15-Epi-16-(Para-Fluorophenoxy)-Lipoxin A₄-Methyl Ester, a Synthetic Analogue of 15-epi-Lipoxin A₄, Is Protective in Experimental Ischemic Acute Renal Failure

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Abstract. Lipoxins are endogenous lipoxygenase-derived eicosanoids, generated during inflammatory, hypersensitivity, and vascular events, that display vasodilatory, antiinflammatory, and pro-resolution activity. Here, we evaluated the efficacy of 15-epi-16-(para-fluorophenoxy)-lipoxin A₄-methyl ester (15-epi-16-(FPhO)-LXA₄-Me), a stable synthetic analogue of aspirin-triggered 15-epi-lipoxin A₄ in ischemic acute renal failure (ARF) in NIH Swiss mice. ARF was induced by 30-min crossclamping of renal pedicles and was associated with elevated serum creatinine, morphologic injury, polymorphonuclear leukocyte (PMN) recruitment, and increased mRNA levels for adhesion molecules (intercellular adhesion molecule–1 [ICAM-1] and vascular cell adhesion molecule–1 [VCAM-1]), chemokines (growth regulated oncogene-1 [GRO1]), and cytokines (interleukin–1β [IL-1β] and IL-6) after 24-h reperfusion. A single bolus of 15-epi-16-(FPhO)-LXA₄-Me afforded striking functional (mean SEM creatinine in mg/dl: sham-operated, 0.77 ± 0.04; ARF vehicle, 2.49 ± 0.19; ARF + 15-epi-16-(FPhO)-LXA₄-Me, 0.75 ± 0.12; P < 0.001) and morphologic protection and reduced PMN infiltration. Treatment with 15-epi-16-(FPhO)-LXA₄-Me was also associated with lower IL-1β, IL-6, and GRO1 mRNA levels, whereas ICAM-1 and VCAM-1 mRNA levels were unchanged. Compatible with these results, LXA₄ blunted chemoattractant-stimulated PMN migration across HK-2 renal epithelial cell monolayers in vitro, but it did not inhibit cytokine-induced HK-2 ICAM-1 expression or adhesiveness for PMN. Interestingly 15-epi-16-(FPhO)-LXA₄-Me–treated animals also displayed increased renal mRNA levels for suppressors of cytokine signaling–1 (SOCS-1) and SOCS-2, but not CIS-1, endogenous inhibitors of cytokine-elicited Jak/Stat-signaling pathways. These results indicate that 15-epi-16-(FPhO)-LXA₄-Me is protective in renal ischemia reperfusion injury in vivo, at least partially by modulating cytokine and chemokine expression and PMN recruitment, and provides a rationale for further exploration of the efficacy of LXA₄ structural analogues in ischemic ARF and other renal diseases.

Ischemic acute renal failure (ARF) remains a formidable clinical problem for which there is no specific treatment (1). The pathophysiology of ARF is multifaceted and includes persistent intrarenal vasoconstriction, hypoxic tubule epithelial cell injury, and polymorphonuclear leukocyte (PMN)–mediated cytotoxicity upon reperfusion (1,2). Despite the impressive efficacy of agents that specifically target these processes in experimental models, none has proved effective in randomized controlled clinical trials (1). These disappointing results have shifted attention toward regimens that simultaneously target two or more of the aforementioned pathophysiologic events.

Lipoxins (LX) are lipoxygenase-derived arachidonate metabolites that are generated in a variety of human and experimental inflammatory, hypersensitivity, and vascular diseases (reviewed in references 3–5). They are generated principally by transcellular routes during cell-cell interactions by biosynthetic pathways initiated through the action of two lipoxygen-
ases (either 5- and 15-lipoxygenase or 5- and 12-lipoxygenase) on arachidonic acid (3–5). In the presence of aspirin, cyclooxygenase-2 (COX-2) retains the enzymatic capacity to generate 15R-HETE (3). In the context of neutrophil-endothelial cell interactions, neutrophils can convert endothelial cell–derived 15R-HETE to epimers of native lipoxins in which the hydroxyl group at the carbon-15 is in the R rather than the S configuration (3). These aspirin-triggered lipoxins (ATLs) retain many of the bioactivities of native LX (see below) (3). The bioactivity profile reported to date for the native LX and ATLs in vitro and in vivo suggests that these eicosanoids may confer benefit in renal ischemia reperfusion injury. Lipoxins are potent intrarenal vasodilators, inhibit PMN chemotaxis, adhesion, and migration across endothelium and gastrointestinal epithelium, promote clearance of apoptotic PMN, and modulate several cytokine responses (6–12). Analogues of the major mammalian lipoxins, namely LXA4 and LXB4, and of ATLs have been synthesized that are relatively resistant to degradation and share many actions of native LX and ATLs in vitro (13). Native LXA4, ATLs, and several synthetic LX analogues have already been demonstrated to have impressive antiinflammatory activity in experimental dermal inflammation, glomerulonephritis, and/or hind limb–induced second organ injury (14–19).

Against this background, we evaluated the effect of a stable analogue of aspirin-triggered 15-epi-LXA4, namely 15-epi-16-(FPhO)-LXA4-Me, in experimental murine ARF in vivo.

Materials and Methods
15-Epi-16-(Para-Fluorophenoxy)-Lipoxin A4-Methyl Ester

The preparation of 15-epi-16-(FPhO)-LXA4-Me by total organic synthesis and its physical properties have been reported previously (15). This compound is a methyl ester of an analogue of 15-epi-LXA4 in which a bulky (para-fluoro)-phenoxy group replaces the ω-chain at C16 (15). It is more stable than LXA4 in murine whole blood ex vivo (15).

Induction and Analysis of Murine Ischemic Acute Renal Failure

ARF was induced in NIH Swiss mice (25 to 35 g) by clamping both renal pedicles for 30 min. Renal function and morphology were assessed 24 h after reperfusion as previously reported (20–22). PMN infiltration was assessed using the specific antibody Gr-1 (Pharmin- gen, San Diego, Ca) and myeloperoxidase (MPO) tissue activity (20). Animals received a 15-μg single bolus injection of 15-epi-16-(FPhO)-LXA4-Me or an equivalent volume of its vehicle into the inferior vena cava 10 min before clamping. Sham-operated animals served as controls.

Renal mRNA levels were assessed by reverse transcriptase–PCR (RT-PCR) for intercellular adhesion molecule-1 (ICAM-1) (5'-CTGTGGGAGGAGATCAGAG-3', 5'-CTTCAGAGGCCAAGACTCAAG-3'), vascular cell adhesion molecule-1 (VCAM-1) (5'-GAGGCTC- CTTGGTTTTGAGG-3', 5'-ACCTAGCGAGGCAAACAAGA-3'), interleukin-1β (IL-1β) (5'-CAGGCGAGCTGATCACATCA-3', 5'-AGCTCATATGGGTCCGACAG-3'), IL-6 (5'-ATATCACAGCCAGAAGATC-3'), GRO1 (murine homologue of human IL-8) (5'-TGTGTGCGGAAAGAG-AGTGC-3', 5'-CGAGACGAGACGAGAGAAA-3'), suppressors of cytokine signaling-1 (SOCS-1) (5'-ATGGTAGACGTAAACCAG-3', 5'-CTCAGCACGCTGAGAAC-3'), SOCS-2 (5'-AAATTAAAGGAAGCCACGAA-3', 5'-AATGTGATCGCCAGAGA-3'), CIS-1 (5'-TCTCCTACTCCGGGAATCT-3', 5'-CCAGTCGGAAGCTGA- GTCG-3'), and GAPDH (5'-CTCATGACAGCTTCATGTC-3', 5'- CCGTGTGCTGTAGCCGTAT-3') using a standard PCR reaction protocol with an annealing temperature of 58°C.

PMN Interactions with HK-2 Tubule Epithelial Cells

PMN were isolated from normal human volunteers as previously reported (8), and their interaction with HK-2 tubule epithelial cells was assessed as previously reported for PMN transmigration across monolayers of T84 colonic carcinoma cells (9). ICAM-1 protein expression was assessed by FACS analysis.

Statistical Analyses

Statistical analyses were performed using the t test in which P < 0.05 was deemed significant.

Results

15-Epi-16-(FPhO)-LXA4-Me Confers Morphologic and Functional Protection against Ischemic Acute Renal Failure

Prior exposure of mice to 15-epi-16-(FPhO)-LXA4-Me (15 μg/mouse) resulted in a protection against histologic damage as determined by relative preservation of tubule epithelial integrity, an increased number of tubular nuclei, and a reduction in the number of intratubular casts (Figure 1A). This morphologic protection was paralleled by functional protection as determined by a lesser increment in serum creatinine levels (Figure 1B).

Treatment with 15-Epi-16-(FPhO)-LXA4-Me Is Associated with Less PMN Accumulation and Reduced mRNA Levels for IL-1β, IL-6, and GRO1, but not ICAM-1 and VCAM-1

Ischemia reperfusion injury was associated with PMN infiltration of the renal parenchyma as determined by an increase in Gr-1–positive intratubular cells in tissue sections (Figure 2A) and increased MPO activity in renal tissue homogenates (Figure 2B). Both indices of PMN infiltration were significantly reduced in mice treated with 15-epi-16-(FPhO)-LXA4-Me (15 μg/mouse) before induction of ischemic ARF (Figure 2).

Renal ischemia reperfusion injury was associated with increased renal mRNA levels for the pro-inflammatory cytokines IL-1β, IL-6, and GRO1 and the adhesion molecules ICAM-1 and VCAM-1, as assessed by semiquantitative RT-PCR. These increments in mRNA levels for IL-1β, IL-6, and GRO1, but not ICAM-1 and VCAM-1, were attenuated by 15-epi-16-(FPhO)-LXA4-Me (Figure 3A).

In keeping with these in vivo studies, LXA4 (10−7 to 10−9 M) attenuated PMN adhesion to and basolateral-apical transmigration across HK-2 tubule epithelial cells triggered by the classic peptide chemoattractant fmet-leu-phe, but it did not attenuate TNF-α–induced (1 to 10 ng/ml for 24 to 48 h)
increments in ICAM-1 expression or epithelial cell adhesive-
ness (data not shown).

**Modulation of Renal mRNA Levels for the Suppressors of Cytokine Signaling SOCS-1 and SOCS-2 by 15-Epi-16-(FPhO)-LXA₄-Me**

The suppressors of cytokine signaling (SOCS) are endogenous inhibitors of Jak/Stat signaling events elicited by engagement of cytokine receptors. Interestingly, treatment of mice with 15-epi-16-(FPhO)-LXA₄-Me (15 µg/mouse) was associated with increased renal mRNA levels for SOCS-1 and SOCS-2, but not the related SOCS family member CIS-1 (Figure 3B).

**Discussion**

Lipoxins are generated in a variety of experimental and human diseases, including glomerulonephritis, rheumatoid arthritis, asthma, and sarcoidosis (3–5). Of particular interest in the setting of ischemic reperfusion injury, LX are also gener-
ated in the coronary vascular lumen after angioplasty (23). A compelling body of evidence, compiled from model systems in vitro and in vivo, suggests that the lipoxins are not just anti-inflammatory braking signals in inflammation but also important stimuli for resolution (3–5). The recognition that lipoxin epimers are generated through a COX-2–dependent pathway in the presence of aspirin and that these ATLs shared many of the bioactivities of native LX in vitro was thus intriguing, as it raised the possibility that some antiinflammatory drugs currently in use may act, at least partially, by influencing the profile of LX and ATLs in an inflammatory milieu. The protective effects of a lipoxin stable analogue against renal ischemia reperfusion injury noted in this study establishes the therapeutic potential of lipoxin bio-mimetics in renal disease and lays the foundation for further exploration of the structural characteristics and temporal requirements for optimal renoprotection in ischemic ARF.

The renoprotective effect of 15-epi-16-(FPhO)-LXA4-Me

Figure 2. Influence of 15-epi-16-(FPhO)-LXA4-Me on polymorphonuclear leukocyte (PMN) staining and myeloperoxidase (MPO) activity after renal ischemia reperfusion injury. PMN infiltration was assessed after 24 h of reperfusion, using the PMN-specific antibody Gr-1 (A) and also using MPO activity (B); n = 3 for sham-operated controls and n = 5 for 15-epi-16-(FPhO)-LXA4-Me (15 μg/mouse) and vehicle-treated ARF; *P < 0.001.
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Figure 3. Differential effects of 15-epi-16-(FPhO)-LXA₄-Me on renal mRNA levels for some key determinants of PMN recruitment and cytokine bioactivity after renal ischemic reperfusion injury. (A) mRNA levels for interleukin–1β (IL-1β), IL-6, growth regulated oncogene–1 (GRO-1), intercellular adhesion molecule–1 (ICAM-1), and vascular cell adhesion molecule–1 (VCAM-1) in ARF after 24-h reperfusion, each lane representing an individual animal; sham-operated controls, 15-epi-16-(FPhO)-LXA₄-Me provides a prototype compound for testing in other renal diseases, such as acute and chronic glomerulonephritis. The latter are logical and attractive targets, given that LXA₄ attenuates PMN recruitment in acute immune complex glomerulonephritis (17) and that LXA₄ also inhibits mesangial cell proliferation triggered by activation of the PDGF receptor (27), a putative central event in the pathogenesis of many forms of chronic glomerulonephritis.

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References

was associated with reduced PMN infiltration, but whether the latter was itself a consequence of less severe injury to the renal parenchyma during hypoxia in LX-treated animals or a direct effect of the LX analogue on PMN in this setting is unclear. Furthermore, our experimental design also did not allow us to distinguish between inhibition of PMN recruitment and stimulation of PMN clearance. It is likely that both contributed. LXA₄ is a potent inhibitor of PMN chemotaxis and beta-2 integrin–mediated PMN adhesion to endothelium (7,8). LXA₄ also blunts endothelial hyperadhesiveness for PMN induced by mobilization of endothelial P-selectin (8). Our observations that 15-epi-16-(FPhO)-LXA₄-Me and LXA₄ modulate intrarenal cytokine expression during ischemic ARF in vivo and attenuate PMN interactions with HK-2 renal epithelial cells in vitro, respectively, builds on previous reports that LXA₄ attenuates PMN interactions with gastrointestinal epithelial cell lines and IL-8 release from cytokine-activated gastrointestinal epithelial cells in vitro and protects epithelial cells in colonic strips against cytokine-triggered cytotoxicity ex vivo (7–11). Regarding PMN clearance, lipoxins are potent stimuli for nonphlogistic phagocytosis of apoptotic PMN by human monocyte-derived macrophages in vitro and in thioglycollate-induced peritonitis in vivo (12,24). A protective role for 15-epi-16-(FPhO)-LXA₄-Me through a direct action on other leukocyte subsets cannot be excluded. The finding of reduced mRNA levels for IL-1β, IL-6, and GRO-1 in 15-epi-16-(FPhO)-LXA₄-Me–treated animals in association with increased expression of SOCS-1 and SOCS-2 is intriguing given the putative role for SOCS as endogenous inhibitors of cytokine bioactivities transduced through Jak/Stat signal transduction pathways (25,26). Indeed, SOCS-1 and SOCS-2 can specifically inhibit IL-6–mediated signaling events (26), suggesting a novel mechanism through which 15-epi-16-(FPhO)-LXA₄-Me could modulate cytokine bioactivity in disease.

Our results do not exclude an additional renoprotective action of 15-epi-16-(FPhO)-LXA₄-Me through modulation of renal hemodynamics. Indeed, given the documented ability of LXA₄ to counter the vasoconstrictive effects of cysteinyl-leukotrienes within the renal vascular bed (6), it is highly likely that the renoprotective efficacy of 15-epi-16-(FPhO)-LXA₄-Me in ischemic ARF is due to multipronged effects on the renal vascular tone, PMN trafficking, and epithelial cell integrity. The small quantities of 15-epi-16-(FPhO)-LXA₄-Me (15 µg/mouse) required to confer renoprotection was particularly impressive in this study. In the broader context of renal disease, the impressive efficacy of 15-epi-16-(FPhO)-LXA₄-Me provides a prototype compound for testing in other renal diseases, such as acute and chronic glomerulonephritis. The latter are logical and attractive targets, given that LXA₄ attenuates PMN recruitment in acute immune complex glomerulonephritis (17) and that LXA₄ also inhibits mesangial cell proliferation triggered by activation of the PDGF receptor (27), a putative central event in the pathogenesis of many forms of chronic glomerulonephritis.