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Resolvin D Series and Protectin D1 Mitigate Acute Kidney Injury

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Omega-3 fatty acid docosahexaenoic acid is converted to potent resolvins (Rv) and protectin D1 (PD1), two newly identified families of natural mediators of resolution of inflammation. We report that, in response to bilateral ischemia/reperfusion injury, mouse kidneys produce D series resolvins (RvDs) and PD1. Administration of RvDs or PD1 to mice before the ischemia resulted in a reduction in functional and morphological kidney injury. Initiation of RvDs and RvD1 administration 10 min after reperfusion also resulted in protection of the kidney as measured by serum creatinine 24 and 48 h later. Interstitial fibrosis after ischemia/reperfusion was reduced in mice treated with RvDs. Both RvDs and PD1 reduced the number of infiltrating leukocytes and blocked TLR-mediated activation of macrophages. Thus, the renal production of Rv and protectins, a previously unrecognized endogenous anti-inflammatory response, may play an important role in protection against and resolution of acute kidney injury. These data may also have therapeutic implications for potentiation of recovery from acute kidney injury.

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Injury to the kidney, even of a relatively modest degree, is a major health burden accounting for many hospital admissions and high morbidity and mortality (1–3). Although there have been major advances in the management of acute kidney injury over the past 50 years, treatment is largely supportive. In many cases, there is complete failure or partial failure of resolution with important implications for mortality and, in some cases, progression to end stage renal disease (4). Over the last decade, it has been appreciated that acute kidney injury is an inflammatory disease, with inflammation contributing to small vessel congestion and the perpetuation of the functional deficiencies (5–9). Even with repair and regeneration following injury, emerging evidence indicates that postinflammatory scarring of the kidney may be a major factor contributing to chronic renal disease (10–12). It has also been postulated that ischemic inflammatory kidney injury is a central component of many progressive diseases of the kidney (13–15). In murine models of ischemic and toxic acute kidney injury, the innate immune response plays a key role in disease progression as demonstrated by the protective effect of Abs that block integrin and integrin receptor interactions necessary for diapedesis of leukocytes as well as the protective effect of depletion of complement factors such as C5a (5, 7). Similar to the innate inflammatory response in other settings, neutrophils (polymorphonuclear neutrophils (PMNs)) predominately in the first 24 h of injury, being replaced subsequently by monocytes/macrophages and T cells (5, 16–18).

To date, several compounds have been shown in animal models to ameliorate acute kidney injury, but in clinical trials these agents have had limited efficacy (19–22). One of the features of injury to the kidney may be redundancy in proinflammatory pathways. Therefore, blockade of one pathway and/or target alone may be ineffective. The focus of therapy has to date been primarily directed at proinflammatory factors and there has been little attention to the understanding of the endogenous factors normally involved in the resolution of inflammation or an attempt to facilitate this process therapeutically.

Recently, it has been recognized that endogenous anti-inflammatory lipid mediators can be generated which are short-lived autacoids derived from precursor omega-3 fatty acids (23, 24). Production of these mediators can be enhanced by aspirin. These novel families of compounds were termed resolvins (Rv) and protectins (25) because they derive from docosahexaenoic acid (DHA) and act to resolve inflammation (23). During the resolution of inflammation in mice treated with aspirin, endothelial-neutrophil (PMN) interactions were required for the generation of some of these compounds by human cells (23). PMNs generate both bioactive D series Rv (RvDs) and protectins from DHA and precursors present in exudates (23, 26).

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4 Abbreviations used in this paper: PMN, polymorphonuclear neutrophil; DHA, docosahexaenoic acid; I/R, ischemia/reperfusion; PD1, protectin D1; 10R,17S-dihydroxy-docosa-4Z,7E,11E,13S,15Z,19Z-hexaenoic acid, also termed neuroprotectin D1 (NPD1) when produced in neural tissue (see Ref. 49); PLP, paraformaldehyde; i-l-lysine-peridate; AA, arachidonic acid; LDH, lactate dehydrogenase; LC, liquid chromatography; MS, mass spectrometer/spectrometry; PAS, periodic acid-Schiff; MPO, myeloperoxidase; Rv, resolvin; RvDs, Rv D series; RvD1, 7S,16,17-trihydroxy-docosa-4Z,9E,11E,13Z,15E,19Z-hexaenoic acid; RvD2, 7Z,16,17-trihydroxy-docosa-4Z,9E,11E,13E,15Z,19Z-hexaenoic acid; RvD3, 7S,16,17-trihydroxy-docosa-4Z,9E,11Z,12E,14Z,19Z-hexaenoic acid; RvD4, 35,11,17,19-tetrahydroxy-docosa-5Z,7E,9E,13Z,15Z,19Z-hexaenoic acid; RvD5, 7S,17S-dihydroxy-docosa-4Z,9E,11Z,13Z,15E,19Z-hexaenoic acid; RvD6, 45,17S-dihydroxy-docosa-5Z,7E,10Z,12Z,14Z,19Z-hexaenoic acid; LDH, lactate dehydrogenase.
In this study, we document endogenous production of anti-inflammatory RvDs and protectin D1 (PD1) in the kidney following ischemic acute kidney injury. We show that the precursor DHA is present in kidneys before and after ischemic acute kidney injury. Increased amounts of Rv and protectins were generated in the post-ischemic kidney, and administration of these compounds protected kidneys from ischemic injury, reducing leukocyte influx and the postischemic increase in serum creatinine as well as reducing post-ischemic kidney fibrosis. It is of particular interest that initiation of administration of RvD1 and RvDs after reperfusion also provides a functional protection of the kidney as measured by serum creatinine 24 and 48 h later. Based on these results, it is likely that endogenous anti-inflammatory compounds directed at resolution of the inflammatory response play an important role in the natural course of acute kidney injury (27). Dysregulation or inadequacy of this response may be responsible for delay in recovery or inability to recover from acute kidney injury as seen in many cases in humans. These newly identified DHA-derived lipid mediators may serve as a new paradigm for the design of effective therapeutics to treat patients with acute kidney injury and hasten their recovery improving morbidity and mortality in these patients.

Materials and Methods

In vivo experimental protocols

Kidney ischemia/reperfusion model. Male 23- to 28-g BALB/c mice (Charles River Laboratories) fed on standard chow were anesthetized with pentobarbital (65 mg/kg i.p., shaved on both flanks, and prepared by cleaning skin with betadine. The animals were placed prone on temperature-controlled heating pads linked to arectal probe (Harvard Apparatus). Core mouse temperature was stabilized between 36.7°C and 37.3°C. Mice were given reagents by tail vein injection. When compounds were administered i.v., the interval between injection and clamping of kidney vessels was 10 min. Longitudinal incisions were made over both kidneys in the mid scapular line, the muscle wall was divided by blunt dissection. The left kidney was exposed. A microaneurysm clamp (Roboz) was placed across the renal pedicle to occlude artery and vein. Dusking of the hue of the kidney was confirmed after which the clamped kidney was returned to the retroperitoneum, and the skin held closed. The right kidney was then exposed and the renal pedicle clamped within 1 min of the placement of the left kidney clamp. For both kidneys, the clamps were allowed to remain for precisely 30 min. Clamps were then removed and reperfusion confirmed visually. Kidneys were again returned to the retroperitoneum, and the skin wound closed with clips. Before closure of the right flank wound, a pocket was created by blunt dissection between the fascial planes separating dermis and muscle, pointing cranially toward the cervical end of the thoracic spine. Primed, warmed, preprepared osmotic minipumps were placed in this space with the exit point facing cranially, and the skin wound closed. Mice were transferred to a warmed cage until they had fully recovered from anesthesia. Kidney ischemia/reperfusion model.

Preparation of kidneys for analysis. Cohorts of mice were euthanized at 24, 48 h, or 15 days. Tissues were either flushed with ice-cold PBS to remove erythrocytes and circulating leukocytes or perfusion fixed in situ with paraformaldehyde-t-lysin-peridate (PLP) solution using techniques previously described (10). Unfixed kidneys were snap-frozen in liquid N2 and stored at −80°C. Fixed tissues were transferred from PLP to 15% sucrose solution in PBS after 2 h. Eighteen hours later, they were frozen in OCT (optimal cutting temperature) compound and stored at −80°C. Tissues for staining were fixed in 10% neutral buffered formalin for 12 h, embedded in paraffin wax. The tissue sections were transferred to 70% ethanol and embedded in paraffin wax. Tissues were either flushed with ice-cold PBS to remove erythrocytes and circulating leukocytes or perfusion fixed in situ with paraformaldehyde-t-lysin-peridate (PLP) solution using techniques previously described (10). Unfixed kidneys were snap-frozen in liquid N2 and stored at −80°C. Fixed tissues were transferred from PLP to 15% sucrose solution in PBS after 2 h. Eighteen hours later, they were frozen in OCT (optimal cutting temperature) compound and stored at −80°C. Tissues for staining were fixed in 10% neutral buffered formalin for 12 h, transferred to 70% ethanol and embedded in paraffin wax.

Characterization of RvDs, PD1, and related products

RvDs and 175-hydroxy-DHA (HDHA) were generated using DHA (Cayman Chemical) and 15-lipoxygenase (24, 30, 31). The enzymatically derived preparations were isolated via C18 solid phase extraction following by HPLC on a C18 column (150 mm × 2 mm × 5 μm) (Phenomenex), which was eluted with 70% methanol. RvD and HDHA were identified using commercial RvD1 and RvD2 collected, and characterized by total organic synthesis and was quantified by both physiochemical and biological properties (32). The solutions were taken to dryness with N2, gas and suspended in ethanol stock solution. Immediately before iRF experiments, each stock solution was diluted with saline (0.9% NaCl) or saline containing delipidated endotoxin-free BSA (0.01% BSA) (33). The composition of the RvDs was 1:2:1 (RvD1:RV2:RvD3), reflecting their relationship in vivo. These results were confirmed with RvD1 prepared by total organic synthesis, matched with enzymatic and biologically generated RvD1 that will be reported elsewhere (Y. P. Sun, J. Uddin, S. F. Oh, K. Gotlinger, E. Campbell, S. P. Colgan, N. A. Petasis, and C. N. Serhan, submitted for publication). The synthetic RvD1 and PD1 were provided by Prof. N. P. Petasis (Department of Chemistry, University of Southern California, Los Angeles, CA). The total organic synthesis of PD1 was reported (34).

Assessment of kidney injury

Preparation of kidneys for analysis. Cohorts of mice were euthanized at 24, 48 h, or 15 days. Tissues were either flushed with ice-cold PBS to remove erythrocytes and circulating leukocytes or perfusion fixed in situ with paraformaldehyde-t-lysin-peridate (PLP) solution using techniques previously described (10). Unfixed kidneys were snap-frozen in liquid N2 and stored at −80°C. Fixed tissues were transferred from PLP to 15% sucrose solution in PBS after 2 h. Eighteen hours later, they were frozen in OCT (optimal cutting temperature) compound and stored at −80°C. Tissues for staining were fixed in 10% neutral buffered formalin for 12 h, transferred to 70% ethanol and embedded in paraffin wax.

Immunofluorescence. Five-micrometer cryostate-cut PLP-fixed sagittal sections were preblocked with Fc-block (BD Pharmingen), then immunolabeled with anti-GR-1 Abs or anti-CD11b Abs (eBioscience) at 1/200 dilution, followed by affinity-purified goat anti-rat Cy3 fluorescent Abs (Jackson ImmunoResearch) and then by anti-CDC68-FITC Abs (Serotec) in 10% rabbit serum for 2 h at room temperature. Sections were washed in PBS three times, then mounted with Vectashield including 4',6'-diamidino-2-phenylindole (200 ng/ml). Sections were viewed by fluorescence microscopy (×200) and serial images captured using identical settings, covering the entire section. All images were assessed quantitatively for percentage area of kidney positive for a particular stain using methods previously described (35). Brieferized (24) PD1 was assessed using Fovia Pro software. A range of hues, saturations, and intensities were selected to selectively include the positively stained cells only. These settings were applied to each image, giving a percentage area of the image positive for
the stain. For each kidney, the average for the entire whole sagittal section was obtained by recording the area for each captured image.

**Staining of tissue sections.** Three-micrometer paraffin sagittal sections were stained with periodic-acid-Schiff (PAS) or the Mason’s trichrome method. PAS-stained sections from each kidney were assessed blindly for histological severity of disease using the following established scale (5): 1+, normal; 2+, mitosis and necrosis of individual cells; 3+, necrosis of all cells in adjacent proximal tubules, with survival of surrounding tubules; 4+, necrosis confined to the distal third of the proximal tubules with a band of necrosis extending across the inner cortex; 5+, necrosis affecting all three segments of the proximal tubules. Each kidney was ascribed a disease severity value. The quantity of blue-stained collagen deposition was assessed in kidneys 15 days after ischemic injury using quantitative morphometry measurements as described above. The quantity of collagen was determined as the percentage area of the total kidney area.

**Quantitative myeloperoxidase (MPO) assay.** Snap-frozen kidney samples were homogenized at 4°C in potassium phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide, sonicated, freeze- thawed three times, then sonicated again. The suspension was centrifuged at 12,000 × g for 15 min, and 20 μl of supernatant was added to 900 μl of potassium phosphate buffer (pH 6.0) containing 0.167 mg/ml o-dianisidine dihydrochloride (Sigma-Aldrich) and 0.0006% hydrogen peroxide (Sigma-Aldrich). The rate of change in absorbance at 460 nm was monitored at 23°C at 90-s intervals (36).

**Creatinine measurements.** Plasma was obtained by either tail bleeds as described or cardiac puncture into preheparinized tubes. Plasma was separated by centrifugation (800 g) for 15 min, and 200 μl of supernatant was collected and cells lysed in the remaining medium with 0.1% Triton X-100. The proportion of total cellular LDH released into the supernatant was used as a measure of injury and LDH was quantitated using a standardized assay ranging from 1 to 3 (1 = Mouse activity score. Mice were assessed 24 h following I/R for well-being using a standardized assay ranging from 1 to 3 (1 = + , lazy, slow movement; 2+, intermediate level of activity; 3+, active movement or searching) (38). This time reflects the peak of the plasma creatinine.

**In vitro assays**

**Tubule cell injury.** LLC-PK1, swine kidney epithelial cells with proximal tubule characteristics, were obtained from American Type Culture Collection and passaged in DMEM (Invitrogen Life Technologies) with glutamine and 10% FCS. For the experiments cells were grown to confluence in 24-well plates (Corning) in 1.0 ml of medium containing 1% FCS. Experiments were conducted in triplicate. Cells were treated with 500 nM LPS (Escherichia coli 0127:B8; Sigma-Aldrich) which was added to each well together with simultaneous addition of one of the active reagents, DHA, PD1, RvD6, or the essential fatty acid, arachidonic acid (AA), in one of the following molar concentrations: 0.5, 5.0, and 50.0 μM. In previous experiments we had determined that 500 μM H2O2 resulted in ~50% lactate dehydrogenase (LDH) release 6 h later (39). Thus, after 6 h of incubation, 100 μl of supernatant was collected and cells lysed in the remaining medium with 0.1% Triton X-100. The proportion of total cellular LDH released from the supernatant was used as a measure of injury and LDH was quantitated using a standard colorimetric LDH-specific assay (39).

**Macrophage TNF-α production.** Primary bone marrow-derived macrophages were cultured as previously described (40). Day 7 mature macrophages were plated into 24-well plates (2.5 × 10⁵/well). Each well had 500 μl of DMEM/F12 containing 10% FCS. Each experiment was performed in triplicate. A total of 100 ng/ml LPS (Escherichia coli 0127:B8; Sigma-Aldrich) was added to each well together with simultaneous addition of one of the active reagents, DHA, PD1, RvD6, or the essential fatty acid, arachidonic acid (AA), in one of the following molar concentrations: 0.5, 0.5, 50, and 500 μM in PBS 0.01% delipidated BSA or an equal volume of vehicle alone. macrophages were cultured for 24 h, supernatants harvested, and assayed for TNF-α production using a mouse TNF-α sandwich ELISA (R&D Systems). The concentration of TNF-α was normalized to the total protein in each well of macrophages which had determined that 500 ng/ml LPS was the optimal concentration for TNF-α production. The proportion of total cellular LDH released from the supernatant was used as a measure of injury and LDH was quantitated using a standard colorimetric LDH-specific assay (39).

**StatISTICS**

Values are expressed as mean ± SEM. Differences among groups were assessed by ANOVA and between groups by Students’ t test. Significant differences between groups are denoted: *, p < 0.05; **, p < 0.01.

**Results**

**I/R acute kidney injury results in biosynthesis and release of D series resolvins and protectins**

To explore the role of endogenously generated omega-3 DHA, and lipids derived from exogenously introduced omega-3 DHA, on recovery after injury to the kidney, DHA (62.5 μg/mouse) or vehicle was administered as a bolus to mice before ischemia and then infused by osmotic pump (dose 62.5 μg/mouse) over the succeeding 24 h. The impact of renal I/R in the presence and absence of concurrent administration of DHA on the biosynthesis and release of D series Rv and protectins was studied using LC-UV-MS-based informatics (Fig. 1). In contrast to sham-treated mice, ischemia followed by 24 h reperfusion triggered in kidney tissue the endogenous biosynthesis and/or release of the precursor DHA into the plasma (Fig. IA). The DHA-derived lipid mediator, PD1, and its biosynthetic intermediate, 17S-HDHA, and to a lesser extent RvD1 and RvD3, were also generated by the posts ischemic kidney, but not by the sham-operated kidney (Fig. 1, C and F). In plasma, endogenous DHA was markedly elevated by I/R (Fig. 1A) and there was an increase in the intermediate 17S-HDHA. In contrast to kidney tissue, however, there was no increase in plasma levels of PD1 or RvD1 in the absence of added DHA, whereas both RvD2 and RvD4 and to a lesser extent RvD3 levels were increased in plasma (Fig. 1, D and E). Both RvD5 and RvD6 were also identified at low levels in plasma after I/R in the vehicle group (Fig. ID). Increased levels of 17S-HDHA were found in posts ischemic kidneys in both the vehicle and DHA-treated groups, suggesting that I/R induced activity of the enzyme 15-lipoxygenase (Fig. 1, A and C), which is instrumental in transforming DHA to 17S-HDHA in vivo in mouse tissues (24, 33).

Exogenous administration of DHA to mice exposed to kidney I/R not only increased the amounts of unesterified DHA in both plasma and renal tissue (Fig. IA), but increased the tissue levels of RvD6 and RvD2. In addition, the level of the precursor 17S-HDHA was increased, as was the level of RvD1 and to a lesser extent RvD3. PD1 was notably not increased by DHA infusion (Fig. IC). Exogenous administration of DHA following ischemic kidney injury led to de novo generation of PD1 and RvD1 in plasma. RvD2 concentration was increased in plasma as were the levels of other Rv including RvD3, RvD5, and RvD6 (Fig. 1C). Of interest, administration of DHA did not directly change RvD4 levels in plasma or kidney tissue within this interval. The levels of 17S-HDHA were also increased in plasma with administration of DHA (Fig. ID). The identification of the DHA-derived bioactive mediators described above was conducted using a LC-MS-MS metabolomics approach generating ion chromatograms from LC-MS-MS (Fig. 1, E and F). The MS/MS spectrum at m/z 375 acquired at a LC retention time of 4.8 min on a representative plasma sample from mice 24 h following I/R is consistent with the structure of RvD2 with diagnostic ions at m/z 109 (129-2H2O-2H), 123 (142-H2O-H), 131 (129 + 2H), 203 (247-CO2-H), 209 (263-3H2O-H), 217 (233 + 2H-H2O), 227 (263-2H2O-H), 233, 241 (276-2H2O-H), 247 (246-H2O), 261 (263-2H), 277 (276-H), 287 (286-H2O-H), 293 (M-H-2H2O), 313 (M-H-2H2O-CO2-H), 331 (M-H-CO2), 339 (M-H-2H2O-CO2-H), 357 (M-H) (Fig. 1E) (24). The complete stereochirality of neuroprotectin D1/PD1 was recently established (41). PD1 was identified in mouse kidney 24 h following ischemia based on the MS/MS spectrum at m/z 359, which possesses diagnostic ions at m/z 235 (M-H), 341 (M-H-H2O), 323 (M-H-2H2O), 315 (M-H-CO2), 297 (M-H-H2O-CO2-H), 289, and 277 (M-H-2H2O-CO2-2H). Ions consistent with the carbon 10 and carbon 17 alcohol-containing positions were observed at m/z 153,
Because local inflammation is a prominent component in the pathophysiology of acute kidney injury (8) and DHA is the precursor of RvDs and PD1, we evaluated whether DHA was an effective therapy for ischemic kidney injury. Mice were given DHA by i.p. injection immediately before induction of bilateral I/R injury. At reperfusion, DHA therapy was continued over 25 h by s.c. osmotic-pump infusion. At 24 h after reperfusion, levels of plasma creatinine (a quantitative marker of renal failure) were measured (Fig. 2A). Neither creatinine levels nor kidney histology (examined at 24 h, data not shown) were different in the DHA-treated mice compared with the vehicle-treated mice. Furthermore, when histological injury was determined by a semiquantitative injury severity score determined by viewing PAS-stained kidney sections, there was no significant difference between DHA-treated and vehicle-treated sections (4.2 ± 0.3 vs 4.0 ± 0.2).

The RvDs display potent anti-inflammatory activity (30, 33, 42). We administered these compounds to mice in amounts related to their endogenous formation (1:2:1, RvD1:RvD2:RvD3; vide infra) 10 min before ischemia of both kidneys and during the reperfusion period. At 24 h after ischemia, we used LC-UV-MS-MS-based informatics to identify and quantify the DHA-derived compounds. Mice received 5 μg of DHA/g body weight or an equivalent volume of its vehicle. Details are shown in Materials and Methods. Sham-operated mice served as control. Data represent the mean from two independent experiments.

**FIGURE 1.** Endogenous DHA, Resolvin D series, and PD1 in murine renal tissues and plasma 24 h after ischemic injury. A, Concentration of DHA in kidney (left panel) and plasma (right panel) from sham-operated mice, mice 24 h following I/R kidney injury (vehicle), and mice 24 h following I/R injury with an infusion of DHA. B, The molecular structures of DHA-derived 17S-HDHA, PD1, RvD1, RvD2, and RvD3. C, DHA-derived products in kidney from sham-operated mice, mice 24 h post-kidney I/R injury, and mice 24 h post-I/R injury with an infusion of DHA. D, DHA-derived compounds in plasma from sham-operated mice, mice 24 h post-kidney I/R injury, and mice 24 h post-I/R injury with an infusion of DHA. Identification and quantification were conducted via LC-UV-MS-MS-based informatics. Mice received 5 μg of DHA/g body weight or an equivalent volume of its vehicle. Details are shown in Materials and Methods. Sham-operated mice served as control. Data represent the mean from two independent experiments. E, MS/MS spectrum at m/z 375 showing the production of RvD2 in mouse plasma 24 h following I/R. F, MS/MS spectrum at m/z 359 showing endogenous generation of PD1 in mouse kidney 24 h after ischemia.
cells. At low power (×4), there is little evidence of necrotic debris or proteinaceous debris. Distal tubules and ascending limbs are normal in appearance without tubule cell flattening and with fewer apoptotic cells. By comparison, kidneys from mice treated with both PD1 and RvDs show much milder disease. Many tubules have an intact brush border and those with necrosis show small amounts of intratubular debris (intense pink). By contrast, kidneys from mice treated with vehicle show widespread proximal tubular necrosis, with complete loss of tubular cells in some areas, lumina filled with debris and marked flattening of tubular cells in distal tubules and thick ascending limbs. In addition, many apoptotic cells are seen in the distal tubules, and interstitial cell infiltrate is visible. At lower power (×4), there is little evidence of necrotic debris or proteinaceous debris.

Subsequent 48-h interval via a s.c. mini pump. Half of the total dose of RvDs was given by infusion pump (Alzet). At both a total dose of 3.5 μg (140 ng/g body weight) or 35 μg (1.4 μg/g body weight), RvDs limited functional renal injury at 24 h as reflected by a lower plasma creatinine when compared with vehicle-treated animals (Fig. 2B). RvDs had no effect on plasma creatinine levels in sham-operated mice (data not shown).

PD1 administered to mice as above also protected kidneys. Both RvDs and PD1 not only attenuated peak creatinine levels at 24 h, but also appeared to have additional actions on the renal resolution process between 24 and 48 h. Plasma creatinine levels returned to near normal at 48 h in PD1-treated mice and in mice treated with 35 μg of RvDs, whereas in vehicle-treated mice plasma creatinine levels remained markedly elevated (Fig. 2C). A lower total dose of 3.5 μg (140 ng/g) had less efficacy compared with the higher dose of 35 μg (1.4 μg/g) at 24 h. Furthermore, PAS-stained sections of postsischemic kidney showed enhanced tubule cell survival, decreased renal inflammation, and decreased capillary occlusion in mice treated with 35 μg of either PD1 or RvDs (Fig. 3, Table I). From a behavioral perspective, mice with acute kidney injury secondary to ischemia exhibited reduced activity during the first 24 h of reperfusion. Mice treated with either RvDs or PD1 exhibited increased activity (Table II) compared with vehicle-treated mice, providing further evidence for the protective actions of these mediators.

**RvDs and PD1 reduce leukocyte accumulation**

Because we and others have strongly implicated leukocytes in the pathophysiology of ischemic kidney disease (5, 9, 12), we used two independent methods to quantify the actions of PD1 and RvDs on leukocyte involvement in kidney injury. Tissue MPO activity in whole kidney was assessed and leukocytes were stained with specific Abs for immunocytochemistry. Kidney MPO activity was decreased in mice treated with DHA, 17S-HDHA, PD1, and RvDs compared with vehicle-treated mice at both 24 and 48 h (Fig. 4). Both exogenous PD1 and RvDs reduced MPO activity by 80% compared with vehicle (Fig. 4B).

**FIGURE 2.** Kidneys are partially protected from ischemic injury with ProtecinD1, Resolvin D series, but not the precursor DHA. A, Mice with renal I/R injury were treated with vehicle (□), with DHA (125 μg/mouse), PD1 (3.5 μg/mouse) or the intermediate 17S-HDHA (17.5 μg/mouse), and plasma creatinine was measured 24 h following injury. Note that DHA does not protect kidneys from renal injury. B, Mice were treated with PD1 (3.5 μg (shaded) or 35 μg/mouse (■) (24)), RvDs (3.5 μg (shaded) or 35 μg/mouse (■) (24)) and plasma creatinine measured 24 h later. Note a dose-dependent increase in protection with both compounds. C, Mice were treated with PD1 (3.5 μg (shaded) or 35 μg/mouse (■) (24)), RvDs (3.5 μg (shaded) or 35 μg/mouse (■) (24)), and plasma creatinine was measured 48 h later. Note that at high concentrations both compounds result in more rapid declines in plasma creatinine from 24 to 48 h than vehicle, but at lower doses, RvDs-treated mice show similar plasma creatinine levels at 24 and 48 h after injury, albeit in both cases lower than vehicle-treated animals (*, p < 0.05, **, p < 0.01 compared with vehicle-treated controls).

**FIGURE 3.** Histological demonstration of protection from ischemic injury by PD1 or RvDs. Forty-eight hours following ischemic injury, kidneys were sectioned and stained by the PAS method. At higher magnification (×20) (upper panels) of the outer medullary region, vehicle-treated kidneys show widespread proximal tubular necrosis, with complete loss of tubular cells in some areas, lumina filled with debris and marked flattening of tubular cells in distal tubules and thick ascending limbs. In addition, many apoptotic cells are seen in the distal tubules, and interstitial cell infiltrate is visible. At lower power (×4) (lower panels), the whole outer medullar area shows confluent necrosis as distinguished by pale pink (arrows), and more proximally many tubules show proteinaceous debris (intense pink). By comparison, kidneys from mice treated with both PD1 and RvDs show much milder disease. Many fewer proximal tubules show necrosis (×20), many tubules have an intact brush border and those with necrosis show small amounts of intratubular debris alongside intact surviving tubule cells. Distal tubules and ascending limbs are normal in appearance without tubule cell flattening and with fewer apoptotic cells. At low power (×4), there is little evidence of necrotic debris or proteinaceous debris.
Furthermore, PD1 reduced MPO activity by 67% 24 h after ischemia. It is important to note that, while high-dose DHA reduced MPO activity by 41% (Fig. 4A), it did not prevent the rise in creatinine seen with I/R as discussed above (Fig. 2).

The degree of monocyte and neutrophil infiltration was assessed quantitatively by morphometry using Abs to CD11b, which is expressed by both PMNs and monocytes, Abs to GR-1, which is expressed by PMNs, and Abs to CD68, which is expressed by monocytes (Fig. 5). Both PD1 and RvDs treatments resulted in quantitatively less area of CD11b, GR-1, and CD68 immunostaining objectively assessed by morphometry (35). Thus, tissue PMNs and monocytes were reduced by both PD1 and RvDs (Fig. 5).

Administration of RvDs or RvD1 after onset of ischemic injury is protective

Our novel compounds demonstrated efficacy in protecting kidneys from severe injury and promoting resolution. In the above experiments, however, the injured kidneys were exposed to the compounds prior and subsequent to onset of injury. To determine whether Rv and protectins retained efficacy when reaching the kidney after the onset of injury, further studies were designed. In these, Rv and protectins were administered after ischemic injury. Furthermore, the compounds were given i.p. rather than i.v. (followed by s.c. infusion) to slow the rate at which they might gain access to the kidneys. Kidneys treated with RvDs or synthesized RvD1 exhibited marked protection from development of acute renal failure (Fig. 6). PD1 given after ischemic injury did not significantly alter the course of acute renal failure compared with vehicle-treated mice.

RvDs and PD1 limit leukocyte activation but do not directly protect proximal tubule cells from oxidative agents

To study the impact of these novel renoprotective DHA-derived mediators in more detail, we addressed their actions in vitro. The generation of oxygen free radicals and hydrogen peroxide are implicated in the pathogenesis of both ischemic and toxic acute kidney injury (43, 44). To model tubular cell injury observed in vivo, we tested whether Rv and protectins protected cultured proximal tubular epithelial cells from H2O2 injury using an established assay (39). The effects of AA and DHA were examined in parallel. None of the anti-inflammatory compounds (RvDs, PD1, or DHA, or AA) at concentrations as high as 500 nM, had any effect on the H2O2-induced release of LDH from cultured tubular cells (Fig. 7A). In each case, despite the presence of relatively high concentrations of RvDs and PD1, ~25% of total LDH was released from cells by H2O2 exposure. By contrast, in cells not treated with H2O2, 7.3 ± 1.9% of total LDH was released in control conditions. LDH release from cells not treated with H2O2 was not affected by these compounds (data not shown). These results suggest that the actions of these mediators in the kidney were not the direct result of tubule cell protection from oxidative influences.

In addition to H2O2, the monocyte/macrophage proinflammatory cytokine TNF-α has also been implicated in the pathogenesis of ischemic acute kidney injury (45, 46). We prepared cultured macrophages derived from bone marrow and tested whether DHA, RvDs, or PD1, administered simultaneously with a proinflammatory stimulus LPS, limited TNF-α generation in response to cell activation (Fig. 7B). At low concentrations, DHA itself had no suppressive effect on TNF-α release in macrophages by LPS. At higher concentrations, DHA reduced TNF-α release by 22.0 ± 1.6% (Fig. 7B). By contrast, RvD1 afforded a dose-dependent reduction in LPS-induced TNF-α release, and had notable actions on TNF-α release at concentrations 100 times lower than those required with its precursor, DHA. PD1 also lowered LPS-induced TNF-α release, although this was less marked than RvD1, suggesting that both active compounds have anti-inflammatory activities directed at monocytes/macrophages.

RvDs and PD1 limit postischemic interstitial kidney fibrosis

Even though there is recovery of the kidney functionally following renal pedicle clamping and reperfusion, as measured by serum creatinine, histology reveals incomplete resolution with progressive interstitial fibrosis over weeks (10). This fibrosis may result

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**Table I. Histological injury score for kidneys of mice treated with vehicle, PD1, or RvDs**

<table>
<thead>
<tr>
<th></th>
<th>Vehicle</th>
<th>PD1</th>
<th>RvDs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Score</td>
<td>4.25 ± 0.17*</td>
<td>3.25 ± 0.29</td>
<td>3.5 ± 2.4</td>
</tr>
</tbody>
</table>

*The dose of PD1 was 35 μg and RvDs was 35 μg. Scores were determined at 48 h postinjury (see Materials and Methods for details). Sham-operated mice had histological injury score of 1.0 irrespective of treatment; *, p = 0.017 (ANOVA).

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**Table II. Mouse activity score following ischemia in mice treated with PD1 or RvDsa**

<table>
<thead>
<tr>
<th></th>
<th>Vehicle</th>
<th>PD1</th>
<th>RvDs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Score</td>
<td>2.1 ± 0.2*</td>
<td>2.8 ± 0.3</td>
<td>2.7 ± 0.4</td>
</tr>
</tbody>
</table>

*The dose of PD1 was 35 μg and RvDs was 35 μg. Activity scores were determined at 24 h. Sham-operated mice had activity scores of 3.0 irrespective of treatment; *, p = 0.03 (ANOVA).
from persistence of inflammatory leukocytes, in particular interstitial macrophages (47). Interstitial fibrosis and persistent leukocyte infiltration (chronic inflammation) are the harbingers of scarring and chronic renal failure. As many as 40% of patients with acute kidney injury are left with worsening of their baseline kidney status and chronic disease after recovery from the acute injury (3, 48). We assessed whether RvDs and PD1 could limit the deposition of interstitial collagens that contribute to fibrosis. Kidney sections stained for collagen by Gomori’s trichrome method were assessed by quantitative computerized morphometry to assess the region defined as the area of collagen deposition 15 days after I/R. RvDs treatment for 72 h after ischemia resulted in 44 ± 17% less deposition of collagen, whereas PD1 was less effective (a nonsignificant reduction of 21 ± 12% in reducing scarring at 15 days (Fig. 8)).

Discussion

We demonstrate for the first time that two families of newly identified anti-inflammatory mediators, D series Rv and PD1, are generated in the kidney in response to I/R. These mediators are generated by stereospecific enzymatic pathways both in the presence and absence of exogenous administration of precursor DHA. When infused peripherally in mice, they markedly attenuate ischemic kidney injury as well as reduce fibrosis. One of the actions of these compounds in the kidney is to limit the influx of leukocytes and another is to limit activation of leukocytes. Therefore, the pathways described are likely to counterregulate the proinflammatory, cellular and molecular signaling that results from I/R. This represents a new paradigm in the pathophysiology of acute kidney injury, which often leads to acute renal failure. Hence, these anti-inflammatory and counterregulatory influences may be critical to the resolution of damage and limitation of chronic fibrosis resulting from this damage.

More PD1 than RvD is produced with I/R, especially in the absence of exogenously administered DHA. RvDs and PD1 displayed profound protective effects within 24 h of I/R (PMN dominated) that persisted for 48 h (monocyte dominated). RvDs were apparently more effective than PD1 in reducing scarring 15 days after I/R injury.
after ischemia and were much more effective at reducing injury when initially administered after reperfusion. Experiments with epithelial cells in vitro suggest that protection in vivo is not the result of a direct effect on the tubular epithelial cells of the kidney but more likely related to the ability of these mediators to down-regulate components of the inflammatory response involving infiltrating cells.

We have now shown that endogenously generated RvDs and PD1 protect from ischemic injury in the kidney. PD1, also known as NPD1 when generated in neuronal tissues (41, 49), is protective in the brain (33). There is currently a need for effective therapies for diseases in humans resulting from ischemia to the kidney as well as brain, both of which are characterized by uncontrolled local inflammation, which is believed to contribute to acute and chronic functional impairment (2, 33). RvDs and PD1 both limit infiltration of leukocytes and also limit activation of leukocytes in both postischemic organs. It is possible that both compounds may have additional cellular sites of actions in the kidney, i.e., on the endothelium and vascular tone, as well as interstitial fibroblasts because they are also antifibrotic.

In an experimental model of stroke, there was active generation of both PD1 and RsD1, the former peaking 10 h after injury, and the latter peaking 24 h after injury (33). Infusion of PD1 into ventricles of the brain following stroke markedly attenuated stroke area as measured 48 h after ischemia (33). RvDs and PD1 in sterile peritonitis are generated during the resolution phase, and administration of both RvDs and PD1 not only limited neutrophil influx but also limited both chemokines and proinflammatory cytokines in the inflammatory exudates (26).

It is notable that we found that the endogenous level of the biosynthetic precursor of RvDs and PD1, DHA, was increased in response to injury. This likely reflects an activation of cellular lipases (i.e., PLA2) enzymes, which can cleave DHA from phospholipids. Because increased levels of DHA in tissue and plasma alone does not account for the increased generation of D series Rv and PD1 found with I/R, renal ischemic injury may up-regulate the local formation of RvDs and PD1 (24, 33). Endogenously generated anti-inflammatory mediators with or without exogenous DHA administration may be present in insufficient quantities in normal mice during acute kidney injury to provide adequate protection of the organ from innate immune-mediated injury.

Our studies also demonstrate that RvDs and synthesized RvD1 are effective in attenuating renal injury when administered after the insult. These findings indicate that these “proresolution-of-inflammation” compounds may not simply block the activation of the inflammatory response pathways but that they are bioactive during the acute injury phase and can actively counteract inflammation...
and injury. The fact that equimolar administration of RvD1 appears more potent in protecting kidneys from injury than the mixture of RvDs in the postinjury administration of compounds suggests that RvD1 might be more potent than RvD2 and RvD3. Further studies will be required to determine whether RvD1 is more potent at blocking neutrophil activation or chemotaxis. Our studies suggest that PD1 in the kidney is not efficacious in promoting resolution once the injury is established. This may reflect the reduced efficacy of PD1 after established disease onset, and point to a distinct mechanism of action from the RvDs series, or possibly reflects inadequate bioavailability. In the studies in which PD1 is effective, doses of both 3.5 and 35 μg were effective, whereas in the postinjury studies, a single dose of 10 μg was chosen due to limitation of availability of the compound. It is possible that after injury onset higher doses of PD1 are required to achieve therapeutic levels in the injured kidneys. Further studies will be required.

Earlier studies focused on the actions of RvDs and PD1 on PMN activity and chemotaxis (24, 27, 33, 41). In the present study, we also implicate these compounds in limiting macrophage activation showing, for the first time, that LPS-induced activation of macrophages is reduced by PD1 and to a lesser extent by RvDs even when administered at the same time as LPS-induced activation, without the necessity for pretreatment. Because LPS acts through TLR4 and to a lesser extent TLR2 it is likely that these mediators play an essential role in resolution of acute kidney injury. Moreover, we uncovered an antifibrotic action of these mediators and recognized endogenous anti-inflammatory response to injury may be of therapeutic importance in treating acute kidney injury.

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Disclosures

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References


