Signaling pathways modulated by fish oil in salt-sensitive hypertension

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Diaz Encarnacion MM, Warner GM, Gray CE, Cheng J, Keryakos HK, Nath KA, Grande JP. Signaling pathways modulated by fish oil in salt-sensitive hypertension. Am J Physiol Renal Physiol 294: F1323–F1335, 2008. First published April 2, 2008; doi:10.1152/ajprenal.00401.2007.—Although many studies have indicated that fish oil (FO) improves cardiovascular risk factors and reduces histopathological manifestations of injury in experimental renal injury models, potential mechanisms underlying this protective effect have not been adequately defined. The objective of this study was to identify potential signaling pathways that confer protection in the Dahl rat model of salt-sensitive hypertension. Male Dahl salt-sensitive rats (n = 10/group) were provided with formulated diets containing 8% NaCl, 20% protein, and 25% FO or 25% corn oil (CO) for 28 days. FO reduced blood pressure (−11% at 4 wk; P < 0.05), urine protein excretion (−45% at 4 wk; P < 0.05), plasma cholesterol and triglyceride levels (−54%, P < 0.001; and −58%, P < 0.05), and histopathological manifestations of renal injury, including vascular hypertrophy, segmental and global glomerular sclerosis, interstitial fibrosis, and tubular atrophy. Interstitial inflammation was significantly reduced by FO (−32%; P < 0.001), as assessed by quantitative analysis of ED1-positive cells in sections of the renal cortex. FO reduced tubulointerstitial proliferative activity, as assessed by Western blot analysis of cortical homogenates for PCNA (−51%; P < 0.01) and quantitative analysis of MiB-1-stained sections of the renal cortex (−42%; P < 0.001). Decreased proliferative activity was associated with reduced phospho-ERK expression (−37%; P < 0.005) and NF-κB activation (−42%; P < 0.05). FO reduced cyclooxygenase (COX)-2 expression (−63%; P < 0.01) and membrane translocation of the NADPH oxidase subunits p47phox and p67phox (−26 and −34%; P < 0.05). We propose that FO ameliorates renal injury in Dahl salt-sensitive rats through the inhibition of ERK, decreased NF-κB activation, inhibition of COX-2 expression, and decreased NADPH oxidase activation.

Hypertensive nephrosclerosis is the second most common cause of end-stage renal disease in the United States (98). Over 70% of the 5.6 million individuals in the United States with elevated serum creatinine levels are hypertensive (16). The risk of end-stage renal disease is directly related to the degree of blood pressure elevation. Hypertension, which occurs in as many as 43 million individuals, is clearly a multifactorial disorder resulting from a variety of environmental and genetic factors. Of the environmental factors, excess dietary salt intake appears to be one of the most important (93, 103). Of note, salt sensitivity is an independent cardiovascular risk factor in patients with hypertension (69).

Given the prevalence of hypertension, nontoxic, easily tolerated therapeutic agents that complement standard antihypertensive therapy may play a critical role in reducing the prevalence of cardiovascular and renal disease secondary to hypertension. A number of studies have provided evidence that dietary fish oil (FO) supplementation ameliorates cardiovascular and renal disease in humans (12, 21–23, 38, 51, 68, 78, 92) and in a variety of experimental model systems, including spontaneously hypertensive rats (SHRs) (44, 50, 91) and Dahl salt-sensitive (SS) rats, which provide a model of human SS hypertension (45, 83, 85, 86, 94, 101). Despite extensive study, potential mechanisms whereby dietary FO supplementation protects against the development of renal disease have not been adequately defined (33, 34).

The protective effects of FO in renovascular hypertension are likely to be at least in part related to the reduction of blood pressure (10, 27, 30, 66, 70) and improvement of serum lipid profiles (39, 50, 59, 82). However, recent studies have indicated that high salt intake promotes renal disease through mechanisms that are independent of blood pressure (89), and there is evidence that FO may have protective effects that are independent of blood pressure reduction alone (91). In vitro studies have uncovered several potential mechanisms whereby FO may play a critical role in ameliorating renal injury. In cultured cells, FO suppresses growth factor-induced proliferation through cell cycle arrest in the G1 phase (14, 32, 95, 107). In vitro studies of cultured cells and ex vivo studies of inflammatory cells have shown that FO has potent anti-inflammatory effects by reducing cytokine production and NF-κB activation (13, 46, 48, 49, 102), cyclooxygenase (COX)-2 expression (60), and NADPH oxidase activation (90). There have been few studies to address the potential in vivo relevance of these findings in models of renal injury.

To address this issue, we used an experimental model of SS hypertension to identify potential pathway(s) through which FO may prevent or ameliorate renal disease. In accord with previous descriptive studies, we demonstrate that FO reduces blood pressure, serum lipids, and histopathological manifestations of renal injury. The reduction of blood pressure by treating SS rats with hydralazine (HYD) failed to prevent renal injury, indicating that other pathways may be at least in part responsible for the protective effects of FO. We demonstrate that FO inhibits ERK signaling and NF-κB activation, reduces interstitial inflammation, and decreases the renal proliferative response to injury. The anti-inflammatory effects of FO are related to decreased COX-2 expression and NADPH oxidase assembly. Our studies have identified several key pathways...
modulated by FO, which may underlie its protective effect in renovascular hypertension.

MATERIALS AND METHODS

Animal model. All animal procedures were performed according to institutional animal care guidelines established by the National Institutes of Health, and the study protocol was approved by the Mayo Clinic College of Medicine Institutional Animal Care and Use Committee. Studies were conducted using 30 male Dahl SS and 10 male Dahl salt-resistant (SR) rats purchased from Harlan Sprague-Dawley (Indianapolis, IN) at 5–6 wk of age. The rats were housed under standard conditions with access to a normal-salt diet [NSD: 0.45% NaCl, 20% protein, and 5% corn oil (CO)] and water ad libitum for 2 wk before the experiments. At 7–8 wk of age, the SS rats were randomly divided into three treatment groups (n = 10/group) and switched to one of three high-salt diet (HSD) formulations prepared by Purina TestDiet (Richmond, IN). The diets, derived from Basal Diet No. 5755, contained 8% NaCl, 20% protein, and one of the following: 5% CO (HSD), 25% CO (HSD + CO), or 25% FO (HSD + FO). FO [160 g/kg eicosapentaenoic acid (EPA) and 100 g/kg docosahexaenoic acid (DHA)] was kindly provided by Pronova Biocare (Lysaker, Norway). All diets delivered the same amount of vitamins, minerals, and fiber per calorie. The SR rats received the HSD and served as the negative control. In a separate experiment, HYD was administered to SS rats on the HSD to determine the effect of lowering blood pressure has on renal damage and signaling pathways. These animals were divided into three groups and given the NSD (n = 3), HSD (n = 3), or HSD + HYD (5 mg·kg body wt⁻¹·day⁻¹) in drinking water (n = 6).

Systolic blood pressure in conscious rats was measured weekly by the tail-cuff method using the XBP1000 Noninvasive Blood Pressure System (Kent Scientific, Torrington, CT). Each week, the rats were weighed and placed in metabolic cages to monitor food intake and urine output and to collect 24-h urine samples. Urine protein was measured using the Lowry method (58). After 28 days, the rats were anesthetized with ketamine-xylazine, blood samples were collected for lipid analysis, and the kidneys and hearts were harvested. Portions of the kidneys were fixed for histopathological analysis and immunohistochemical staining. Additional portions were snap frozen in liquid nitrogen and stored at −80°C for Western blot analysis.

Histology and immunohistochemistry. Renal tissue was fixed in 10% neutral-buffered Formalin, dehydrated, and embedded in paraffin per standard techniques. Sections were cut at a thickness of 4 μm and stained with hematoxylin-eosin, Masson’s trichrome, or Sirius red. Immunostains were performed using the following antibodies: α-smooth muscle actin (α-SMA; DakoCytomation, Carpinteria, CA), Mib-1 (Ki67; NovoCastra), and ED1 (Fitzgerald, Concord, MA). Slides were deparaffinized, followed by antigen retrieval via heat treatment in EDTA for 30 min using a vegetable steamer. Trypsin treatment was performed for the ED1 stain. Commercially available kits (Vectastain ABC Kit; Vector, Burlingame, CA; and Envision Plus HRP Kit; DakoCytomation) were used for the blocking, secondary antibody and amplification steps. NovaRed (Vector) was used for color development, followed by the hematoxylin counterstain. To facilitate consistency between staining batches, the slides were stained on the Dako Autostainer (DakoCytomation), an automated staining machine.

Assessment of histopathological features. Quantitative analysis of histopathological manifestations of renal injury was performed, in a blinded fashion, with the MetaVue Image Analysis System (Universal Imaging, Downingtown, PA). Glomerular, interstitial, tubular, and vascular features of renal tissue, including glomerular size and vascular lumen-to-wall thickness ratios, were analyzed on hematoxylin-eosin-stained slides. Interstitial fibrosis was determined by quantitative assessment of the extracellular matrix in trichrome- and Sirius red-stained slides observed with both white and polarized light, the latter being an index of fibrillar collagen deposition (19). Glomerular and tubulointerstitial staining for α-SMA, a marker of epithelial-to-mesenchymal transformation, were expressed as a percentage of their respective surface areas. The proliferation marker Mib-1 and the macrophage marker ED1 were assessed as the number of positively staining glomerular or interstitial cells per ×200 field.

Western blot analysis. For preparation of whole cell lysates, renal cortical tissue was homogenized in 1× lysis buffer (Cell Signaling Technology, Beverly, MA) with protease inhibitors (protease inhibitor cocktail; Roche Applied Science, Indianapolis, IN). Homogenates were then centrifuged at 10,000 g for 10 min at 4°C, and the resulting supernatants were used for Western blot analysis. Microsomal fractions were prepared as previously described (15). In brief, tissues were homogenized in a buffer containing 0.25 M sucrose and 20 mM Tris·HCl (pH 7.2). The homogenates were centrifuged at 2,000 g for 10 min to remove insoluble debris and then ultracentrifuged at 100,000 g for 60 min. The pellets were resuspended in homogenizing buffer and used for the detection of levels of COX-2, p47phox, p67phox, and heme oxygenase (HO)-1. Protein concentrations were determined using the Lowry method. Equal amounts of whole cell lysate or microsomal protein (~100 μg) were denatured for 5 min at 100°C in SDS loading buffer (Bio-Rad, Burlingame, CA) and subjected to SDS-PAGE in the Criterion System (Bio-Rad) at a constant current (200 mA/gel), followed by a transfer to polyvinylidene difluoride membranes (Bio-Rad). The membranes were blocked with 1× casein (Vector) in Tris-buffered saline containing 0.5% Tween 20 and incubated with primary antibodies for phospho-(p)-ERK and PCNA (Santa Cruz Biotechnology, Santa Cruz, CA), HO-1 (Stressgen Biotechnologies, Ann Arbor, MI), COX-2 (Cayman Chemical, Ann Arbor, MI), p47phox and p67phox (BD Transduction, Franklin Lakes, NJ), followed by horseradish peroxidase-conjugated secondary antibodies (Southern Biotech, Birmingham, AL; and Stressgen). The blots were then visualized by exposure to X-ray film using the ECL Western blotting detection reagents and analysis system (Amer sham Biosciences, Piscataway, NJ). The blots were stripped and reprobed with antibodies for total ERK (Santa Cruz) or GAPDH (Novus Biologicals, Littleton, CO) to normalize for gel loading and transfer efficiency; microsomal blots were stained with a MemCode Reversible Protein Stain Kit (Pierce, Rockford, IL). Densitometric analyses were performed with the Kodak ds Image Station 440.

Electrophoretic mobility shift assay. Tissue nuclear extracts were prepared and assessed for the activation of NF-κB by electrophoretic mobility shift assay (EMSA), as previously described by Rangan et al. (84) and modified by Nath et al. (73). Approximately 75 mg of the rat renal cortex were homogenized on ice in 400 μl of buffer A containing (in mM) 10 HEPES (pH 7.9), 10 KCl, 2 MgCl₂, and 0.1 EDTA and protease inhibitors (0.5 mM DTT + Roche protease inhibitor cocktail), followed by the addition of 50 μl of 10% Nonidet P-40. The extracts were vortexed extensively and centrifuged at 13,000 g for 5 min at 4°C. The pellets were resuspended in 60 μl of buffer B containing (in mM) 7.5 HEPES, 300 NaCl, and 50 KCl and 10% glycerol and protease inhibitors, vortexed, and centrifuged at 13,000 g for 10 min. The extracted nuclear protein in the supernatants was assessed using the Lowry method. The NF-κB probe used in the EMSA, a double-stranded consensus oligonucleotide from Promega (Madison, WI), was labeled with [γ-³²P]ATP, the unincorporated nucleotide was removed using a BioSpin6 column (Bio-Rad). Five micromgrams of nuclear extract was combined with binding buffer containing (in mM) 7.5 HEPES (pH 8.0), 100 NaCl, 1 MgCl₂, 0.05 EDTA, and 0.5 DTT and 3% glycerol, 0.2 mg/ml poly(dI·dC), and 50,000 counts per minute of labeled NF-κB probe and incubated for 30 min at room temperature. The binding reactions were analyzed by electrophoresis on a 5% nondenaturing polyacrylamide gel and visualized by autoradiography. A supershift analysis was performed using a concentrated anti-p65 antibody (Santa Cruz); 1 μl of antibody was added to 5 μg nuclear extract and allowed to incubate on ice for 15 min before the binding reaction.

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Statistical analysis. Data are expressed as means ± SE. Statistical significance was evaluated by one-way ANOVA, followed by a Bonferroni multiple comparisons test and Mann-Whitney test for unpaired observations. Differences were considered to be significant when \( P < 0.05 \).

RESULTS

FO improves serum lipid profiles in SS + HSD rats. Male Dahl SS rats (\( n = 10 \)/group) were provided with one of the following formulated diets: 0.45% NaCl and 5% CO (SS + NSD), 8% NaCl and 5% CO (SS + HSD), 8% NaCl and 25% CO (SS + HSD + CO), or 8% NaCl and 25% FO (SS + HSD + FO). SR rats were provided with the 8% NaCl and 5% CO diet (SR + HSD). No significant differences in daily food intake between the CO- and FO-treated rats were observed, and total weight gain during the experimental period was similar in all groups (data not shown). At the end of the experimental period, serum triglyceride and total cholesterol levels were obtained in nonfasted rats. As previously reported, serum cholesterol levels increased by twofold in SS rats fed the HSD compared with those of the SR rats fed the HSD (Table 1). Although the 25% CO diet had no significant effect on serum cholesterol or triglyceride levels in SS rats, the 25% FO diet reduced serum cholesterol and triglyceride levels >50% (Table 1).

FO reduces blood pressure and heart weight in SS + HSD rats. There were no significant differences in systolic blood pressure before the administration of formulated diets containing high salt (Fig. 1A). As expected, systolic blood pressure was significantly increased in SS + HSD rats, whereas the systolic blood pressure did not significantly increase throughout the experimental period in SR + HSD rats (Fig. 1A). Blood pressure elevation in SS rats on the HSD containing 25% CO (SS + HSD + CO) was similar to that observed in SS rats on the HSD containing 5% CO (SS + HSD; data not shown). FO supplementation significantly reduced systolic blood pressure compared with that in with SS + HSD rats (Fig. 1A). An additional group of SS + HSD rats was treated with HYD (5 mg·kg body wt⁻¹·day⁻¹), and blood pressure was measured after 4 wk. This treatment (SS + HSD + HYD) significantly reduced systolic blood pressure compared with that in SS + HSD (141 vs. 193 mmHg; \( P < 0.05 \); Fig. 1B). This reduction of blood pressure was greater than that observed in SS + HSD + FO rats (141 vs. 172 mmHg; \( P < 0.05 \)). As expected, the systolic blood pressure of SS + HSD and SS + NSD rats remained at basal levels (Fig. 1B). Heart weight is the best measure of myocardial hypertrophy in response to systemic hypertension. The mean heart weight-to-body weight ratio of SS + HSD + FO rats was found to be significantly lower than that of the SS + HSD or SS + HSD + CO rats (Table 1). SS + HSD + HYD rats had ratios similar to those of the SS + HSD + FO rats (Table 1).

FO decreases urinary protein excretion in SS + HSD rats. Urine protein excretion is a well-recognized marker of glomerular damage. In accordance with previous observations, the development of renovascular hypertension in SS + HSD rats was associated with the development of proteinuria (Fig. 2A). Although blood pressure was significantly reduced in SS + HSD + HYD rats, urine protein excretion progressively increased with time (Fig. 2A). Urine protein excretion in SS + HSD and SS + HSD + HYD rats was not significantly different. SS rats on an NSD (SS + NSD) did not develop significant proteinuria (Fig. 2A). FO treatment (SS + HSD + FO) significantly decreased urine protein excretion compared with that of SS + HSD + CO rats (Fig. 2B). SR + HSD rats did not develop significant proteinuria (Fig. 2B).

FO decreases histopathological manifestations of renal injury in SS + HSD rats. A summary of histopathological alterations identified in hypertensive SS + HSD rats is provided in Table 1. SS + HSD rats developed segmental and global glomerular sclerosis with glomerular enlargement, as assessed by the quantitative evaluation of glomerular planar surface area with the MetaVue Image Analysis System (Table 1 and Fig. 3). The addition of 25% CO to the diet had no effect on the extent of glomerular sclerosis and hypertrophy (SS + HSD + CO vs. SS + HSD). However, the addition of 25% FO to the diet significantly reduced glomerular sclerosis and hypertrophy (SS + HSD + FO vs. SS + HSD + CO; Table 1 and Fig. 3). Arcuate-sized arteries from the SS + HSD group showed a modest increase in diameter and a reduction in lumen-to-wall thickness ratio. FO, but not CO, prevented the development of fibromuscular hyperplasia in SS rats (SS + HSD + FO vs. SS + HSD + CO; Table 1 and Fig. 3). Both interlobular-sized arteries and arterioles showed striking fibromuscular proliferation in SS + HSD rats, leading to a significant increase in vessel diameter and a significant decrease in the lumen-to-wall thickness ratio (Table 1 and Fig. 3). Although HYD reduced blood pressure to levels significantly lower than those observed in SS + HSD + FO rats, the extent of glomerular and vascular hyperplasia was similar to that observed in SS + HSD + FO rats.

Table 1. Serum lipids, heart weight-to-body weight ratio, and histopathological manifestations of renal injury

<table>
<thead>
<tr>
<th></th>
<th>SR + HSD</th>
<th>SS + HSD</th>
<th>SS + HSD + CO</th>
<th>SS + HSD + HYD</th>
<th>SS + HSD + FO</th>
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<tbody>
<tr>
<td>Cholesterol, mg/dl</td>
<td>52±2</td>
<td>103±5†</td>
<td>116±6†</td>
<td>122±16†</td>
<td>49±4*‡§</td>
</tr>
<tr>
<td>Triglycerides, mg/dl</td>
<td>123±10</td>
<td>129±14</td>
<td>141±25</td>
<td>92±17</td>
<td>59±6*‡§</td>
</tr>
<tr>
<td>Heart weight-to-body weight ratio, ×10⁻³</td>
<td>3.86±0.08</td>
<td>4.86±0.11†</td>
<td>4.67±0.11†</td>
<td>4.01±0.06‡§</td>
<td>4.18±0.15‡§</td>
</tr>
<tr>
<td>Segmental sclerosis, %glomeruli</td>
<td>0</td>
<td>4.87±0.77†</td>
<td>4.38±0.98†</td>
<td>5.30±0.52§</td>
<td>1.46±0.27‡§</td>
</tr>
<tr>
<td>Glomerular area, µm²</td>
<td>10,723±380</td>
<td>16,416±316†</td>
<td>17,238±613</td>
<td>15,085±369*‡§</td>
<td>12,591±382*‡§</td>
</tr>
<tr>
<td>Blood vessels, lumen-to-wall thickness ratio</td>
<td>0.98±0.07</td>
<td>0.80±0.05</td>
<td>0.94±0.04*</td>
<td>0.93±0.03**</td>
<td>1.03±0.07*†</td>
</tr>
<tr>
<td>Arcuate arteries</td>
<td>0.83±0.05</td>
<td>0.50±0.04†</td>
<td>0.45±0.04</td>
<td>0.40±0.03‡</td>
<td>0.84±0.08‡§</td>
</tr>
<tr>
<td>Interlobular arteries</td>
<td>0.61±0.03</td>
<td>0.35±0.03†</td>
<td>0.32±0.03</td>
<td>0.35±0.03‡</td>
<td>0.63±0.02‡§</td>
</tr>
<tr>
<td>Arterioles</td>
<td>0.25±0.10</td>
<td>13.23±1.27†</td>
<td>11.36±1.41‡</td>
<td>11.36±0.86†</td>
<td>6.06±0.47‡§</td>
</tr>
</tbody>
</table>

Values are means ± SE; \( n = 6 \) salt sensitive (SS) + high-salt diet (HSD) + hydroalazine (HYD) and \( n = 10 \) salt resistance (SR) + HSD, SS + HSD, SS + HSD + corn oil (CO), and SS + HSD + fish oil (FO) rats. HSD, 8% NaCl; CO and FO, 25%; HYD, 5 mg·kg⁻¹·day⁻¹. *\( P < 0.05 \) vs. SS + HSD; †\( P < 0.05 \) vs. SR + HSD; ‡\( P < 0.05 \) vs. SS + HSD + CO; §\( P < 0.05 \) vs. SS + HSD + HYD.
SR + HSD rats did not develop any significant glomerular enlargement or sclerosis, vascular hyperplasia, interstitial fibrosis, or tubular atrophy (Table 1 and Figs. 3 and 4). α-SMA is a marker of mesangial cell activation and interstitial myofibroblast transformation (104). Both glomerular and tubulointerstitial α-SMA expression were markedly increased in SS + HSD and SS + HSD + CO rats (Fig. 5, A and B). This response was significantly blunted in SS + HSD + FO rats (Fig. 5, A and B).

**FO decreases tubular epithelial cell proliferation in SS + HSD rats.** The development of hypertension in SS + HSD rats was associated with a 2.4-fold increase in the proliferation of tubular epithelial cells, as assessed by quantitative analysis of histological sections stained with the proliferation marker Mib-1. Tubular epithelial cell proliferation was 42% lower in SS + HSD + FO compared with SS + HSD or SS + HSD + CO rats (Fig. 6A). The number of glomerular Mib-1-positive

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**Figure 1.** Fish oil (FO) decreases salt-sensitive (SS) hypertension in Dahl SS rats. A: systolic blood pressure was measured in salt-resistant (SR) and SS rats at baseline and at weekly intervals during administration of a high-salt diet (HSD) alone (8% NaCl; SR + HSD and SS + HSD) or supplemented with FO (25%; SS + HSD + FO). Values are means ± SE (n = 10). *P < 0.05 vs. SR + HSD; †P < 0.05 vs. SS + HSD. B: rats were treated with a normal-salt diet (NSD; 0.45% NaCl; SS + NSD, n = 3), HSD alone (SR + HSD and SS + HSD, n = 10), or HSD supplemented with hydralazine (HYD; 5 mg·kg⁻¹·day⁻¹; SS + HSD + HYD, n = 6) or FO (SS + HSD + FO, n = 10). Blood pressure was measured at baseline and after 4 wk on diet. Values are means ± SE. #P < 0.05 vs. SS + NSD; *P < 0.05 vs. SS + HSD; †P < 0.05 vs. SS + HSD + HYD.

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**Figure 2.** FO reduces proteinuria in Dahl SS rats. A: 24-h urine protein excretion was assessed at baseline and at weekly intervals in SS rats fed diets containing NSD (0.45% NaCl; SS + NSD, n = 3), HSD (8% NaCl; SS + HSD, n = 10), or high salt with HYD (5 mg·kg⁻¹·day⁻¹; SS + HSD + HYD, n = 6). Values are means ± SE. #P < 0.05 vs. SS + HSD; *P < 0.05 vs. SS + HSD. B: 24-h urine protein was measured at baseline and after 4 wk in rats on NSD (SS + NSD, n = 3), HSD alone (SR + HSD and SS + HSD, n = 10), HSD supplemented with HYD (SS + HSD + HYD, n = 6), or FO (SS + HSD + FO, n = 10). Values are means ± SE. #P < 0.05 vs. SS + NSD; *P < 0.05 vs. SS + HSD; †P < 0.05 vs. SS + HSD + HYD.

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Observed in hypertensive rats (SS + HSD + HYD vs. SS + HSD and SS + HSD + CO; Table 1). However, HYD appeared to limit the extent of glomerular enlargement that developed in hypertensive rats (SS + HSD + HYD vs. SS + HSD and SS + HSD + CO; Table 1). SS + HSD and SS + HSD + CO rats developed tubular atrophy, with dilated tubules containing proteinaceous casts involving up to 13% of the cortical surface area (Table 1 and Fig. 3). FO significantly arrested the development of these tubular lesions (SS + HSD + FO vs. SS + HSD + CO). SS + HSD rats developed interstitial fibrosis, as determined by the quantitative assessment of extracellular matrix in trichrome- and Sirius red-stained slides (Fig. 4). The extent of interstitial fibrosis was significantly lower in SS + HSD + FO compared with SS + HSD and SS + HSD + CO rats (Fig. 4). As expected, the
cells was increased in SS + HSD or SS + HSD + CO rats, but FO did not significantly reduce the number of positive cells. To confirm these findings, Western blots prepared from homogenates of the renal cortex were probed with an antibody directed against the proliferation marker PCNA. As expected, proliferative activity was increased in cortical homogenates obtained from SS + HSD and SS + HSD + CO but not SS + HSD + FO rats (Fig. 6B). Although HYD significantly reduced blood pressure in SS + HSD rats, it did not decrease the PCNA expression in SS + HSD rats (Fig. 6C). In our previous in vitro studies, we demonstrated that the antiproliferative effect of DHA was associated with reduced ERK activity (107). To test the in vivo relevance of this observation, we assessed pERK expression in renal cortical homogenates from the rats in the present study. The development of hypertension in SS + HSD rats was associated with the increased expression of pERK. The dietary administration of FO, but not CO, completely abolished this effect (SS + HSD + FO vs. SS + HSD + CO; Fig. 6D).

**FO reduces inflammation in SS + HSD rats.** Interstitial inflammation is a well-recognized feature of renal lesions in SS + HSD rats. The interstitial influx of macrophages, lymphocytes, and plasma cells may directly contribute to the chronic histopathological lesions associated with this model. In vitro and ex vivo studies have shown that FO has potent anti-inflammatory effects (2, 13, 24, 102). We therefore sought to determine whether macrophage influx was lower in SS + HSD + FO compared with SS + HSD + CO rats. Histological sections were stained with the macrophage marker ED1, and the number of ED1-positive interstitial cells per 200× field was quantitated. SS + HSD rats had nearly six times more ED1-positive cells compared with those of SR + HSD rats (Fig. 7A). The number of positive cells in SS + HSD + FO rats was 23% lower than that in SS + HSD or SS + HSD + CO rats (P < 0.05). The administration of 25% CO did not reduce the number of ED1-positive cells (Fig. 7A).

**FO reduces NF-κB activity in SS + HSD rats.** A variety of proinflammatory cytokines promotes the activation of effector cells through activation of the NF-κB signaling pathway. We sought to test the hypothesis that the anti-inflammatory effects of FO were associated with reduced NF-κB signaling. Nuclear extracts prepared from the renal cortex were subjected to EMSA using a 32P-labeled NF-κB probe (73). Extracts from SS + HSD and SS + HSD + CO rats showed increased binding to the NF-κB probe compared with those of the SR + HSD rats, as evidenced by increased band density on autoradiographs (Fig. 7B). The band was supershifted following incubation with a p65 antibody (data not shown). Binding to the NF-κB probe was significantly decreased in SS + HSD + FO rats, indicative of reduced NF-κB activity (Fig. 7B).

**Fig. 4.** FO reduces interstitial fibrosis in Dahl SS rats on an HSD. SR and SS rats were maintained for 4 wk on HSD alone (SR + HSD and SS + HSD) or supplemented with CO (SS + HSD + CO), HYD (SS + HSD + HYD), or FO (SS + HSD + FO) as indicated. Histological sections of renal tissue were stained with Sirius red or Masson’s trichrome; staining was quantitated, relative to cortical surface area, with the MetaVue Image Analysis System. Values are means ± SE (n = 10). #P < 0.05 vs. SR + HSD; *P < 0.05 vs. SS + HSD; §P < 0.05 vs. SS + HSD + CO; %P < 0.05 vs. SS + HSD + HYD. Images are representative trichrome stains.
FO reduces COX-2 production in SS + HSD rats. In vitro studies indicate that NF-κB activates the COX pathway, leading to the production of proinflammatory mediators including prostaglandins, leukotrienes, and HETEs (81). We therefore sought to test the hypothesis that the FO-mediated reduction in NF-κB activation is associated with reduced COX-2 expression, as assessed by Western blot analysis of microsomal preparations of the rat renal cortex. As expected, COX-2 was reduced in hypertensive SS + HSD rats. FO, but not CO, prevented the induction of COX-2 (SS + HSD + CO vs. SS + HSD + FO; Fig. 7C).

FO prevents membrane translocation of the NADPH oxidase components p47phox and p67phox in SS + HSD rats. The development of hypertension in SS + HSD rats is associated with increased NADPH oxidase activity and oxidative stress. To test the hypothesis that FO prevents the membrane translocation of the NADPH oxidase subunits p47phox and p67phox, we compared the relative abundance of each in renal cortical membranes from SS + HSD vs. SS + HSD + CO and SS + HSD + FO rats by Western blot analysis. The development of hypertension in SS + HSD rats was associated with the membrane translocation of both subunits (SS + HSD + FO vs. SS + HSD + CO; Fig. 8). FO, but not CO, significantly decreased the translocation of both subunits (SS + HSD + FO vs. SS + HSD + CO; Fig. 8).

FO does not induce HO-1 expression in SS + HSD rats. HO-1, an inducible isozyme of HO, has been shown to have potent anti-inflammatory effects in a variety of injury models (5, 73). We therefore sought to determine whether the protective effect of FO in SS + HSD rats is associated with the induction of HO-1, as assessed by Western blot analysis of microsomal preparations of the rat renal cortex. We found that the level of HO-1 expression appeared to reflect the extent of renal damage, in that the highest level of HO-1 expression was observed in SS + HSD and SS + HSD + CO rats and was reduced in SS + HSD + FO rats (Fig. 9).

**DISCUSSION**

Although a number of in vitro studies have identified several pathways through which FO may prevent the development of renal disease, the in vivo relevance of these observations has not been established. The primary objective of this study was to determine whether pathways modulated by FO in vitro may provide a mechanistic basis for its renoprotective effect in SS hypertension. In accordance with previous studies in hypertensive rats (6, 50, 91), we found that FO partially reduces blood pressure, improves lipid profiles, and decreases proteinuria.

In human studies, the reduction of blood pressure is thought to underlie at least some of the cardioprotective effects of FO (66, 68). We found that FO partially but not completely reduced the elevated blood pressure that develops in SS + HSD rats. The reduction of blood pressure observed in this study was associated with decreased left ventricular hypertrophy, as assessed by heart weight. Similar effects of FO on left ventricular hypertrophy have been identified in SHR rats (99). EPA and DHA, the predominant fatty acid constituents in FO, exert vasorelaxant effects in aortas from hypertensive animals (26, 27) through a nitric oxide-independent mechanism that involves the modulation of intracellular calcium release (25, 67).

Although FO reduced blood pressure in Dahl SS rats fed the HSD, the blood pressure remained significantly elevated compared...
FISH OIL AMELIORATES HYPERTENSIVE RENAL INJURY

A

SR+HSD  SS+HSD  SS+HSD+CO  SS+HSD+FO

B

PCNA

GAPDH

C

PCNA

GAPDH

D

pERK

ERK

Relative to SS+NSD

Relative to SS+HSD

Relative to SR+HSD
pared with that of the SR controls. This incomplete reduction of blood pressure has been observed in SHRs treated with DHA (50, 86). Additionally, SHRs treated with menhaden oil for 12 wk showed less renal fibrosis but no differences in systolic blood pressure (91). Although HYD was more effective than FO in reducing blood pressure in SS/HSD rats, this treatment failed to prevent the development of glomerular, interstitial, and vascular alterations that are characteristic of SS/HSD rats. Based on these considerations, we propose that the protective effects of FO in SS/HSD rats are largely independent of blood pressure reduction.

We demonstrate that FO significantly reduces serum cholesterol and triglyceride levels in SS/HSD rats. Similar lipid-lowering effects of FO have been reported in SHRs (50, 91, 99). HYD treatment had no effect on serum lipid levels. Lipids are recognized risk factors for the development of progressive renal disease (80). It is thought that the reduction of serum lipid levels is one of the mechanisms underlying the cardioprotective effects of FO in humans (68). A meta-analysis of 21 clinical trials has shown that FO promotes a dose-dependent beneficial effect on serum triglycerides (8). Studies indicate that FO lowers serum triglyceride levels through the suppres-
expression of α-SMA is a marker of epithelial-to-mesenchymal transformation and a marker of the profibrotic state (31, 47, 55). The tubulointerstitial expression of α-SMA was significantly reduced in SS + HSD + FO rats. By quantitative analysis of trichrome- and Sirius red-stained slides, we demonstrate that FO significantly reduces interstitial fibrosis. Arteriopathic alterations, characterized by vascular smooth muscle hyperplasia and luminal constriction, are characteristic histopathological features of renovascular hypertension in Dahl SS rats and in SHRs (88, 97, 101, 106). We found that FO reduced vascular fibromuscular hyperplasia in SS + HSD rats, as evidenced by an increased lumen-to-wall thickness ratio of interlobular-sized arteries and arterioles.

Although we and others (6, 44, 62, 91) have shown that FO decreases the development of renal injury in Dahl SS + HSD rats, relatively few studies have addressed potential signaling pathways whereby FO confers a protective effect. In vitro studies of cultured cells treated with DHA or EPA have provided evidence for several potential signaling pathways. We have previously demonstrated that low-dose DHA inhibits the proliferation of renal cells in vitro through the inhibition of growth factor-stimulated ERK activity and suppression of cyclin E kinase activity (107). In the anti-rat thymocyte serum model of mesangial proliferative glomerulonephritis, we have previously demonstrated that FO suppresses the mesangial proliferative response to injury (32).

In hypertensive SS + HSD and SS + HSD + CO rats, we demonstrate that the tubulointerstitial expression of the proliferation marker Mib-1 is markedly increased. Tubulointerstitial Mib-1 expression was significantly decreased in SS + HSD + FO rats. Furthermore, the expression of the proliferation marker PCNA in cortical homogenates was also increased in SS + HSD and SS + HSD + CO rats, whereas FO significantly reduced PCNA expression to levels observed in SR + HSD rats. In SHRs (6, 44, 62, 91), we demonstrate that FO prevents the development of chronic glomerular, tubulointerstitial, and vascular alterations in SS + HSD rats. These extensive histopathological lesions were also observed in rats treated with HYD to lower blood pressure (SS + HSD + HYD). We found that FO prevented the development of glomerular hypertrophy and segmental sclerosis. Similar effects of FO on glomerular hypertrophy have been reported in SHRs (6). Tubulointerstitial lesions were also observed in rats treated with HYD to lower blood pressure (SS + HSD + HYD). We found that FO prevented the development of glomerular hypertrophy and segmental sclerosis. Similar effects of FO on glomerular hypertrophy have been reported in SHRs (6). Tubulointerstitial
HSD rats. We found that the antiproliferative effect of FO in SS + HSD rats was associated with a significant inhibition of ERK activity. In SS + HSD rats, the development of hypertension is associated with chronic ERK activation (37, 77). The effect of FO on ERK activity in Dahl SS rats has not been previously defined. However, the ANG II type 1 receptor antagonist candesartan decreased ERK activity and renal injury in Dahl SS rats through a mechanism that appeared to be independent of blood pressure reduction (77). In a murine model of type 2 diabetes, EPA ameliorates renal damage through the suppression of the proinflammatory cytokine monocyte chemoattractant protein-1 and ERK (36). In the RAW 264.7 murine macrophage cell line, FO reduced LPS-stimulated TNF-α production through the inhibition of ERK activity (56). These studies indicate that FO-mediated inhibition of ERK activity may lead to the suppression of renal proliferation as well as inflammation in Dahl SS rats.

We found that interstitial inflammation was significantly reduced in SS + HSD + FO rats, as assessed by immunostaining for the macrophage marker ED1. Both in vitro studies of cultured cells and ex vivo studies of leukocytes have shown that DHA and/or EPA decreases the production of proinflammatory cytokines (2, 11, 13, 18, 24, 41, 48, 102). These studies have provided the rationale for clinical trials of FO in patients with a variety of inflammatory/autoimmune diseases (13, 38, 75). Of note, the reduction of interstitial inflammation by mycothenol molefil alone significantly reduced blood pressure in Dahl SS rats (61).

The activation of NF-κB may play an important role in mediating interstitial monocyte infiltration (84) and may thereby provide an important target of FO in the treatment of glomerulonephritis (57). The development of hypertension in Dahl SS rats is associated with a marked upregulation of glomerulonephritis (57). We found that NF-κB activation was significantly suppressed in SS + HSD + FO rats, as evident by the decreased NF-κB binding of nuclear extracts subjected to EMSA. The relevance of this observation is underscored by a study of adriamycin nephrosis, in which the inhibition of NF-κB with pyrrolidine dithiocarbamate reduced interstitial inflammation and the extent of renal injury (84). Our findings are in accord with a number of in vitro studies, which demonstrate that FO decreases the production of proinflammatory cytokines through the downregulation of NF-κB signaling (11, 54, 79, 108). Sodium directly stimulates NF-κB activation in cultured renal proximal tubular epithelial cells (35). In cultured endothelial cells (60), rat vascular smooth muscle cells (11), and renal tubular epithelial cells (54), the EPA- and/or DHA-mediated decrease in NF-κB signaling appears to occur through the inhibition of ERK activity. Based on these considerations, we hypothesize that FO decreases NF-κB activation and renal inflammation through the downregulation of ERK. However, we cannot exclude the possibility that the effect of FO on NF-κB activation is due to reduced inflammation in SS + HSD + FO rats.

In Dahl SS rats, we demonstrate that FO significantly decreases COX-2 expression. It is well recognized that arachidonic acid metabolites, produced through the action of COX-2, lead to the production of a variety of metabolites, which promote inflammation and increase vascular tone. In SHRs, the development of hypertension is associated with the increased production of the COX-2 product thromboxane A₂, a potent vasoconstrictor. In this model, FO reduces blood pressure and vascular reactivity (87, 105). In Dahl SS rats, the COX-2 inhibitor celecoxib reduces interstitial inflammation and histopathological manifestations of renal injury (42). In vitro studies indicate that FO, in addition to competing with arachidonic acid as a substrate for COX, can directly suppress COX-2 induction (60). The inhibitory effect of DHA on COX-2 expression was due to decreased NF-κB activation, as assessed by EMSA, and decreased binding of NF-κB to the COX-2 promoter (60). In this study, DHA decreased NF-κB activation by decreasing cytokine-stimulated reactive oxygen species generation and ERK activation through effects on NADPH oxidase activity (60).

We found that FO prevented the assembly of the NADPH oxidase complex, as assessed by the decreased membrane translocation of the NADPH oxidase subunits p47phox and p67phox in hypertensive SS + HSD + FO rats. Other investigators have shown that FO decreases lipid peroxidation and enhances antioxidant status in SHRs (29). The development of hypertension in SS rats is associated with increased oxidative stress (9, 52, 65, 96) and increased NADPH oxidase-mediated superoxide production (3, 40). The importance of NADPH oxidase activation in the development of hypertension is underscored by studies employing p47phox (−/−) mice. In these animals, ANG II infusion fails to elicit hypertension and superoxide generation is markedly attenuated (53). The antioxidant α-tocopherol prevents the development of hypertension and renal dysfunction in SS rats (7, 28, 96). The superoxide dismutase mimetic tempol reduces glomerular injury (43, 63) and MAPK activity in Dahl SS rats (76). DHA attenuates the development of hypertension by decreasing NADPH oxidase activity in rats infused with ANG II (20). Based on these observations, we propose that FO ameliorates renal injury in SS hypertension at least in part by inhibiting the assembly of NADPH oxidase subunits. However, we cannot exclude the possibility that the effect of FO on the membrane translocation of p47phox and p67phox in SS + HSD rats is due to the fact that FO prevents the influx of circulating inflammatory cells into the kidney. Further studies are needed to address this issue.

HO has been shown to have a number of critical antioxidant, anti-inflammatory, and cytoprotective functions (5, 71–73). Induction of HO-1 occurs as an adaptive response to a variety of injurious stimuli, including ischemia, cytokines, and oxidative stress (1, 4, 5, 71–74). We found that HO-1 expression was decreased in SS + HSD + FO compared with SS + HSD or SS + HSD + CO rats. The lack of induction of HO-1 in FO-treated rats suggests that FO does not directly regulate HO-1 expression and that the level of HO-1 expression in SS + HSD rats reflects the underlying extent of renal injury.

In summary, we demonstrate that FO improves lipid profiles and reduces histopathological manifestations of renal injury in SS hypertensive rats. This effect appears to be largely independent of decreases in blood pressure in SS + HSD + FO rats. We have identified several potential targets that underlie these therapeutic effects of FO in preventing renal damage. FO markedly decreases the proliferative response to renal injury through the downregulation of ERK activity. Complementary in vitro studies previously performed in our laboratory demonstrate that the FO-mediated decrease in ERK activity is associated with the reduction in cyclin E kinase activity (107).
FO exerts potent anti-inflammatory effects through the down-regulation of NF-κB. This reduction in NF-κB signaling may underlie the reduction in COX-2 expression, since the proximal promoter region of the COX-2 gene contains an NF-κB binding site (60). Finally, FO inhibits the assembly of NADPH oxidase subunits (p47phox and p67phox), an effect that may underlie the previously reported antioxidant effects of FO in renal injury models (20, 29). The FO-mediated modulation of these pathways may underlie the reported protective effects of FO in other experimental renal disease models and in human renal disease.

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