Omega-3 fatty acid supplementation attenuates oxidative stress, inflammation, and tubulointerstitial fibrosis in the remnant kidney

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Omega-3 fatty acid supplementation attenuates oxidative stress, inflammation, and tubulointerstitial fibrosis in the remnant kidney. Am J Physiol Renal Physiol 297: F895–F903, 2009. First published August 5, 2009; doi:10.1152/ajprenal.00217.2009.—Significant reduction of renal mass initiates a series of hemodynamic and nonhemodynamic events which lead to proteinuria, glomerulosclerosis, tubulointerstitial injury, and end-stage renal failure. Lipid mediators derived from fatty acids participate in regulation of renal hemodynamic and nonhemodynamic processes that influence progression of renal disease. Composition of cellular fatty acids and hence related signaling responses are influenced by their dietary contents. Consumption of omega-3 fatty acids (O-3FA) has proven effective in mitigating atherosclerosis. We tested the hypothesis that O-3FA supplementation may retard progression and attenuate upregulation of pathways involved in oxidative stress, inflammation, and fibrosis in rats with renal mass reduction. Sprague-Dawley rats were subjected to 5/6 nephrectomy [chronic renal failure (CRF)] and randomly assigned to the untreated and O-3FA-treated (0.3 g · kg−1 · day−1 by gastric gavage for 12 wk) groups. Sham-operated rats served as controls. The untreated CRF rats exhibited proteinuria, hypertension, azotemia, upregulations of renal tissue NAD(P)H oxidase, MCP-1, PAI-1, TGF-β, Smad2, α-smooth muscle actin, fibronectin, and hepatocyte growth factor, activation of ERK1/2 and NF-κB, downregulation of Smad7, intense mononuclear leukocyte infiltration, tubulointerstitial fibrosis, and glomerulosclerosis. O-3FA supplementation significantly lowered COX-2, NAD(P)H oxidase (NOX-4, gp91phox, p47phox, p22phos), PAI-1, TGF-β, connective tissue growth factor, α-smooth muscle actin, fibronectin, Smad2, and MCP-1, raised Smad7, and attenuated ERK1/2 and NF-κB activation, tubulointerstitial fibrosis, and inflammation. Thus, long-term O-3FA supplementation can reduce or reverse upregulation of prooxidant, proinflammatory, and profibrotic pathways and attenuate tubulointerstitial fibrosis in the remnant kidney.

chronic kidney disease; NAD(P)H oxidase; TGF-β; Smad; epithelial-mesenchymal transition; MAP Kinase

Lipid mediators formed from fatty acids play an important role in regulation of renal hemodynamic and nonhemodynamic processes and as such their modifications may influence progression of renal disease. Arachidonic acid-derived eicosanoids including prostanooids participate in both hemodynamic and inflammatory events in the kidney (13, 16). Omega-3 fatty acid (O-3FA) supplementation has been shown to lower cell membrane content of arachidonic acid whose metabolites participate in many inflammatory, oxidative, and thrombotic phenomena and raise those of docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) whose metabolites attenuate these processes (7, 10). In fact, O-3FA supplementation has been used in the treatment/prevention of cardiovascular disease and IgA nephropathy among other disorders (9, 39).

Available data on the effect and the potential mechanisms of action of O-3FA supplementation on progression of chronic kidney disease are limited and somewhat contradictory. In an earlier study, Clark et al. (6) showed that dietary O-3FA supplementation significantly reduced renal tissue incorporation of arachidonic acid, increased incorporation of EPA and DHA, and limited glomerulosclerosis and macrophage infiltration in 5/6 nephrectomized rats. In addition, EPA administration was shown to attenuate oxidative stress, lower TGF-β abundance, and ameliorate tubulointerstitial fibrosis and albuminuria in KKAy/Ta mice with type 2 diabetic nephropathy (15, 40). Moreover, EPA was shown to suppress phosphorylation of ERK1/2 in cultured murine mesangial cells and in the mice with diabetic nephropathy (40). Similarly, Barcelli et al. (2) reported favorable effects of fish oil on progression of renal disease and kidney histology in rats with renal mass reduction. In contrast, Scharshmidt et al. (35) found decreased glomerular filtration rates, reduced filtration fraction, increased proteinuria, and greater glomerular sclerosis with fish oil supplementation in subtotal nephrectomized rats.

In view of the above observations, the present study was undertaken to determine the effect of chronic (12 wk) O-3FA supplementation on renal function and structure and expression of key molecules involved in the pathogenesis of inflammation, oxidative stress, and fibrosis in an animal model of chronic renal disease induced by renal mass reduction.

MATERIALS AND METHODS

Study groups. Male Sprague-Dawley rats with an average body weight of 311 g (Harlan Sprague-Dawley, Indianapolis, IN) were used in this study. Animals were housed in a climate-controlled vivarium with 12-h day and night cycles and were fed a standard laboratory diet (Purina Rat Chow; Purina Mills, Brentwood, MO) and water ad libitum. The animals were randomly assigned to the chronic renal failure (CRF) group, O-3FA-treated CRF group, and sham-operated normal control group. Six animals were used in each group. The CRF groups underwent 5/6 nephrectomy by surgical resection of the upper
and lower thirds of left kidney, followed by right nephrectomy 5 days later. The control group underwent sham operation. The procedures were carried out under general anesthesia using intraperitoneal injection of a solution containing ketamine (50 mg/kg), xylazine (4 mg/kg), and acepromazine maleate (1 mg/kg).

The CRF rats assigned to the O-3FA-treated group were given 0.3 g·kg⁻¹·day⁻¹ O-3FA by gastric gavage for 12 wk using a Gastright micro-syringe (Gastight, Hamilton, Reno, NV). The given dose was chosen because it was proven effective in improving insulin resistance in Otsuka Long-Evans Tokushima Fatty rats (24) and Dhahlsalt-sensitive rats (26). OmegaBrite (Omega Natural Science, Waltham, MA) containing the EPA-to-DHA ratio of 7:1, 90% pure O-3FA was used. O-3FA supplementation was initiated 7 days after 5/6 nephrectomy.

At the conclusion of the 12-wk treatment period, animals were placed in individual metabolic cages for a 24-h urine collection and measurement of food and water intake. The animals were then anesthetized (50 mg/kg ip pentobarbital sodium injection) and euthanized by exsanguination using cardiac puncture. The kidney was immediately harvested, a section was separated and fixed in 10% formalin, 4% paraformaldehyde, and the remainder was cleaned with PBS, snap-frozen in liquid nitrogen, and stored at −70°C until processed. The experimental protocol was approved by the Institutional Animal Care and Use Committee of the University of California (Irvine, CA).

**Measurement of arterial pressure.** Blood pressure was noninvasively measured by a volume pressure recording sensor and an occlusion tail-cuff (CODA System, Kent Scientific, Torrington, CT). In brief, the conscious animal was placed in a restrainer and permitted to rest for 10 to 15 min. The cuff was then placed on the tail and was inflated and released several times to condition the animal to the procedure. After stabilization, blood pressure was measured three times, and the average of the recorded values was used.

**Blood and urine chemistries.** Urine and plasma creatinine was measured with a Quantichrom creatinine assay kit (Bioassay Systems, Hayward, CA). Blood urea nitrogen (Bioassay Systems), total cholesterol (Wako Chemicals USA, Richmond, VA), triglyceride (Stanbio Laboratory, Boerne, TX), high-density lipoprotein (HDL), cholesterol, and glucose (Wako Chemicals USA) were measured using kits purchased from the specified manufacturers. Urine protein was measured with the rat urinary protein assay kit (Chondrex, Redmond, WA). Plasma insulin level was determined using a kit purchased from Linco Diagnostics (St. Charles, MO). Plasma monocyte chemotactic protein-1 (MCP-1) was determined using a kit obtained from Millipore (St. Charles, MO). Creatinine, urea nitrogen, and total cholesterol concentrations, albumin was used as a standard.

**Histology and immunohistology.** Light microscopy was performed in the formalin-fixed sections stained with periodic acid-Schiff and hematoxylin and eosin. Glomerulosclerosis was graded by a score index used in our previous studies (30): glomeruli were graded from 0 to 4 (grade 0 = normal, grade 1 = <25% involvement of the glomerular tuft, grade 2 = 25−50% involvement of the glomerular tuft, grade 3 = 50−75% involvement of the glomerular tuft, and grade 4 = sclerosis occupying >75% of the glomerular tuft). The glomerulosclerosis score was obtained as follows: [(1 × number of glomeruli with grade 1) + (2 × number of glomeruli with grade 2) + (3 × number of glomeruli with grade 3) + (4 × number of glomeruli with grade 4)] × 100/total number of glomeruli examined.

**Statistical analysis.** Data are expressed as means ± SE. ANOVA and Tukey posttests for multiple groups were used in statistical evaluation of the data using SPSS software version 12.0 (SPSS, Chicago, IL). P values <0.05 were considered significant.

**RESULTS**

**General data.** Results obtained at 12 wk after 5/6 nephrectomy are summarized in Table 1. Compared with the control group, the untreated CRF group and O-3FA-supplemented CRF group exhibited significantly elevated systolic arterial pressure, plasma creatinine, urea nitrogen, and total cholesterol concentrations, increased urinary protein excretion, and reduced body weight, creatinine clearance, and HDL-cholesterol-to-total cholesterol ratio. Triglyceride level was not significantly different among control group and O-3FA-supplemented CRF groups. Plasma
12-wk observation period

Table 1. General data obtained at the conclusion of the 12-wk observation period

<table>
<thead>
<tr>
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<th>CTL (n = 6)</th>
<th>CRF (n = 6)</th>
<th>CRF-OM (n = 6)</th>
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<tr>
<td>Body wt, g</td>
<td>459.8±8.8</td>
<td>405.9±21.0*</td>
<td>406.0±7.6*</td>
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<td>SBP, mmHg</td>
<td>123.5±6.0</td>
<td>168.8±2.0*</td>
<td>153.7±8.4*</td>
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<td>BUN, mg/dl</td>
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<td>53.6±2.2*</td>
<td>52.4±4.8*</td>
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<td>Plasma Cr, mg/dl</td>
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<td>1.56±0.23*</td>
<td>1.46±0.28*</td>
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<tr>
<td>Proteinuria, mg/24 h</td>
<td>6.7±0.6</td>
<td>80.3±3.7*</td>
<td>76.4±7.7*</td>
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<td>Cr clearance, ml/min</td>
<td>5.62±0.53</td>
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<td>Total cholesterol, mg/dl</td>
<td>71.2±4.0</td>
<td>221.2±10.3*</td>
<td>195.2±25.1*</td>
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<tr>
<td>Triglyceride, mg/dl</td>
<td>45.8±8.2</td>
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<td>HDL/total cholesterol</td>
<td>0.69±0.03</td>
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<td>Plasma glucose, mg/dl</td>
<td>169.10±26.80</td>
<td>168.05±37.87</td>
<td>168.15±26.17</td>
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<tr>
<td>Plasma insulin, ng/ml</td>
<td>1.03±0.45</td>
<td>6.65±2.35*</td>
<td>1.55±0.74†</td>
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<td>HOMA-IR index</td>
<td>0.42±0.17</td>
<td>2.87±0.92*</td>
<td>0.72±0.40†</td>
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</table>

Data are expressed as means ± SE. CTL, sham-operated control; CRF, 5/6 nephrectomized rat; CRF-OM, chronic renal failure omega-3 fatty acid supplementation; SBP, systolic blood pressure; BUN, blood urea nitrogen; Cr, creatinine; HDL, high-density lipoprotein; HOMA-IR, homeostasis model assessment-insulin resistance index. *P < 0.05 vs. control group. †P < 0.05 vs. CRF group.

glucose concentration was comparable among the study groups. However, plasma insulin concentration was significantly elevated and HOMA-IR index was markedly increased pointing to significant insulin resistance in the CRF group. O-3FA supplementation significantly reduced plasma insulin concentration was significantly higher in the untreated CRF rats and marked improved HOMA-IR index. Plasma MCP-1 concentration was significantly elevated and HOMA-IR index was markedly increased in the untreated CRF group (Fig. 1).

**NF-κB, COX-2, MCP-1, and PAI-1 data.** Compared with the control group, the untreated CRF group showed significant upregulation of NF-κB, COX-2, MCP-1, and PAI-1 in the remnant kidneys. O-3FA supplementation significantly attenuated upregulations of COX-2, MCP-1, PAI-1, and NF-κB in the treated CRF group (Fig. 2).

**TGF-β and α-SMA data.** Data are illustrated in Figs. 3 and 4. The untreated CRF group showed significant upregulation of TGF-β and α-SMA in the remnant kidneys. O-3FA supplementation significantly attenuated upregulations of TGF-β and α-SMA in the treated CRF group.

**HGF, CTGF, and fibronectin data.** Compared with the control group, the untreated CRF group exhibited significant upregulation of HGF and fibronectin with no change in CTGF abundance in the remnant kidneys (Fig. 5). O-3FA supplementation attenuated upregulation of HGF and fibronectin and lowered CTGF abundance in the remnant kidneys.

**Smad2, Smad7, and ERK1/2 data.** The untreated CRF group exhibited significant upregulation of Smad2 and downregulation of Smad7 in the remnant kidneys (Fig. 6). O-3FA supplementation significantly attenuated upregulation of Smad2 and partially restored Smad7 abundance in the remnant kidney. Compared with the control group, the untreated CRF group showed a significant increase in the ratio of phospho-ERK1/2 to ERK1/2 in the remnant kidney. O-3FA supplementation...
significantly lowered phospho-ERK1/2-to-ERK1/2 ratio in the remnant kidneys (Fig. 7).

**Renal histology.** Data are shown in Fig. 8. As expected, the remnant kidneys in the untreated CRF rats showed significant glomerulosclerosis, mononuclear leukocyte infiltration (untreated CRF vs. sham-operated control, 78.8 ± 3.8 vs. 23.1 ± 1.9 positive cells/mm²), tubular atrophy, tubulointerstitial injury, and fibrosis. O-3FA supplementation significantly attenuated the severity of tubulointerstitial abnormalities and lowered tubulointerstitial mononuclear cell infiltration (50.4 ± 11.7 cells/mm²) but did not significantly affect glomerulosclerosis index in the remnant kidney.

**DISCUSSION**

The CRF animals exhibited hypertension, proteinuria, azotemia, glomerulosclerosis, tubulointerstitial fibrosis, and heavy mononuclear leukocyte infiltration in the remnant kidney. This was
associated with upregulation of oxidative, inflammatory, and fibrotic pathways. In confirmation of earlier studies (36, 37), the remnant kidney in the untreated CRF animals showed marked upregulation of prototypical phagocytic and tissue-specific NAD(P)H oxidase isoforms which are a major source of reactive oxygen species and oxidative stress in the kidney and cardiovascular tissues. As demonstrated in previous studies (12, 36), the untreated CRF rats exhibited significant activation of NF-κB in the remnant kidney as evidenced by increased nuclear translocation of p65, the active subunit of this transcription factor. NF-κB is the general transcription factor for a slew of proinflammatory cytokines and chemokines and as such its activation plays a major role in the pathogenesis of inflammation, tissue injury, and progression of renal disease. This assertion is supported by the observation that pharmacological inhibition of NF-κB activation can retard progression of renal disease in 5/6 nephrectomized rats (12). Activation of NF-κB was associated with upregulation of MCP-1 which is critical for migration and infiltration of circulating monocytes in the target tissue. These events can account for heavy interstitial mononuclear leukocyte infiltration seen in the remnant kidney of our untreated CRF group.

In confirmation of previous reports (11, 13, 36), the remnant kidneys in our untreated CRF animals showed significant upregulation of COX-2. COX-derived products can contribute to oxidative stress and inflammation and upregulation of COX-2 can play an important role in the pathogenesis of progressive nephropathies in rats following 5/6 nephrectomy (11, 13).

TGF-β1 is a well-known profibrotic cytokine which is markedly upregulated in the remnant kidney (3, 19). Binding of TGF-β to its receptor leads to activation of downstream signal transducers, Smad2 and Smad3 (38), which mediate fibrosis by stimulating extracellular matrix (ECM) production, inhibiting ECM degradation (upregulation of metalloproteinase inhibitors), and inducing tubular epithelial and mesenchymal cell transformation to myofibroblasts (4). CTGF is a downstream mediator of TGF-β which modulates renal fibrosis and EMT in progressive kidney disease (4, 14). Besides TGF-β, several other mediators can independently promote fibrosis by activating Smad2/3. For instance, advanced glycation end products and angiotensin II can activate Smad2/3 via the ERK/p38 MAP kinase-dependent crosstalk pathway (23, 24, 31). These observations illustrate the role of Smads as signal integrators,
forming the crosstalk pathways among diverse fibrogenic systems. The profibrogenic effects of Smad2 and 3 are counteracted by Smad7 which is an inhibitory Smad induced by TGF-β (38). Smad7 exerts its negative feedback regulatory function by inhibiting TGF-β-mediated phosphorylation of Smad2/3 and targeting TGF-β receptors for proteasomal degradation via recruitment of the ubiquitin ligases, Smurf-1 and Smurf-2 (20). In addition to TGF-β, activation of signal transducer and activator of T cell 1 (STAT1) pathway by interferon-γ, interleukin-7, or TNF-α-activated NF-κB can induce Smad7 (1).

The untreated CRF rats employed in the present study exhibited marked tubulointerstitial fibrosis, glomerulosclerosis, fibronectin accumulation, and increased α-SMA expression. This was associated with marked upregulations of TGF-β and Smad 2, and significant downregulation of Smad7. As illustrated in Fig. 9, these events can work in concert to promote progressive tubulointerstitial fibrosis and glomerulosclerosis in the remnant kidney. It is of interest that HGF abundance was significantly increased in the remnant kidneys of our untreated CRF rats. HGF has been reported to exert antifibrotic action by counteracting TGF-β and attenuating CTGF induction in the 5/6 nephrectomized animals (19). Consequently upregulation of HGF in the remnant kidney represents a compensatory response to the prevailing profibrotic events.

Earlier studies showed marked upregulation of intrarenal angiotensin II/AT1 receptor system in the remnant kidney in 5/6 nephrectomized rats (13, 36). As noted above, angiotensin II can directly activate Smad2 and Smad3 via the ERK/p38 MAP kinase signaling pathway (31). In fact, our untreated CRF rats exhibited activation of ERK1/2 as evidenced by a marked increase in phospho-ERK1/2 in the remnant kidney.

Activation of the profibrotic pathways in our untreated CRF rats was compounded by marked upregulation of PAI-1 in the remnant kidney. PAI-1 is a prominently expressed TGF-β1-induced transcript which blocks conversion of plasminogen to plasmin by inhibiting tissue plasminogen activator (33). Plasmin is a potent antifibrotic metalloproteinase that catalyzes proteolytic degradation of ECM proteins. Consequently, by limiting plasmin production, upregulation of PAI-1 must have contributed to the observed ECM accumulation and tubulointerstitial fibrosis in the study animals.

O-3FA supplementation resulted in significant attenuation of renal tubulointerstitial injury and fibrosis in the remnant kidney. This was associated with significant reduction of TGF-β, Smad2, CTGF, α-SMA, PAI-1, and phosphorylated ERK1/2, and partial restoration of Smad7 abundance. It thus appears that O-3FA supplementation may attenuate tubulointerstitial fibrosis by restraining both TGF-β-dependent and ERK1/2 MAP kinase-dependent fibrogenic signaling pathways in this model. Amelioration of renal interstitial fibrosis with O-3FA supplementation in rats with renal mass reduction shown here is consistent with the findings recently reported in animal models of diabetic nephropathy and salt-sensitive hypertension treated with EPA or fish oil (8, 15, 25). In this context, plasma insulin concentration was significantly elevated and HOMA-IR index was markedly increased in the untreated CRF animals pointing to presence of insulin resistance. O-3FA administration resulted in significant reduction of plasma insulin and improvement of HOMA-IR index.

O-3FA supplementation significantly attenuated CRF-induced upregulation of oxidative and inflammatory pathways in the remnant kidney. For instance, O-3FA supplementation reversed or attenuated upregulation of NOX-4, gp91phox, p47phox, p22phox, MCP-1, and COX-2 expressions and lowered NF-κB activation in the remnant kidney.

Consumption of O-3FA has been shown to result in a rise in EPA and DHA and a reciprocal fall in omega-6 fatty acid content of plasma membrane (17). These observations suggest that the exogenous O-3FA can compete with the omega-6 fatty acids (e.g., arachidonic acid) for incorporation in the cell membrane. Arachidonic acid plays a major role in signal
transduction pathways involved in inflammation, reactive oxygen species generation, cell proliferation, and ECM production. In addition, arachidonic acid is converted by cyclooxygenase, lipoxygenase, or cytochrome P-450 AA monoxygenase to a series of potent eicosanoids such as thromboxane A2 and leukotriene A4, B4, and D4 which are highly proinflammatory, prooxidant, and prothrombotic. In contrast, interaction of these enzymes with O-3FA results in formation of less inflammatory or anti-inflammatory products. It therefore appears that the anti-inflammatory/antioxidant effects of O-3FA supplementation shown in this and other studies may be, in part, mediated by relative limitation of arachidonic acid availability for participation in the related signal transduction and enzymatic pathways. In fact, O-3FA supplementation appears to lower markers of inflammation in patients with end-stage renal disease and reduce cardiac arrhythmia and sudden death in the general population (22, 32).

O-3FA supplementation did not significantly alter arterial pressure, creatinine clearance, urinary protein excretion, or glomerulosclerosis. The reason for the favorable effect of O-3FA supplementation on progression of tubulointerstitial injury and lack thereof on glomerulosclerosis is not entirely clear. However, induction of glomerulosclerosis by renal mass reduction is heavily driven by maladaptive hemodynamic events including glomerular capillary hypertension and hyperfiltration. In contrast, EMT, oxidative stress, and inflammation are the driving forces in the pathogenesis of tubulointerstitial injury. As noted above, O-3FA supplementation did not significantly affect arterial pressure or creatinine clearance, whereas it significantly suppressed oxidative and inflammatory pathways and lowered CTGF and α-SMA.

The precise mechanism by which O-3FA supplementation attenuated upregulation of profibrotic pathways and mitigated tubulointerstitial fibrosis in the remnant kidney is not known and requires further investigation. Amelioration of tubulointerstitial fibrosis and attenuation of profibrotic pathways with long-term O-3FA supplementation in this model were accompanied by significant reversal of upregulation of the inflammatory and oxidative pathways. It is of note that inflammation and its constant companion oxidative stress are powerful promoters of tissue fibrosis. Therefore, the observed attenuation of tissue fibrosis and the related pathways in the treated CRF animals must be, at least in part, due to the anti-inflammatory actions of O-3FA. In fact, some of the effects seen with O-3FA have been shown in experiments using a variety of antioxidant/anti-inflammatory agents. For instance, in a recent study we found significant reduction of TGF-β, PAI-1, and matrix protein accumulation with long-term administration of niacin which possesses strong antioxidant and anti-inflammatory properties.
(5) Similarly pharmacological inhibition of NF-κB and (13) and long-term administration of melatonin (28) which possesses potent antioxidant/anti-inflammatory properties have been shown to reduce interstitial fibrosis and retard progression of renal disease in this model. Thus, the seemingly diverse effects of O-3FA supplementation seen in the remnant kidney could be, in part, mediated by a combination of its antioxidant, anti-inflammatory actions. It is of note that in the present study O-3FA supplementation was initiated 1 wk after 5/6 nephrectomy. Further studies are required to determine whether initiation of therapy later in the course of the disease would be equally effective.

In conclusion, long-term O-3FA supplementation attenuates tubulointerstitial injury in animals with chronic renal disease by mitigating oxidative stress, inflammation, fibrosis, and EMT. These observations point to the potential role of O-3FA supplementation as an adjunct in the therapeutic strategies aimed at retarding chronic kidney disease progression.

GRANTS

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