ± 0.24</td>
<td>1.63 ± 0.52</td>
<td>0.50 ± 0.002</td>
<td>1.38 ± 0.38</td>
<td>1.25 ± 0.32</td>
</tr>
<tr>
<td>Outer medullary tubules</td>
<td>0.56 ± 0.16</td>
<td>1.25 ± 0.32</td>
<td>0.50 ± 0.002</td>
<td>1.37 ± 0.13</td>
<td>1.00 ± 0.29</td>
</tr>
<tr>
<td>Inner medullary tubules</td>
<td>0.44 ± 0.21</td>
<td>1.25 ± 0.25</td>
<td>0.75 ± 0.25</td>
<td>2.00 ± 0.29</td>
<td>1.00 ± 0.35</td>
</tr>
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a HNE, 4-hydroxy-2-nonenal. Immunostaining was semiquantitatively scored from 0 to +3 in all of the compartments.
b P < 0.05 versus 8-wk-old ZDF rats.
c P < 0.05 versus 22-wk-old ZDF rats.
d P < 0.05 versus 8-wk-old ZL rats.
e P < 0.05 versus 22-wk-old ZDF rats.
f P < 0.001 versus 22-wk-old ZL rats.
prevented the accumulation of lipid peroxidation products; and improved renal function and histologic damage.

**Discussion**

ZDF rats develop obesity, hyperlipidemia, insulin resistance, and hyperglycemia by 6 to 8 wk of age (8,10) and a progressive nephropathy by 12 wk of age, earlier than in most other models of diabetes (10), with increasing proteinuria resulting in chronic renal insufficiency by 22 wk of age. The nephropathy in this model has previously been described as FSGS associated with glomerulomegaly and mesangial expansion, findings characteristically seen in patients with obesity and the meta-
bolic syndrome (23,24) associated with type 2 diabetic milieu. Except for an isolated report describing the tubulointerstitial changes in Zucker obese rats (7), most investigators have mentioned these changes only in passing and as secondary pathology (9,10). Renal vascular pathology has not been described discretely.

FSGS in ZDF rats has been variably ascribed to glomerular hyperperfusion (9) or early podocyte injury induced by hyperlipidemia and cholesterol-induced chemotactic monocyte/macrophage influx into the glomeruli by some (8) and diabetic milieu but not hypertension or obesity by other investigators (10). The role of oxidative and nitrosative stress in the pathogenesis of glomerulopathy and nephropathy in these animals, although suggested (8), has not been evaluated. The data presented herein provide morphologic and functional characterization of progressive nephropathy, including the extraglo-

Figure 8. Representative photomicrographs of endothelial 3-nitrotyrosine (3-NT) staining from 22-wk-old rats display minimal to absent staining in glomeruli (A) and peritubular capillaries (D) in 22-wk-old ZL rats, prominent glomerular (B) and peritubular capillary (E) staining in 22-wk-old ZDF rats, and significantly ameliorated staining in glomeruli (C) and peritubular capillaries (F) in ebselen-treated ZDF rats. Magnification, ×100 in A through C, ×200 in D through F, immunoperoxidase.

Figure 9. ZDF rats at 22 wk of age also displayed strong staining for 3-NT in the outer medullary tubules (A), vasa rectae in the inner medulla (B), arterial endothelium (C), and arterial media (D). Magnification, ×100, immunoperoxidase.
merular pathology in ZDF rats; offers several lines of evidence in support of renal oxidative and nitrosative stress hypothesis; and delineates a pathogenetically based pharmacologic approach to ameliorate both the stress and nephropathy. Furthermore, these findings address two major questions: (1) What might be the origin of oxidative and nitrosative stress in the kidney of ZDF rats? (2) Does oxidative and nitrosative stress to the kidney contribute to the nephropathy and functional demise, or is it a byproduct of the latter?

Coimbra et al. (8) demonstrated pronounced hyperlipidemia with elevated triglycerides and cholesterol and progressive lipid accumulation focally in glomeruli and abundantly in the tubulointerstitium, starting at 6 wk of age, before the development of FSGS or tubulointerstitial damage. They suggested a pathogenetic role for hyperlipidemia in progressive podocytic injury. We, too, observed focal lipid deposits in tubular cells of 8-wk-old ZDF rats with widespread accumulation in tubules and focally in glomerular tufts and podocytes in 22-wk-old ZDF rats. It is interesting that at this age, we also observed significant but focal intra- and/or extracellular lipid deposits in the vasculature affecting larger perihilar arteries, thickened parenchymal arteries of all sizes, and the peritubular capillary endothelium, suggesting a lipid-induced vasculopathy.

There is a body of evidence linking hyperlipidemia to progressive renal injury in various experimental models (25–27), and the injury is believed to be mediated via oxidized LDL (28,29). We evaluated the role of oxidative stress in ZDF rat kidneys that contained abundant lipid deposits by assessing HNE, a marker of lipid peroxidation. HNE immunostaining was greater in the inner medullary tubules by 8 wk of age in ZDF rats compared with the age-matched ZL rats and progressively increased in glomeruli, peritubular capillaries, and arteries in the 22-wk-old ZDF rats. It was significantly greater in the cortical distal and outer and inner medullary tubules at this age compared with the age-matched ZL rats. These findings suggest progressive lipid peroxidation in ZDF starting at 8 wk, before the onset of nephropathy. Oxidative stress with increased plasma lipid peroxidation levels has also been reported in streptozotocin-induced rat model of diabetes (30). A striking renal deposition of HNE with glomerulosclerosis, tubulointerstitial disease, and thickened small parenchymal arteries has also been described in another model of type 2 diabetes (LA/N-fas) with lipid-induced nephropathy (31). Glucose promotes lipoprotein oxidation by a pathway that involves superoxide (32), and increased levels of lipid peroxidation markers are reported in patients with type 1 diabetes (33). Glomerular HNE-reactive products in our 22-wk-old ZDF rats were focal but most prominent in the podocytes and suggested that lipid peroxides may participate in the early podocytic injury as described by others (8,10). Oxidative stress in our animals was further confirmed by chemical analysis of the renal parenchyma showing decreased GSH and BH4 levels in the older ZDF rats. A corresponding increment in BH2 starting at 8 wk of age also suggested early accumulation of oxidative products in the renal tissue of the ZDF rats. Early onset of oxidative stress before the development of kidney lesions and functional renal impairment was reported by other investigators also in Zucker obese rats, in the absence of hyperglycemia, hypertension, and inflammation, in agreement with our findings (34).

A potential pathway to injury involving oxidative stress proceeds with the reaction between superoxide anion and nitric oxide (NO), resulting in the formation of a strong oxidant, peroxynitrite. Nitrosative stress therefore was evaluated in ZDF rats, as scavenging of peroxynitrite ameliorates lipid
peroxidation and DNA damage in other experimental models of renal failure or brain ischemia (17,35,36). Hallmarks of nitrosative stress were noticed on immunohistochemical staining in glomeruli and outer medulla and by chemical analysis of the renal parenchyma for accumulated 3-NT–modified proteins by 8 wk of age in ZDF rats with further dramatic increase by 22 wk of age in all segments of the kidney. This may relate to reduced renal parenchymal levels of BH4 in 22-wk-old ZDF rats as described above. BH4 is known to bind to the oxidase domain of NO synthase (NOS) and is an essential co-factor for the synthesis of NO (37). The presence of suboptimal levels of BH4, as seen in 22-wk-old ZDF rats, causes uncoupling of NOS with generation of both NO and superoxide anions, resulting in the formation of peroxynitrite (38), which is considered vasotoxic (39,40). Peroxynitrite thus formed in turn releases zinc from the zinc-thiolate cluster of endothelial NOS with further decrease in NO synthesis and increase in superoxide anion production (41) and a resultant vicious circle. These investigators also showed that peroxynitrite that was generated by endothelial cells that were exposed to elevated glucose increased the cellular content of 3-NT. It is noteworthy in this respect that there was strong immunostaining of the 3-NT products in the glomerular and vascular endothelium in the 22-wk-old ZDF rats in our study. Increased endothelial NOS–induced superoxide and peroxynitrite have also been reported by other investigators in human endothelial cells that were exposed to elevated glucose (42), in the aortas of other experimental models of diabetes (43,44), and in blood vessels of patients with diabetes (45,46). That reduced bioavailability of BH4 results in the uncoupling of the enzyme with endothelial dysfunction is shown by the improvement in the endothelial dysfunction by the administration of BH4 in other animal models of diabetes (43,47,48), as well as in the coronary arteries of insulin-resistant patients (49). Endothelial dysfunction is considered a major risk factor for the cardiovascular complications in diabetes (50). Our findings suggest that enhanced peroxynitrite production in the glomerular and vascular endothelium may play a role in the vasculopathy and the nephropathy in ZDF rats. Indeed, we recently demonstrated that premature endothelial cell senescence and macrovascularopathy in 22-wk-old ZDF animals can be prevented by ebselen (13,51).

Ebselen, an antioxidant of low toxicity, reportedly acts as a glutathione mimic (52) via the thioredoxin system (53) and reportedly reduces oxidative damage in foci of cerebral ischemia (35,36) and in an experimental model of acute renal failure (17). Chronic ebselen treatment in ZDF rats from 8 to 22 wk of age did not affect the body weight, the severity of hyperglycemia, or the MBP but ameliorated proteinuria and decline in Ccr. We previously reported that chronic ebselen therapy in ZL rats from 8 to 22 wk of age is without any effect on any of the functional parameters examined (13). Consistent with the functional improvement in ZDF rats, the morphologic parameters of the nephropathy improved considerably, too. There was significant amelioration of FSGS, glomerulosclerosis, and tubulointerstitial scarring indices and foci of tubulointerstitial scars and inflammation despite the persistence of glomerular and tubular lipid deposits. The vascular lipid deposits, including in the endothelial lining, however, dissipated with prevention of vasculopathy without changes in MBP. Ebselen treatment also resulted in significantly less HNE immunostaining in the inner medullary tubules, and the staining was virtually abolished from the blood vessels. In parallel, renal tissue levels of GSH and BH4 in ZDF rats were restored to levels comparable to the age-matched ZL rats after chronic ebselen treatment. These findings suggest that oxidation of lipids plays an important role in the nephropathy and vasculopathy in ZDF rats.

Ebselen, in addition to its antioxidant properties, is an effective scavenger of peroxynitrite (15). Chronic ebselen treatment resulted in marked prevention in the accumulation of the 3-NT immunoreactive products in the entire renal parenchyma and in the perihilar arteries compared with the untreated 22-wk-old ZDF rats. Immunostaining was notably decreased in the glomerular and vascular endothelium relative to the untreated ZDF rats. Likewise, the renal tissue levels of 3-NT–modified proteins measured by HPLC were restored in ebselen-treated 22-wk-old ZDF rats to the levels comparable in 8-wk-old ZDF rats. These effects could also be related to restoration of the tissue levels of GSH and BH4 in ebselen-treated 22-wk-old ZDF rats. BH4 as stated earlier improves endothelial dysfunction in experimental (43,44,48) and clinical diabetes (46,47) as a result of reversal of the uncoupling and restored NOS activity with consequent increased NO production (48) and thus decreased peroxynitrite formation.

In addition to FSGS and vasculopathy, 22-wk-old ZDF rats display tubulointerstitial disease, which in the past was considered secondary to FSGS and tubulointerstitial protein leak (9,10). Lipid-induced damage, however, cannot be excluded in its pathogenesis, as tubulointerstitial scarring was disproportionately greater than the degree of FSGS, and progressive tubular deposits of lipids, HNE-immunoreactive lipid peroxides, and 3-NT proteins were observed by us in ZDF rats starting at 8 wk of age. Moreover, chronic ebselen treatment ameliorated not only foci of tubulointerstitial scars but also inflammation in conjunction with downregulation in the tubular immunomarkers for oxidative and nitrosative stress. That tubulointerstitial lesions are not necessarily dependent on glomerulosclerosis is also suggested by aggravation of tubulointerstitial but not glomerular lesions in Zucker obese rats that are on a high-fat diet (54), the strain related to ZDF. Furthermore, prominent, and de novo α-SMA immunoeexpression, a marker for activated mesangial cells and the myofibroblastic transformation of interstitial cells, was also observed mostly in the interstitium in the absence of significant mesangial staining in the 22-wk-old ZDF rats, suggesting a mutually independent pathogenesis between glomerular and tubulointerstitial disease. α-SMA immunoeexpression paralleled and co-localized in the areas of tubulointerstitial scarring. Similar findings including lack of significant mesangial de novo expression of α-SMA despite glomerular hypertrophy and FSGS have been previously reported in ZDF rats (8). It could be speculated that interstitial α-SMA upregulation may also be mediated via oxidative and nitrosative stress. Chronic ebselen treatment
indeed not only ameliorated tubulointerstitial scarring and inflammation but also attenuated α-SMA immunoperoxidase staining even in areas of tubulointerstitial scars, suggesting a correlation with oxidative/nitrosative stress.

In conclusion, we have confirmed that ZDF rats with the metabolic syndrome develop nephropathy with FSGS, proteinuria, and renal failure and in addition demonstrated the occurrence of lipid-induced vasculopathy and tubulointerstitial disease in this rat model. Lipid peroxidation and 3-NT-modified products accumulate in the kidneys starting at 8 wk of age, before the onset of the nephropathy, with further accentuation in 22-wk-old ZDF rats, suggesting these to be (1) the origin of the oxidative and nitrosative stress and (2) the cause and not the consequence of the nephropathy in ZDF. Ebselen, a scavenger of peroxynitrite and an antioxidant, resulted in improvement of renal function (proteinuria and Ccr), morphology (FSGS, tubulointerstitial scars and inflammation, and vasculopathy), and amelioration in chemical and immunomarkers of nitrosative and oxidative stress. The beneficial effects of ebselen, without any effect on or any additional therapy for hyperlipidemia, lipidosis, obesity with the associated metabolic syndrome, hyperglycemia, or MBP, strongly argue that oxidative and nitrosative stress are the primary culprits in the pathogenesis of the nephropathy, including the tubulointerstitial pathology and the microvascular complications in the ZDF rats.

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