Acute Changes in Dietary ω-3 and ω-6 Polyunsaturated Fatty Acids Have a Pronounced Impact on Survival following Ischemic Renal Injury and Formation of Renoprotective Docosahexaenoic Acid-Derived Protectin D1

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Acute Changes in Dietary ω-3 and ω-6 Polyunsaturated Fatty Acids Have a Pronounced Impact on Survival following Ischemic Renal Injury and Formation of Renoprotective Docosahexaenoic Acid-Derived Protectin D1

Iram R. Hassan* and Karsten Gronert2*†

Exacerbated inflammation plays an important role in the pathogenesis of ischemic renal injury (IRI), which is the major cause of intrinsic acute renal failure. Clinical studies suggest that long-term treatment with ω-3 polyunsaturated fatty acids (PUFA) improves renal function and lowers the risk of death or end-stage renal disease. Docosahexaenoic acid, a principle ω-3 PUFA of fish oils, is of particular interest as it is found in most human tissues and is converted to protectin D1 (PD1), which exhibits antiinflammatory and proresolving bioactions. We set out to investigate the impact of acute dietary modulation of ω-3 or ω-6 PUFA on IRI and renal lipid autacoid circuits, using an established mouse model and liquid chromatography-mass spectroscopy/mass spectroscopy-based lipidomics. Thirty minutes of renal ischemia significantly elevated serum creatinine in the ω-3 diet group while renal function remained normal in the matched ω-3 diet group. Notably, extending ischemia to 45 min caused 100% mortality in the ω-6 group, in sharp contrast to 0% mortality in the ω-3 group. Protection against IRI in the ω-3 group correlated with decreased polymorphonuclear leukocyte recruitment, chemokine and cytokine levels, abrogated formation of lipoxigenase- and cyclooxygenase-derived eicosanoids, and increased renal levels of PD1. Systemic treatment with PD1 reduced kidney polymorphonuclear leukocyte influx and, more importantly, amplified renoprotective heme-oxygenase-1 protein and mRNA expression in injured and uninjured kidneys. These findings suggest therapeutic or dietary amplification of PD1 circuits restraints acute renal injury and that short-term changes in dietary ω-3 and ω-6 PUFA dramatically impacts renal lipid autacoid formation and outcome of IRI. The Journal of Immunology, 2009, 182: 3223–3232.

Acute renal failure (ARF) affects 5% of all hospitalized patients with a high mortality, as there is no specific therapy, despite advances in preventative strategies (1). Renal ischemia reperfusion injury is the major cause of intrinsic ARF in native kidneys, and it is associated with a mortality rate of >50% (1). The pathophysiology of ARF includes persistent intrarenal vasoconstriction, hypoxic tubule epithelial cell injury, and polymorphonuclear leukocyte (PMN)-mediated cytocytotoxicity upon reperfusion (1, 2). Ischemic renal injury (IRI) triggers downstream effects such as postischemic leukocyte recruitment, endothelial dysfunction, and tubule cell injury, which is the predominant cause of reduced glomerular filtration rate and ischemic ARF. Inflammation is a key feature of the pathophysiology of IRI (1–3), and hence it is a target in the search for new therapeutic interventions.

Lipid autacoids, such as eicosanoids, which are derived from the ω-6 polyunsaturated fatty acid (PUFA) arachidonic acid (AA, C20:4), play important roles in renal physiology and, more importantly, are some of the earliest signals triggered by injury and stress. Clinical evidence demonstrates that dietary supplementation with the ω-3 PUFA eicosapentaenoic acid (EPA, C20:5) and docosahexaenoic acid (DHA, C22:6) has beneficial effects in acute and chronic inflammatory conditions (4–7) and lowers the risk of death or end-stage renal disease in patients with glomerulonephritis (8). In particular, the ω-3 PUFA DHA, which unlike EPA is present at significant concentrations in most human tissues (9), has been shown to exert antiinflammatory and immunosuppressive effects (10, 11) and in the kidney reduces ischemic ARF in mice (12) and dogs (13). However, there is no clear mechanism to account for the protective properties of ω-3 PUFA, especially not for DHA, and the impact of acute dietary amplification of ω-3 PUFA on renal lipid autacoid circuits remains to be clearly defined.

Considerable interest has focused on novel protective DHA-derived autacoids, in particular protectin D1 (PD1), which regulates critical events in the acute inflammatory/reparative response and its resolution, and thus may provide a mechanism for the dietary requirement and antiinflammatory actions of ω-3 PUFA (14–17). 15-Lipoxigenase (15-LOX) initiates the biosynthesis of PD1 (10R,17S-dihydroxy-docosa-4Z,7Z,11E,13E,15E,19Z-hexaenoic acid), which includes the intermediate, 17S-hydro(peroxy)-DHA; the complete structure of PD1 has been assigned (18). 17S-hydro(peroxy)-DHA is

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*Sunny Medical College, Department of Pharmacology, Valhalla, NY 10595; and †University of California, Berkeley, Center for Eye Disease and Development, School of Optometry, Berkeley, CA 94720

†Address correspondence and reprint requests to Dr. Karsten Gronert, Center for Eye Disease and Development, School of Optometry, Berkeley, CA 94720-2020. E-mail: kgronert@berkeley.edu

1 Abbreviations used in this paper: ARF, acute renal failure; AA, arachidonic acid; ATLa, aspirin-triggered 15-epi-16-ρ-fluorophenoxy-LXA4; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; GC/MS, gas chromatography/mass spectroscopy; HETE, hydroxyeicosatetraenoic acid; HO, heme-oxygenase; IRI, ischemic renal injury; LC-MS/MS, liquid chromatography-mass spectroscopy/mass spectroscopy; LOX, lipoxigenase; LXA4, lipoxin A4; MPO, myeloperoxidase; PD1, protectin D1; PMN, polymorphonuclear leukocyte; PUFA, polyunsaturated fatty acid; Rv, resolvin; 17S-HDHA, 17S-hydroxy-docosa-4Z,7Z,11E,13E,15E,19Z-hexaenoic acid.

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also a substrate for the leukocyte 5-LOX and, in a mechanism analogous to lipoxin biosynthesis, forms several distinct 13-series resolvins (RvDs), which exhibit antiinflammatory actions (14, 15). Recent reports have shown that treatment with PD1 and RvDs can reduce acute kidney injury (19, 20). The impact of dietary ω-3 PUFA on the endogenous formation of DHA-derived lipid autocoids and molecular mechanisms that account for their antiinflammatory properties remain to be elucidated.

Recent in vivo studies have demonstrated that PD1 and 17-HDHA (173-hydroxy-DHA) can amplify expression of the heat-shock protein heme-oxygenase-1 (HO-1) in human corneal epithelial cells (21). HO-1 has emerged as an inducible and essential cytoprotective gene that generates antioxidant and antiinflammatory signals (22, 23). The HO-1 pathway has been shown to be renoprotective in several models of acute renal injury such as IRI, glycerol-induced renal injury, glomerulonephritis, cisplatin nephrotoxicity, and rapamycin-induced kidney injury (24–29). However, an interaction between DHA-derived autocoids and the HO-1 pathway in the kidney has not been investigated.

Herein, we report that a short-term increase in dietary ω-3 PUFA and concomitant decrease in ω-6 PUFA is protective against IRI, which correlates with a dramatic shift in the lipid autocoid profile, namely increased endogenous renal formation of PD1 and 17-HDHA and abrogated formation of eicosanoids. More importantly, systemic treatment with PD1 attenuates renal inflammation and amplifies cytoprotective HO-1 expression in injured and healthy kidneys.

Materials and Methods

Animals

All animal studies were approved by New York Medical College Vertebrate Animal Committee. Male BALB/c mice (6–8 wk) were purchased from Taconic, and male C57BL/6J stock 000664 mice (6–8 wk) were purchased from The Jackson Laboratory.

Ischemic renal injury

Mice were anesthetized by i.m. administration of ketamine/xylazine (80/12 mg/kg) and the abdominal area was shaved and wiped with an alcohol swab. The kidneys were exposed through a midline laparotomy and renal pedicles (artery and vein) were unilaterally or bilaterally occluded with microaneurism clamps (Harvard Apparatus) for 0–90 min. The mice were kept at a constant temperature (37°C) in a heated humidified chamber for the duration of ischemia. At the end of the ischemic time period, the clamp was released and the kidney was observed for 1 min to confirm reperfusion. The wound was sutured, and animals were allowed to recover. Mice were sacrificed at 24 h postischemia; blood samples were obtained via cardiac puncture, and kidneys were harvested for further analysis.

Dietary modulation of ω-3 and ω-6 PUFA

Male 23- to 28-g C57BLJ6 mice (6–8 wk) were fed ad libitum a standard rodent diet (laboratory rodent diet no. 5001; LabDiet), which contained 5 g% fat with 7% ω-3 PUFA (percentage of total fat). To assess the impact of acutely modifying ω-3 and ω-6 PUFA, mice were placed on a custom balanced rodent diet (Research Diets) containing either corn oil (AIN-76A rodent diet no. D10001, 5 g% fat with 2% ω-3 PUFA (percentage of total fat)) or fish oil (AIN-76A rodent diet no. D02032702E with menhaden oil, 5 g% fat with 28% ω-3 PUFA (percentage of total fat)) as a fat source. The ω-3-enriched diet was kept at 4°C in the dark and was replaced every 2 days. See Table I for details of composition of the custom diets. Animals were placed on the diets for 4 wk before being subjected to IRI.

Pharmacological amplification of antiinflammatory lipid autocoids

BALB/c mice (males, 23–26 g, 6–8 wk) were treated systemically with a lipoxin A₄ (LXA₄)-stable analog, PD1, 173-HDHA (173-hydroxy-docosa-4,7,11,14,17-penta-19-hexanoic acid), or vehicle alone (125 μl of sterile HBSS (pH 7.4)) via tail vein injection at a dose of 0.4 mg/kg given once before clamping of the renal pedicle, and a second dose after clamp release. All solutions for treatment were prepared immediately before the injection by removing the solvent (EtOH) from selected lipid autocoids stocks under a gentle stream of nitrogen and immediate resuspension in 125 μl of sterile HBSS. 175-HDHA and PD1 were prepared by biogenic synthesis and purified by HPLC as previously described (30, 31), and the LXA₄-stable analog (ATLa, aspirin-triggered 15-epi-16-fluorophenoxy-LXA₄) was kindly provided by Drs. John F. Parkinson and William J. Guilford (Bertlex Biosciences).

Myeloperoxidase (MPO) activity

MPO activity, an index of tissue leukocyte infiltration, was measured in 24-h postischemic kidneys using a method adapted from that of Meldrum et al. (32). In brief, kidney tissues (one-half kidney) were homogenized for 30 s in 1 ml of 20 mM potassium phosphate buffer (PPB) (pH 7.4) with a hand-held homogenizer ( Fisher PowerGen model 125; Thermo Fisher Scientific). Homogenates were centrifuged for 30 min (40,000 × g, 4°C) and the resulting pellets resuspended in 1 ml of PPB and centrifuged a second time. These pellets were homogenized in 450 μl of 50 mM PPB (pH 6.0) containing 0.5% hexadecyltrimethyl ammonium bromide, followed by sonication, freeze-thaw, and a second sonication. The homogenates were incubated at 60°C for 2 h and then centrifuged at 14,000 × g for 5 min. The resulting supernatant was measured by spectrophotometry using o-dianisidine dihydrochloride oxidation as a colorimetric indicator. Calibration curves for MPO activities were established with PMN collected from zymosan A-induced peritonitis exudates in BALB/c mice.

Chemokine and cytokine measurement

Kidney tissue (one-half kidney) was homogenized on ice in tissue protein extraction reagent (TPER; Pierce Biotechnology) containing protease inhibitors (one protease inhibitor cocktail tablet per 10 ml of TPER, Complete Mini; Roche Diagnostics). The particulate matter was removed by centrifugation, and supernatants were analyzed for mouse chemokines (MIP-2 and KC) and cytokines IL-1β, IL-6, and TNF-α using a custom SearchLight quantitative multiplexed sandwich ELISA proteome array (Pierce Biotechnology). The panel of cytokines and chemokines was selected because they are established inflammatory markers that are up-regulated within 24 h after renal ischemic insult (33).

Assessment of renal function

At 24 h postischemia or sham surgery, blood was collected from the heart with heparinized needle syringes and centrifuged (800 × g for 10 min). Serum creatinine, a marker of the glomerular filtration rate, was measured using an Infinity creatinine kit (Thermo Electron).

Cell culture

Rat glomerular mesangial cells were a kind gift from Dr. Michal Laniado-Schwartzman (New York Medical College, Valhalla, NY) and are a primary cell line isolated from male Sprague Dawley rat renal cortical glomeruli according to an established method (34). Rat glomerular mesangial cells from passages 5–10 were grown in DMEM-F12 media supplemented with 10% FBS and 1% penicillin-streptomycin (American Type Culture Collection). Complete media was replaced with serum-free media at 60% confluency. After 12 h of serum starvation, rat glomerular mesangial cells were treated with 17-HDHA or NPD1 (10 nM or 100 nM) or vehicle alone (500 μl of sterile HBSS) for 8 h. Treatments were prepared by removing

Table I. Composition of open source diets from research diets

<table>
<thead>
<tr>
<th>Components (g/kg)</th>
<th>Corn Oil Diet</th>
<th>Fish Oil Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein, 30 mesh</td>
<td>200</td>
<td>200</td>
</tr>
<tr>
<td>rt-methionine</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Corn starch</td>
<td>150</td>
<td>150</td>
</tr>
<tr>
<td>Sucrose</td>
<td>500</td>
<td>500</td>
</tr>
<tr>
<td>Cellulose, BW200</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Corn oil</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Menhaden oil</td>
<td>0</td>
<td>45</td>
</tr>
<tr>
<td>Mineral mix S10001</td>
<td>35</td>
<td>35</td>
</tr>
<tr>
<td>Vitamin mix V10001</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Choline bitartrate</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Total ω-6 PUFA</td>
<td>30</td>
<td>6</td>
</tr>
<tr>
<td>Total ω-3 PUFA</td>
<td>1</td>
<td>14</td>
</tr>
</tbody>
</table>

The corn oil and fish oil diets are based on a widely used nutritionally balanced purified rodent diet (AIN-76A) containing 20.8 kcal% protein (20.3 g%), 67.7 kcal% carbohydrates (66 g%), and 11.5 kcal% fat (5 g%).
HO-1 protein and gene expression

For Western immunoblot analyses, kidneys were homogenized in TPER (Pierce Biotechnology) containing protease inhibitors (one protease inhibitor cocktail tablet per 10 ml of TPER, Complete Mini; Roche Diagnostics). Samples were separated on a 12% SDS-PAGE gel and then electrothermally transferred to a polyvinylidene difluoride membrane (Bio-Rad), with one lane containing a sample from LPS-treated RAW 264.7 macrophages as a positive control for HO-1. Proteins of interest were detected by specific Abs: mouse anti-HO-1 (StressGen Biotechnologies) and mouse anti-β-actin (Sigma-Aldrich). Immunostaining was detected using HRP-conjugated anti-mouse Ig (Kirkegaard & Perry Laboratories). Immunoreactive bands were revealed with chemiluminescent substrate (Kirkegaard & Perry Laboratories) and quantitated by Scion software image analyzer (National Institutes of Health Image).

To measure HO-1 gene expression, total RNA was extracted from kidneys using TRizol reagent (Invitrogen), and RNA integrity was verified by agarose gel electrophoresis and quantitated by spectrophotometry. RNA was reverse-transcribed using a SuperScript III first-strand synthesis system (Invitrogen). Specific primer pairs were selected from a real-time PCR primer and probe database (medgen.ugent.be/rtprimerdb/) and verified by National Institutes of Health GenBank. Rat HO-1 was amplified using 5'-CAACCCCACCAAGTTCAAACA-3' and 3'-AGGCCGCTTACGCCTTCTG-5', and rat ribosomal 18S was used as a reference gene and amplified using 5'-CACGGCGTTACGAGAGAGGAGGCGGCGACAGACC-3' and 3'-AGAGCACCCGCACTGTAAGAAA-5'. Real-time PCR was performed using a Quantitect SYBR Green PCR kit (Qiagen) and a MX3000P quantitative PCR system (Stratagene) as previously described (21). PCR efficiency for each primer pair was determined by quantitating amplification with increasing concentrations of template DNA, and specific amplification was verified by subsequent analysis of melt curve profiles for each amplification. Data are expressed as the fold change in HO-1 mRNA and was calculated by MxPro analytical software (Stratagene).

Lipid autocad analysis

For endogenous product analysis, kidneys were immediately homogenized using a hand-held homogenizer (Fisher PowerGen model 125; Thermo Fisher Scientific) in 66% methanol (4°C), and 0.5 ng of PGB2 was added as an internal standard for recovery. Kidneys were collected 24 h after initiating ischemia/reperfusion injury (see Fig. 5) to match the time point for analyses of inflammatory endpoints (i.e., myeloperoxidase activity, cytokine/chemokine array) and renal function (i.e., plasma creatine). We also quantified DHA-derived mediators in sham-operated mice to assess if renal levels of the inflammatory DHA-derived autocoids are modulated by 3 days of in injured kidney before initiation of the ischemic insult (see Fig. 6). Eicosanoids and docosanoids were extracted by solid phase using Sep-Pak C18 cartridges as described previously (35). Eicosanoids and DHA-derived metabolites were identified and quantified by lipid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS)-based lipidomics and gas chromatography (GC)/MS analysis (18, 31, 36, 37). In brief, extracted samples were analyzed by a triple quadrupole linear ion trap LC-MS/MS system (MDS Sciex 3200 QTRAP) equipped with a Luna C18-2 minibore column using a mobile phase (methanol-water-acetate, 65:35:0.03 (v/v/v)) with a 0.35 ml flow rate. MS/MS analyses were conducted in negative ion mode, and prominent fatty acid metabolites were quantitated by multiple reaction monitoring (MRM mode) using established transitions for PGE2 (351→271 m/z), PGB2 (333→175 m/z), 5-hydroxyeicosatetraenoic acid (5-HETE) (319→115 m/z), 15-HETE (319→175 m/z), LXA4 (351→115 m/z, 351→217 m/z), 4-HDHA (343→101 m/z), 17-HDHA (343→245 m/z), and 10,17-diHDHA (359→153 m/z) (18, 36, 37). Calibration curves (1–1000 pg) and LC retention times for each compound were established with synthetic standards. Lipid autocad levels were corrected for PGB2 recovery and wet kidney weight. Structures were confirmed by MS/MS analysis using enhanced product ion mode with appropriate selection of the parent ion in quadrupole 1.

GC/MS analysis was performed as described in Gronert et al. (31) with a 6890N GC system with a HP5MS cross-linked ME silicic column (30 m × 0.25 mm × 0.25 μm), an autosampler, and a 5973N mass-selective detector (Agilent Technologies). The helium flow rate was 1.5 ml/min, and the initial temperature was 150°C, followed by 230°C (2 min) and 280°C (10 min). For selected samples, HPLC fractions that correspond to established retention times of authentic standards were collected and derivatized to generate pentfluorobenzyl esters and trimethylsilyl ether derivatives, and 10–1000 pg was injected in 2 μl of iso-octane for GC/MS analysis.

**FIGURE 1.** Prolonged ischemia time correlates with increased formation of inflammatory markers and impaired renal function. Mice on a regular diet (ω-3 PUFA = 7% of total fat) were subjected to 0–90 min of ischemia, and kidneys and serum were collected after 24 h of reperfusion. A, MPO activity was measured as a quantitative measure of PMN content in the injured kidney (see Materials and Methods; n = 4–7). B, Serum creatinine was measured as a marker of renal function (n = 4–7). C, Western blot analysis for HO-1 and protein expression in kidneys 24 h after ischemic injury (n = 3). *, p < 0.05 vs 0 min of ischemia.

Statistics

All data are expressed as means ± SEM unless otherwise indicated. Student’s t test was used to evaluate the significance of differences between groups, and multiple comparisons were performed by regression analysis and one-way ANOVA with Statistica (version 6.1; StatSoft). Values of p < 0.05 were considered significant.

Results

Duration of ischemia correlates with increased inflammation and impaired renal function

Clamping of the renal pedicle is a well-established mouse model for renal ischemia-reperfusion injury, as this model mimics pathophysiological events of human ARF (1, 3). Experiments with mice on the standard rodent diet (ω-3 PUFA, 7% of total fat) demonstrated that prolonged duration of ischemia directly correlates with increased PMN infiltration 24 h postischemia, measured by MPO activity (Fig. 1A, p < 0.05 vs 0 min, n = 4–7), and increased serum creatinine as a marker of impaired renal function (Fig. 1B, p < 0.05 vs 0 min, n = 4–7). The HO-1 pathway is recognized as an essential and renoprotective stress response and its expression is relatively low under physiologic conditions but strongly induced after hypoxic, ischemic, and/or inflammatory insult (38). Western immunoblot data in Fig. 1C (n = 3) demonstrates that increased duration of renal ischemia correlates to increased HO-1 protein expression, confirming HO-1 induction as a fundamental renal stress response (39).

Acute dietary increase in ω-3 PUFA and concomitant decrease in ω-6 PUFA correlates with increased survival and reduced renal inflammation

We set out to assess the impact of an acute increase in dietary ω-3 PUFA on the sequelae of IRI. To this end, mice were placed on a standard balanced laboratory rodent diet (Research Diets, AIN-76A) where the fat source (11.5 kcal%, 5% g%) was replaced with either menhaden oil (ω-3 PUFA group, ω-3 PUFA = 28% of total fat) or corn oil (ω-6 PUFA group, ω-3 PUFA = 2% of total fat).
Mice were placed on the custom diets for 4 wk to model an acute increase (menhaden oil) or decrease (corn oil) in dietary -3 PUFA (see Table I for detailed diet comparison). Serum creatinine was selected as an established clinical index of renal function. Thirty minutes of bilateral ischemia in C57BL/J6 mice on the -6 PUFA diet correlated with a significant elevation in serum creatinine (Fig. 2A, p < 0.03 vs sham, n = 9–18); in sharp contrast, renal function (i.e., change in serum creatinine) remained normal in the -3 PUFA group (ω-3 PUFA = 28% of total fat). Only severe ischemia (45 min) induced a significant elevation in serum creatinine in the -3 PUFA group (Fig. 2A, p < 0.03 compared with sham, n = 9–18). Notably, mice in the -6 PUFA group (ω-3 PUFA = 2% of total fat) did not survive 45 min of IRI, compared with 100% survival in the -3 PUFA group (Fig. 2B, n = 8–12). Mice on the -6 PUFA diet (ω-3 PUFA = 28% of total fat) also demonstrated improved renal function (creatinine levels, ω-6 diet 0.43 ± 0.11 mg/dl vs standard diet 0.83 ± 0.19 mg/dl) compared with mice on the standard diet (Lab Diet 5001, ω-3 PUFA = 7% of total fat) with 45 min of ischemia. However, despite decreased renal function, mice on the regular diet exhibited low mortality (Fig. 2B) following 45 min of ischemia (mortality in ω-3 PUFA diet = 0%, standard diet = 12%, and ω-6 PUFA diet = 100%).

Inflammation and increased formation of lipid autacoids are key features in the pathophysiology of IRI. Hence, we assessed whether an acute increase in dietary ω-3 PUFA and concomitant decreased in ω-6 PUFA reduces renal inflammation and stress in response to IRI. Selected cytokines/chemokines levels, PMN content, and expression of the stress gene HO-1 were assessed as established markers of renal inflammation (22, 33). Thirty minutes of renal ischemia induced marked infiltration of PMN, evidenced by significant MPO activity in the kidney 24 h after IRI. This hallmark inflammatory response was significantly inhibited by 62% in the ω-3 PUFA group when directly compared with mice in the ω-6 PUFA group (Fig. 3A, p < 0.04, n = 6). Additionally, the ω-3 PUFA group demonstrated attenuated levels of renal chemokines MIP-2 (p < 0.01, n = 5) and KC (p < 0.01, n = 5) by ~30%, and cytokines IL-1β (p < 0.004, n = 5), IL-6 (p < 0.004, n = 5), and TNF-α (p < 0.02, n = 5) by 36%, 47%, and 35%, respectively (Fig. 3B). Note that these selected cytokines and chemokines are established mediators of inflammation and prominent markers of renal inflammation during the first 24 h of reperfusion (33).

HO-1 is an early response gene and is recognized as a fundamental cytoprotective system, which is induced in response injury, oxidative stress, and inflammation. In this sense, HO-1 is a direct marker of increased renal stress and injury (22) (Fig. 1). Consistent with this notion, data in Fig. 3C demonstrate that mice in the ω-6 PUFA group had 74% higher expression of renal HO-1 protein when directly compared with the ω-3 PUFA group (Fig. 3C, p < 0.03, n = 3–4). Increased expression of the HO-1 system in the ω-6 PUFA group correlated with impaired renal function, high
mortality, and increased inflammation (Figs. 2 and 3). Taken together, these data indicate that dietary ω-3 PUFA affords significant protection against IRI in mice.

**Dietary ω-3 PUFA attenuate renal eicosanoid formation in response to IRI**

Autacoids derived from the ω-6 PUFA arachidonic acid, namely eicosanoids, have well-established roles in normal renal physiology and in the pathophysiology of renal injury and disease. To assess the impact of acute dietary increase in ω-3 PUFA on renal eicosanoid formation after ischemic renal injury, we employed LC-MS/MS-based lipidomic analysis (see Materials and Methods). Analyses of kidneys from mice subjected to IRI (30 min/24 h of ischemia/reperfusion) demonstrated that an acute increase in dietary ω-3 PUFA abrogated renal formation of 5-HETE (Fig. 4A, p < 0.02, n = 3–4), 12-HETE (Fig. 4B, p < 0.02, n = 3–4), 15-HETE (Fig. 4C, p < 0.03, n = 3–4), LXA₄ (Fig. 4D, p < 0.001, n = 3–4), and PGE₂ (Fig. 4E, p < 0.003, n = 3–4). Moreover, basal levels (uninjured kidney) of 5-HETE (Fig. 4A, p < 0.02, n = 3) and 15-HETE (Fig. 4C, p < 0.03, n = 3) were also attenuated in the ω-3 PUFA group when directly compared with the ω-6 PUFA group. LXA₄ levels in uninjured kidneys and in kidneys from mice in the ω-3 PUFA group were below current LC-MS/MS detection limits (~1 pg). These data provide evidence that an acute increase in ω-3 PUFA correlates with abrogated formation of prominent eicosanoids from both cyclooxygenase and LOX pathways.

**Increase in dietary ω-3 PUFA amplifies endogenous formation of DHA-derived autacoids**

Next we set out to investigate whether the renoprotective effects of dietary ω-3 PUFA were associated with formation of DHA-derived protective autacoids. Initial diode array detector reversed phase HPLC analysis (n = 3) identified the presence of unique peaks that matched the retention times and characteristic UV chromophores of authentic 17S-HDHA and PD1 standards (Fig. 5, A and B, respectively). Fractions corresponding to the retention time of authentic 17S-HDHA and PD1 standards were isolated by reversed phase HPLC, derivatized, and analyzed by GC/MS using selective ion monitoring (see Materials and Methods). Full scanning analysis of the 17S-HDHA fraction (Fig. 5C) revealed an anion of strong intensity at m/z 415 with a weaker ion at m/z 325, which is consistent with a monohydroxy-DHA structure, whereas the PD1 fraction (Fig. 5D) revealed an anion of strong intensity at m/z 503 with two weaker ions at m/z 413 and m/z 323, which is consistent with a dihydroxy-DHA structure.

LC-MS/MS analysis was employed to profile formation of prominent DHA-derived autacoids and to obtain MS/MS data for additional structural confirmation. Multiple reaction monitoring for established and specific transition ions (see Materials and Methods) demonstrated significant endogenous renal formation of PD1, 17-HDHA, 14-HDHA, and 4-HDHA in mice from the ω-3 PUFA group (Fig. 5E, n = 3). Enhanced product ion mode was selected to obtain MS/MS spectra for molecular ion m/z 359. The MS/MS spectrum (Fig. 5E, inset) displayed diagnostic fragment ions at m/z 341 (M-H-H₂O), 315 (M-H-CO₂), 297 (M-H-H₂O-CO₂), and, consistent with a 10,17-dihydroxy-DHA structure, fragment ions of m/z 153, 261, 217 (261-CO₂), and m/z 199 (261-H₂O-CO₂) for the compound that matched the retention time of 10R,17S-dihydroxy-docosa-4Z,7Z,11E,13E,15Z,19Z-hexaenoic acid (18). Taken together, these physical data provide strong evidence for the endogenous renal formation of PD1 and 17-HDHA in injured kidneys.

We recently reported that the basal tone of resident antiinflammatory or protective autacoids directly impacts the outcome of acute inflammatory/reparative responses (40). Hence, we set out to assess whether an increase in dietary ω-3 PUFA increases basal formation of DHA-derived autacoids, which have established antiinflammatory and renoprotective actions, namely PD1 and 17-HDHA. LC-MS/MS analysis of uninjured kidneys from sham mice revealed that endogenous formation of DHA-derived PD1 (Fig. 6A, p < 0.05, n = 7) and 17-HDHA (Fig. 6B, p < 0.04, n = 3) was significantly amplified in the ω-3 PUFA group when directly compared with the ω-6 PUFA group. Endogenous formation of PD1 was increased by 160 pg/kidney in the ω-3 diet group, while 17-HDHA, a metabolic marker for 15-LOX activity and the formation of PD1 and 17S-series resolvins, increased by 2.6 ng/kidney. Of interest, renal formation of 4-HDHA, a 5-LOX metabolite and pathway marker for formation of renoprotective DHA-derived resolvin (14), increased ~4-fold in the ω-3 PUFA group (Fig. 6C, p < 0.03, n = 3). 5-LOX is a key enzyme in the formation of RvDs by stereospecific oxygenation, at either the number 4 or 7 carbon, of 17-hydroxy-DHA (14). It was recently demonstrated that the 5-LOX metabolite RvD1 has potent renoprotective action in mice (19). Taken together, these findings provide evidence that an acute increase in dietary ω-3 PUFA and concomitant decrease in ω-6 PUFA affords protection against IRI, which is associated with increased endogenous formation of antiinflammatory DHA-derived autacoids.

**PD1 inhibits PMN recruitment and amplifies renal expression of cytoprotective HO-1**

We next assessed whether systemic treatment with DHA-derived antiinflammatory autacoids attenuates renal injury. To this end, male BALB/c mice on a balanced standard laboratory rodent diet were subjected to 30 min of IRI and treated i.v. at a dose of 0.4
mg/kg with either 17-HDHA or PD1. A stable analog of LXA₄, ATLa, was also selected since this LXA₄ mimetic when given systemically has proven antiinflammatory and renoprotective actions in mice (41). Ischemia/reperfusion (30 min/24 h) triggered robust infiltration of PMN in comparison to sham-operated mice (Fig. 7A, p < 0.001, n = 3–4). Systemic treatment with the LXA₄ analog reduced PMN recruitment by 49% (Fig. 7A, p < 0.03, n = 3). PD1 was as potent as ATLa, as it blunted PMN influx to the injured kidney by 64% (Fig. 7A, p < 0.005, n = 4). These data are in agreement with a recent report that demonstrated that systemic treatment with either 17S-HDHA or PD1 significantly attenuates MPO activity in mouse kidneys after IRI (19).

The molecular mechanism of action for the protective effect of DHA-derived autacoids remains to be fully defined. Induction of HO-1 in models of IRI is renoprotective (22, 24, 25, 38), and we recently demonstrated that topical LXA₄ can amplify endogenous expression of HO-1 in the eye, which correlates with increased wound healing and reduced inflammation (21). Hence, we set out to determine whether 17-HDHA and PD1 can increase renal expression of HO-1. Western blot analysis of kidneys demonstrated that ischemic injury increased expression of the cytoprotective early response gene HO-1 (Fig. 7B, n = 3). As expected, the level of HO-1 protein expression in kidneys of sham-operated mice was very low (data not shown, n = 3), which is consistent with results shown in Figs. 1C and 7C. Systemic treatment with ATLa significantly increased renal HO-1 expression 204% over saline treatment alone (Fig. 7B, p < 0.05, n = 3), while 17-HDHA, the precursor to 17S-series resolvins such as RvD1 (14), induced a 525% increase in renal HO-1 expression (p < 0.003, n = 3), and PD1 was as potent as ATLa (p < 0.05, n = 6). These results provide the first evidence that systemic treatment with DHA-derived autacoids can amplify HO-1 expression in the injured kidney. To examine the impact of DHA-derived autacoids on basal HO-1 expression in uninjured kidneys, we subjected male BALB/c mice to sham operation and treated them i.v. at a dose of 0.4 mg/kg with PD1 or saline. Western blot analysis demonstrated that PD1 increased renal basal HO-1 expression by 100% when directly compared with saline treatment alone (Fig. 7C, p < 0.02, n = 4). These
results indicate that PD1 treatment not only amplifies HO-1 expression in injured kidney, but that it induces expression of this cytoprotective response in healthy kidneys.

Mesangial cells play a key role in the sequelae of events in kidney-related pathology after IRI (42). Hence, we investigated, in vitro, if DHA-derived autacoids can modulate HO-1 expression in primary cultured rat mesangial cells. Quantitative real-time PCR analyses demonstrated that 17-HDHA and PD1 significantly increased basal HO-1 mRNA at concentrations of 10 and 100 nM (Fig. 7, *p* < 0.05, *n* = 4). Taken together, these findings provide the first in vivo and in vitro evidence for regulation of renal HO-1 by DHA-derived antiinflammatory autacoids.

**Discussion**

The present study demonstrates for the first time that short-term changes in dietary intake of ω-3 and ω-6 PUFA are sufficient to dramatically alter the sequelae of renal IRI. Specifically, short-term dietary decrease in ω-3 PUFA and concomitant increase in ω-6 PUFA was associated with increased renal inflammation, impaired renal function, and high mortality due to IRI. In stark contrast, mice on a balanced high ω-3 PUFA and low ω-6 PUFA diet were protected against IRI and mortality even with 45 min of renal ischemia. Protection against IRI was associated with decreased renal formation of AA-derived eicosanoids and increased formation of antiinflammatory and proresolving DHA-derived autacoids, PD1 and 17-HDHA, in mouse kidneys. The endogenous protective role of these recently discovered DHA-derived autacoids, specifically PD1, were supported by in vivo experiments, which demonstrated that systemic treatment with PD1 attenuated renal inflammation and amplified expression of the cytoprotective HO-1 system.

The Western diet contains 20- to 25-fold more ω-6 than ω-3 PUFA (43). This predominance in the diet is due to the abundance of linoleic acid (ω-6, 18:2) in the diet present in soy, corn, safflower, and sunflower oils. The paucity of dietary ω-3 PUFA correlates with a higher incidence of coronary heart disease in the Western populations in comparison with Japanese and Greenland Eskimo populations (44). In recent years, ω-3 PUFA have been specifically recommended for the prevention and treatment of cardiovascular disease, as well as for disorders with an inflammatory component such as type 2 diabetes, irritable bowel syndrome, macular degeneration, rheumatoid arthritis, asthma, cancer, and psychiatric disorders (45). This recommendation came forth after population studies and clinical trials had demonstrated that dietary fish
oils, enriched in DHA and EPA, are cardio- and renoprotective and that DHA, in particular, has antiinflammatory and immune regulatory actions (7, 8, 46–49). Our present findings with mice underscore the striking impact of even short-term dietary PUFA manipulation in the sequelae of acute ischemic renal injury and are in agreement with experimental and clinical studies that provide a strong rationale for a beneficial role of increasing the ω-3 PUFA/ω-6 PUFA ratio in renal diseases (6, 8, 46, 47, 50). Our finding that mice on a corn oil diet (ω-3 PUFA = 2% of total fat), unlike mice on a fish oil diet (ω-3 PUFA = 28% of total fat) or the standard rodent diet (ω-3 PUFA = 7% of total fat), had 100% mortality due to IRI provides experimental support for meta-analyses that show an inverse correlation with cardiovascular disease, coronary heart disease, and stroke mortality in human populations and their dietary intake of ω-3 PUFA and ω-6 PUFA; namely, countries with the lowest ω-3 PUFA and highest ω-6 PUFA dietary intake appear to have the highest risk for disease mortalities (45).

The consequence of acutely modifying dietary intake of ω-3 vs ω-6 fatty acids on renal IRI, to the best of our knowledge, has not yet been investigated. Our findings indicate that a short-term increase in the dietary ratio of ω-3 to ω-6 PUFA has significant effects on IRI, which includes improved surgical survival and attenuated inflammation and correlates with a distinct shift in the renal lipid autacoid profile. In stark contrast, acute dietary decrease in ω-3 PUFA and concomitant increase in ω-6 PUFA was associated with worsened outcome of IRI and a remarkable impact on mortality. In addition to our study, independent reports provide evidence that ω-3 PUFA in general are renoprotective in ischemic injury, namely that dietary supplementation with EPA and DHA ameliorates ischemic ARF in dogs (13) and that systemic treatment with DHA alone can ameliorate ischemic ARF and protect renal function in mice (12, 50).

The renoprotection observed with acute dietary amplification of ω-3 PUFA correlated with reduced levels of eicosanoids and increased formation of DHA-derived autacoids. Mice fed the ω-3 diet had lower renal levels of both LOX- and cyclooxygenase-derived eicosanoids, PGE2, 5-HETE, 15-HETE, 12-HETE, and LXA4. In contrast, endogenous levels of DU1 and metabolic markers for the formation of RvDs were significantly amplified by the fish oil diet in uninjured kidneys. Kidney levels of PD1 (0.24 ng/kidney) measured in our study are lower than those recently reported for mouse lungs (45 ng) (51), and this likely reflects organ- and model-specific formation/metabolism and mouse strain differences. More importantly, in selected mouse models of inflammation, PD1 at i.v. doses as low as 2 ng/mouse has proven antiinflammatory actions (51), and thus it is likely that an increase in local endogenous formation of PD1 by 0.16 ng is physiologically relevant and can evoke marked renoprotective bioactions.

Our analyses did not detect formation of established eicosapentaenoic acid-derived antiinflammatory mediators such as resolvin E (RvE1). However, significant renal levels of 17-HDHA, 14-HDHA, and 4-HDHA were detected, which increased ~4-fold in the ω-3 diet group. These DHA-derived products are metabolic markers for LOX pathways that generate PD1 (i.e., 17-HDHA) and other members of the recently described family of DHA-derived mediators (i.e., 17S-series resolvins), which have documented antiinflammatory bioactions in mice (14). More importantly, these 17S-series resolvins (i.e., RvDs) and 17-HDHA exhibit renoprotective actions in vivo (19). Hence, in addition to PD1 and 17-HDHA, other bioactive DHA-derived resolvins are formed in healthy or injured kidneys and likely contribute significantly to the marked protection against IRI in the ω-3 diet group. Moreover, striking reduction of eicosanoid levels in uninjured and injured kidney reflects a fundamental shift in the activation or formation of lipid mediator pathways that have established roles in normal renal physiology and inflammatory responses; hence, abrogated activation of ω-6 PUFA lipid circuits likely has a key role in limiting the sequelae of IRI.

It is well established that 4 wk of dietary ω-3 supplementation is sufficient to significantly increase DHA and EPA content of phospholipid pools in virtually all tissues in mice (52–59). However, despite a relative large percentage increase in DHA and EPA levels, AA levels are only slightly decreased and AA remains as the most abundant tissue PUFA in all reported short-term diet studies in mice (52–59). A marked increase in eicosanoid production, especially PGE2 and LXA4 generation, is induced as a direct consequence of tissue injury and inflammation, due to robust activation of phospholipases and downstream biosynthetic pathways. Hence, it is likely that the striking absence or reduction in renal eicosanoid levels after ischemia/reperfusion reflects attenuated activation of cells, which leads to, or is a consequence of, reduced inflammation and/or renal injury, rather than substrate competition of AA with either DHA or EPA. This notion is supported by the markedly lower induction of the HO-1 following IRI in the ω-3 diet group, as expression of this stress gene is a marker of renal injury and inflammation. Restrained activation of the inflammatory response may be in part mediated by an increase in the basal levels of antiinflammatory DHA-derived signals such as PD1 (Fig. 6). Hence, it is tempting to speculate that increased basal formation of ω-3 PUFA-derived autacoids may provide an elevated tone of antiinflammatory mediators, which raise the threshold for full activation of the renal inflammatory cascade.

Amplification of the PD1 pathway by systemically treating mice with PD1 provides strong evidence for an endogenous antiinflammatory action of this circuit, since IRI-induced renal PMN recruitment was markedly reduced. These findings are in agreement with a recent report that demonstrates that combined systemic injection and s.c. infusion of either PD1 or RvD5s affords both functional and morphological protection against IRI (19). It is now recognized that antiinflammatory lipid autacoids are generated endogenously from both AA and DHA, namely LXA4 and PD1, respectively (14–16, 41). Pharmacological amplification of these endogenous antiinflammatory circuits by systemic treatment with either lipid autacoid or their stable analogs is renoprotective (16, 19, 41), as is genetic amplification (60) by renal transfection with the key biosynthetic enzyme, 15-LOX, for PD1, RvDs, and LXA4. The present study demonstrates for the first time that an acute dietary increase in ω-3 PUFA significantly increases endogenous basal levels of renoprotective PD1 and RvDs. PD1 shares several bioactions with the well-studied antiinflammatory eicosanoid LXA4. Specifically, in the kidney PD1 maintains glomerular filtration rate, suppresses PMN recruitment and inflammation, and reduces postischemic interstitial kidney fibrosis after IRI (Fig. 7) (16, 19).

The potent renoprotective action of PD1 and RvDs and their general antiinflammatory action in distinct models of acute inflammation provide a compelling argument that formation of DHA-derived autacoids contributes to the beneficial effects of dietary ω-3 PUFA.

The pharmacokinetics for systemically, i.p., or orally delivered PD1, 17-HDHA, or related endogenous lipid autacoids have not been defined. However, studies investigating their metabolic inactivation demonstrate that these classes of lipid autacoids are rapidly metabolized (14), a notion that is supported by detailed pharmacokinetic studies (61) with analogs of the antiinflammatory lipid mediator LXA4 (e.g., ATLa), which demonstrated rapid clearance and relatively short plasma half-life (i.e., 0.3 h for an i.v. dose of 3 μg/mouse). PD1 and/or 17-HDHA have demonstrated...
antiinflammatory actions in mice when administered topically, i.e., or i.p. at doses ranging from 0.02 to 35 μg/mouse (19, 31, 52, 62, 63). We selected the dose of 10 μg/mouse based on two renal ischemia reperfusion studies in mice, which used PD1 or 17-HDHA (19) at doses of 3.5–35 or 17.5 μg/mouse, respectively, and an earlier study that used a stable LXA₄ analog at a dose of 15 μg/mouse (64). These studies demonstrated that despite rapid metabolic inactivation and clearance, i.e., administered PD1, 17-HDHA, or LXA₄ analogs are bioavailable and provide significant renoprotection in mice.

The molecular mechanism for the protective effects of PD1 and RvDs remain to be defined. Hence, it is of particular interest that several recent reports provide evidence for an interaction of LXA₄ and its biosynthetic pathway with the essential cytoprotective HO system (21, 65). The HO system has well-established antiinflammatory roles and is renoprotective in several models of acute renal injury (24–29). However, it is important to recognize that even though HO-1 induction as a consequence of injury, inflammation, and oxidative stress is a fundamental protective response, it is not sufficient to prevent tissue injury. More importantly, physiological HO-1 activity and expression can be greatly amplified by pharmacological agents (i.e., metalloporphyrins) to enhance its in vivo cytoprotective actions. An impressive body of work (22) has demonstrated that amplification of physiological HO-1 expression and activity by pharmacological agents correlates with antiproliferative, anti-inflammatory, and antiapoptotic actions, while HO-1 inhibition or genetic deletion exacerbates tissue injury. Hence, it is of particular interest that systemic treatment with PD1, an endogenous renal antiinflammatory autacoid, can amplify expression of the HO-1 system. It is notable that prominent antiinflammatory lipid autacoids (21, 65, 66), such as LXA₄, 15-deoxy PGJ₂, and RvDs remain to be defined. Hence, it is of particular interest that systemic treatment with PD1, an endogenous lipid autacoid, which in part account for its remarkable renoprotective actions (41). Mesangial cells, even though they are not immediately affected by renal ischemia, are relevant in the pathophysiology of IRI because the hemodynamic changes triggered by ischemic insult to the kidney can cause mesangial cell contraction, proliferation, and a subsequent reduction in glomerular filtration rate (42). Hence, it is of interest that PD1 amplifies HO-1 expression in mesangial in a nanomolar concentration range. In view of the documented antiapoptotic and anti-oxidant role of the HO-1 system in mesangial cells (67, 68), it is tempting to speculate that amplified HO-1 expression in mesangial cells may inhibit their pathophysiological response to IRI.

Taken together, our findings demonstrate that acute changes in dietary ω-3 and ω-6 PUFA dramatically alter the outcome of IRI and that increased dietary ω-3 PUFA are associated with increased formation of antiinflammatory DHA-derived lipid autacoids in mouse kidneys. Pharmacological amplification of this endogenous pathway, by systemic treatment with PD1, significantly amplified expression of the renal cytoprotective HO system, thus providing evidence for a potential interaction of antiinflammatory lipid au-
tacoids and the essential HO-1 system. These data add to a rapidly evolving paradigm, namely that formation of DHA-derived signals constitutes a resident antiinflammatory pathway that is amenable to amplification by altering dietary ω-3 PUFA intake.

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Disclosures

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References


