

Reduction of Renal Ischemia-Reperfusion Injury in 5-Lipoxygenase Knockout Mice and by the 5-Lipoxygenase Inhibitor Zileuton

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ABSTRACT

The role of 5-lipoxygenase (5-LOX) in the pathophysiology of renal ischemia/reperfusion (I/R) injury is not known. Here we investigate the effects of 1) the 5-LOX inhibitor zileuton and 2) 5-LOX gene knockout (5-LOX^{-/-}) mice on renal dysfunction and injury caused by I/R of the kidney in mice. Wild-type mice treated with zileuton (3 mg/kg i.v.) or 5-LOX^{-/-} mice were subjected to bilateral renal artery occlusion (30 min) followed by reperfusion (24 h). Plasma urea, creatinine, and aspartate aminotransferase (AST) were measured as markers of renal dysfunction and reperfusion injury. Kidneys were used for histological evaluation of renal injury. Renal myeloperoxidase activity was measured and used as an indicator of polymorphonuclear leukocyte (PMN) infiltration and renal expression of intercellular adhesion molecule-1 (ICAM-1) was determined us-

ing immunohistochemistry. Administration of zileuton before I/R significantly reduced the degree of renal dysfunction (urea, creatinine) and injury (AST, histology). In addition, zileuton reduced the expression of ICAM-1 and the associated PMN infiltration caused by I/R of the mouse kidney. Compared with wild-type mice, the degree of renal dysfunction, injury, and inflammation caused by I/R in 5-LOX^{-/-} mice was also significantly reduced, confirming the pathophysiological role of 5-LOX in the development of renal I/R injury. We propose that 1) endogenous 5-LOX metabolites enhance the degree of renal injury, dysfunction, and inflammation caused by I/R of the kidney by promoting the expression of adhesion molecules, and 2) inhibitors of 5-LOX may be useful in the treatment of conditions associated with I/R of the kidney.

Leukotrienes (LT) are metabolites of arachidonic acid formed from the 5-lipoxygenase (5-LOX) pathway and exert potent vasoactive and pro-inflammatory effects. LTs play a pivotal role in the pathophysiology of asthma (Samuelsson, 1983; Wenzel, 2003) and psoriasis (Brain et al., 1982; Wedi and Kapp, 2001), as well as in conditions associated with ischemia-reperfusion (I/R) of skin (Dolan et al., 1998a), brain (Ciceri et al., 2001), and kidney (Klausner et al., 1989; Carter et al., 1991). LTs also play a physiological role in the host defense against microbial infections (Demitsu et al., 1989). The activation of 5-LOX is calcium-dependent (Rouzer and Samuelsson, 1985), and 5-LOX acts together with 5-LOX-activating protein to form LTA₄ (Dixon et al., 1990). LTA₄ is unstable and is rapidly converted to either LTB₄ or the cys-

teinyll-LTs, LTC₄, LTD₄, and LTE₄. 5-LOX is predominantly expressed by cells of myeloid origin, particularly neutrophils, eosinophils, macrophages/monocytes, and mast cells (Miller et al., 1990; Reid et al., 1990).

LTB₄ is a key mediator in the pathophysiology of the renal dysfunction caused by I/R of the kidney as well as the associated infiltration of the kidney with polymorphonuclear cells (PMN) (Noiri et al., 2000). LTB₄ activates PMNs, thus changing their shape and promoting their binding to endothelium by inducing the expression of cell-adhesion molecules. After PMN transmigration into ischemic renal tissue, PMNs release reactive oxygen species, proteases, elastase, myeloperoxidase (MPO), cytokines, and various other mediators (Rabb et al., 1997), all of which exacerbate inflammation and contribute to tissue injury (positive feedback). For instance, ROS will react with the polyunsaturated membrane lipids (Rao et al., 1983), and lipid per-

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ABBREVIATIONS: LT, leukotriene; 5-LOX, 5-lipoxygenase; I/R, ischemia-reperfusion; PMN, polymorphonuclear cell; MPO, myeloperoxidase; AST, aspartate aminotransferase; MOPS, 3-(N-morpholino)propanesulfonic acid; ICAM-1, intercellular adhesion molecule-1.

oxidation, in turn, will enhance the tissue levels of free arachidonic acid (Sevanian and Kim, 1985). The 5-LOX inhibitor, zileuton, is highly effective at preventing LT formation in vitro, ex vivo, and in vivo (Carter et al., 1991) and is currently in clinical use for the treatment of patients with asthma (1997).

In this study, we investigated the effects of zileuton on the renal dysfunction and injury caused by I/R of the kidney of the mouse in vivo. To ensure that an enhanced formation of LTs from 5-LOX does indeed contribute to renal I/R injury, we have compared the degree of injury/dysfunction caused by I/R in wild-type mice treated with zileuton with that in mice in which the gene for 5-LOX is absent (*5-LOX*^{-/-}).

Materials and Methods

Animals. Thirty-three male C57BL/6J wild-type and 12 male *5-LOX*^{-/-} mice (25–30 g, purchased from Harlan Nossan, Milan, Italy) were used in this study. Mice were allowed access to food and water ad libitum and were cared for in compliance with Italian regulations on protection of animals used for experimental and other scientific purposes (D.M. 116192), as well as with the European Economic Community regulations (O.J. of E.C. L358/1 1986 Dec 18).

Renal Ischemia/Reperfusion. Mice were anesthetized using chloral hydrate (125 mg/kg, i.p.), and core body temperature was maintained at 37°C using a homeothermic blanket. After performing a midline laparotomy, mice were then divided into the following six groups: 1) I/R wild-type group, wild-type mice that underwent renal ischemia for 30 min followed by reperfusion for 24 h (*n* = 13); 2) I/R wild-type zileuton (3 mg/kg) group, wild-type mice that were administered zileuton (3 mg/kg i.v. bolus via the tail vein) 15 min before I/R and after 12 h of reperfusion (*n* = 7); 3) I/R *5-LOX*^{-/-} group, *5-LOX* knockout mice that underwent renal ischemia for 30 min followed by reperfusion for 24 h (*n* = 8); 4) sham wild-type group, wild-type mice that were subjected to the surgical procedures described above but were not subjected to renal I/R (*n* = 9); 5) sham wild-type zileuton (3 mg/kg) group, wild-type mice that were treated identically to sham wild-type mice except for the administration of zileuton (3 mg/kg i.v. bolus via the tail vein) 15 min before sham-I/R and after 12 h of reperfusion (*n* = 4); and 6) sham *5-LOX*^{-/-} group, *5-LOX* knockout mice that were subjected to the surgical procedures described above but were not subjected to renal I/R (*n* = 4).

The route of administration and dose of zileuton used in this study were based on those previously used in the mouse (Qian et al., 2001). Because of the short half-life of zileuton (1–2 h), a further dose of zileuton (3 mg/kg i.v.) was administered at the midpoint of the reperfusion period (12 h after renal ischemia). Mice that did not receive zileuton were administered 8 ml/kg 1% dimethyl sulfoxide (vehicle for zileuton) at equivalent time points (15 min before renal ischemia and 12 h after). Mice were maintained under anesthesia for the duration of ischemia (i.e., 30 min). After performing a midline laparotomy, mice from the I/R groups were subjected to bilateral renal ischemia for 30 min, during which the renal arteries and veins were occluded using microaneurysm clamps (Chatterjee et al., 2003a). The time of ischemia chosen was based on that found to maximize reproducibility of renal functional impairment while minimizing mortality in these animals (Chatterjee et al., 2003a). After the renal clamps were removed, the kidneys were observed for a further 5 min to ensure reflow, after which 1 ml of saline at 37°C was injected into the abdomen and the incision was sutured in two layers. Mice were then returned to their cages, where they were allowed to recover from anesthesia and observed for 24 h. Sham-operated mice underwent surgical procedures identical to those of I/R mice except that microaneurysm clamps were not applied.

Measurement of Biochemical Parameters. At the end of the reperfusion period, 1-ml blood samples were collected from anesthetized mice via cardiac puncture. The samples were centrifuged (2213g for 3 min) to separate plasma. All plasma samples were

analyzed for biochemical parameters within 24 h after collection. Plasma urea and creatinine concentrations were used as indicators of renal (glomerular) function (Chatterjee et al., 2003a). The rise in the plasma levels of aspartate aminotransferase (AST), an enzyme located in the proximal tubule, was used as an indicator of reperfusion-injury (Chatterjee et al., 2003a). Plasma LTB₄ levels were determined as an indicator of 5-LOX activity by using a colorimetric, commercial enzyme-linked immunosorbent assay kit (Calbiochem-Novabiochem Corporation, San Diego, CA).

Histological Evaluation. Kidneys were removed from mice at the end of the experimental period after tying the renal pedicle and cut in a sagittal section into two halves. These tissue samples were fixed by immersion in 10% (w/v) formaldehyde in phosphate-buffered saline (0.01 M; pH 7.4) at room temperature for 1 day. After dehydration using graded ethanol, the tissue was embedded in Paraplast (Sherwood Medical, Mahwah, NJ), cut in fine (8 μm) sections, and mounted on glass slides. Sections were then deparaffinized with xylene, counterstained with hematoxylin and eosin, and viewed under a light microscope (Dialux 22; Leitz, Milan, Italy).

For histological scoring, renal sections were prepared as described previously and used for the assessment of renal I/R injury (Thiemermann et al., 2003). In brief, 100 intersections were examined for each kidney and a score from 0 to 3 was given for each tubular profile involving an intersection: 0, normal histology; 1, tubular cell swelling, brush border loss, nuclear condensation, with up to one third of the tubular profile showing nuclear loss; 2, as for score 1, but greater than one third and less than two thirds of tubular profile show nuclear loss; 3, more than two thirds of tubular profile shows nuclear loss. The total score for each kidney was calculated by addition of all 100 scores with a maximum score of 300.

Myeloperoxidase Activity. MPO activity in kidney samples was determined as an index of PMN accumulation, as described previously (Chatterjee et al., 2003a). Kidneys were homogenized in a solution containing 0.5% hexadecyltrimethylammonium bromide and 10 mM MOPS dissolved in 80 mM sodium phosphate buffer, pH 7, and centrifuged for 30 min at 20,000g and 4°C. An aliquot of the supernatant was then allowed to react with a solution of tetramethylbenzidine (16 mM) and 1 mM hydrogen peroxide. The rate of change in absorbance was measured by a spectrophotometer at 650 nm. MPO activity was defined as the quantity of enzyme degrading 1 μmol of peroxide/min at 37°C and was expressed in units per weight (in grams) of wet tissue.

Polymorphonuclear Leukocyte Influx into Renal Tissues. Because it has become apparent that the MPO and naphthol-AS-D-chloracetate esterase assays can cross-react with monocytes and macrophages (Ysebaert et al., 2000), standard hematoxylin-eosin staining was performed to estimate the presence of PMNs, based on the morphology of the nucleus. The total number of infiltrating leukocytes (e.g., neutrophils and mononuclear cells) in cortical interstitial spaces was assessed quantitatively by counting the number of PMNs in 20 high-power fields.

Immunohistochemical Analysis of Intercellular Adhesion Molecule-1. Localization of intercellular adhesion molecule-1 (ICAM-1) in kidney sections was determined as described previously (Cockerill et al., 2001). In brief, sections were incubated overnight at 4°C with primary anti-ICAM-1 (CD54) antibody [1:500 (v/v) in phosphate-buffered saline] (DBA, Milan, Italy). Controls included kidney sections incubated with buffer alone or nonspecific purified IgG (DBA). After blocking endogenous avidin and biotin, specific labeling of antigen-antibody complex was visualized using chromogen diaminobenzidine. Immunohistochemistry photographs (*n* = 5) were assessed by densitometry as described previously (Cuzzocrea et al., 2000) with the use of Optolab Graftek software on a Macintosh personal computer.

Materials. Unless otherwise stated, all compounds used in this study were purchased from Sigma-Aldrich Company Ltd. (Milan, Italy). Zileuton was obtained from Sequoia Research Products (Oxford, U.K.). All solutions used in vivo were prepared using nonpyro-

genic saline [0.9% (w/v) NaCl; Baxter Healthcare Ltd., Thetford, Norfolk, UK].

Statistical Analysis. All values described in the text and figures are expressed as mean \pm S.E.M. for n observations. Each data point represents biochemical measurements obtained from 4 to 13 separate animals. For histological scoring, each data point represents analysis of kidneys taken from 4 to 13 individual animals. One-way analysis of variance with Dunnett's post test was performed using Prism v.4.00 for Windows (GraphPad Software, San Diego CA), and a P value of less than 0.05 was considered significant.

Results

Plasma LTB₄ Levels in Wild-Type Mice Treated with Zileuton and in 5-LOX^{-/-} Mice. Compared with sham-operated mice, I/R caused a significant increase in the plasma level of LTB₄ (from 87 ± 8 to 189 ± 12 ng/ml; $P < 0.05$) in wild-type mice, suggesting a significant increase in 5-LOX activity (Fig. 1). Administration of zileuton significantly attenuated LTB₄ synthesis (118 ± 12 ng/ml; $P < 0.05$) caused by I/R in wild-type mice (Fig. 1). Compared with wild-type mice subjected to I/R, the plasma level of LTB₄ (<1 ng/ml; $P < 0.05$) and, therefore, 5-LOX activity, was abolished in 5-LOX^{-/-} mice subjected to I/R (Fig. 1).

Renal Dysfunction (Plasma Urea and Creatinine) in Wild-Type Mice Treated with Zileuton and in 5-LOX^{-/-} Mice. Compared with sham-operated mice, I/R caused a significant increase in the plasma levels of urea (from 32.1 ± 3.2 to 382.8 ± 3.5 mg/dl; $P < 0.05$) and creatinine (from 0.7 ± 0.02 to 1.8 ± 0.05 mg/dl; $P < 0.05$) in wild-type mice (Fig. 2, A and B), suggesting a significant degree of renal dysfunction. Administration of zileuton significantly attenuated the renal dysfunction (urea, 311.4 ± 20.2 mg/dl, $P < 0.05$; and

creatinine, 1.4 ± 0.12 mg/dl, $P < 0.05$) caused by I/R in wild-type mice (Fig. 2, A and B). Compared with wild-type mice subjected to I/R, plasma levels of urea (205.0 ± 17.0 mg/dl; $P < 0.05$) and creatinine (0.65 ± 0.02 mg/dl; $P < 0.05$)

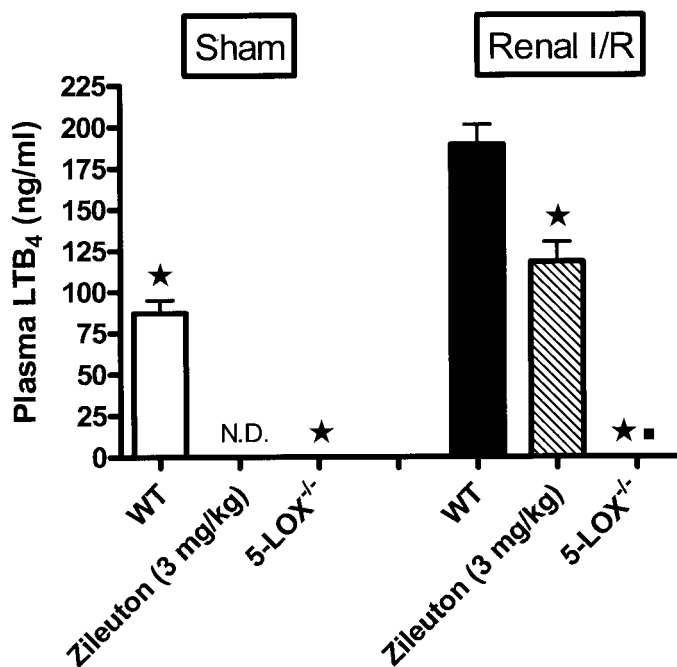


Fig. 1. Plasma LTB₄ levels in wild-type mice treated with zileuton and in 5-LOX^{-/-} mice. Plasma LTB₄ levels were measured as a biochemical marker of 5-LOX activity after sham operation [sham wild-type (WT), $n = 9$; sham 5-LOX^{-/-}, $n = 4$] or renal I/R [I/R WT, $n = 13$; I/R zileuton (3 mg/kg), $n = 7$; I/R 5-LOX^{-/-}, $n = 8$]. N.D., not determined. Data represent mean \pm S.E.M. for n observations; *, $P < 0.05$ versus renal I/R WT group; ■, $P < 0.05$ versus renal I/R zileuton group.

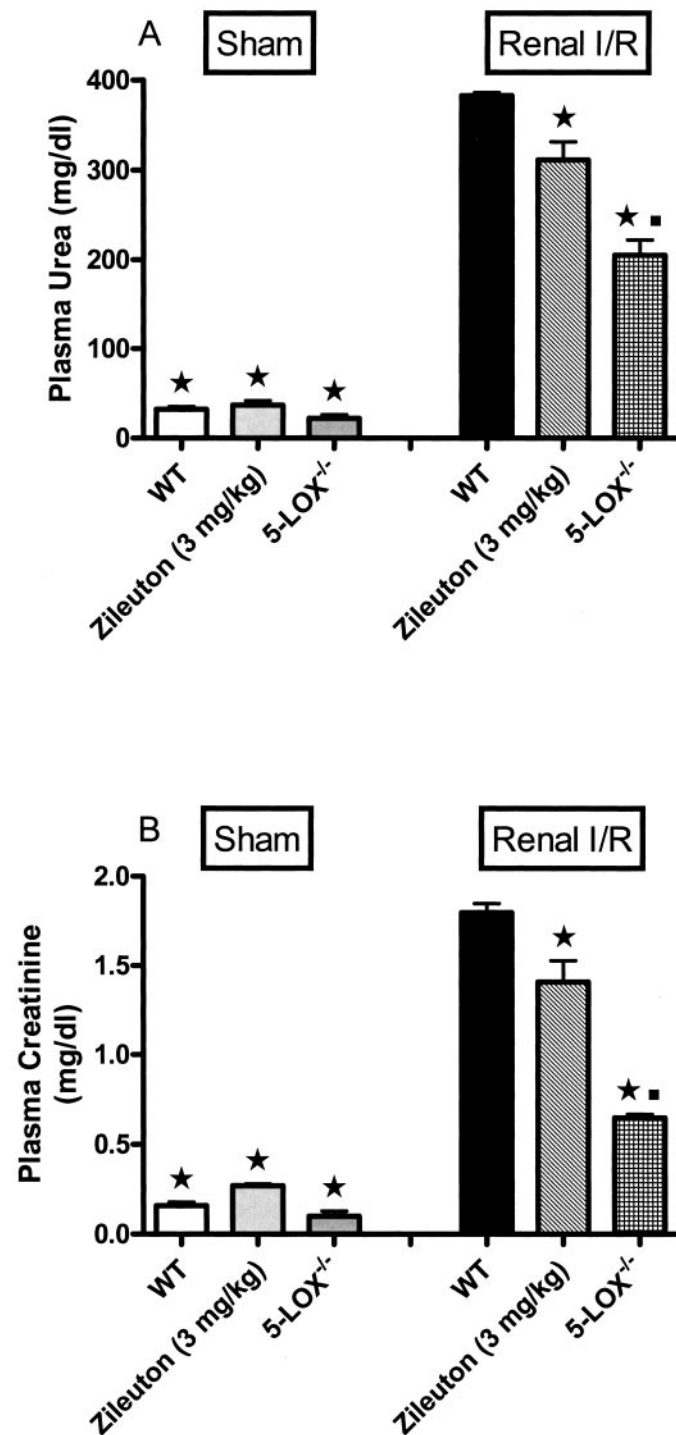


Fig. 2. Renal dysfunction in wild-type mice treated with zileuton and in 5-LOX^{-/-} mice. Plasma urea (A) and creatinine levels (B) were measured as biochemical markers of renal dysfunction after sham-operation [sham wild-type (WT), $n = 9$; sham zileuton (3 mg/kg), $n = 4$; sham 5-LOX^{-/-}, $n = 4$] or renal I/R [I/R WT, $n = 13$; I/R zileuton (3 mg/kg), $n = 7$; I/R 5-LOX^{-/-}, $n = 8$]. Data represent mean \pm S.E.M. for n observations; *, $P < 0.05$ versus renal I/R WT group; ■, $P < 0.05$ versus renal I/R zileuton group.

and, therefore, renal dysfunction, were significantly lower in $5\text{-LOX}^{-/-}$ mice subjected to I/R (Fig. 2, A and B).

Reperfusion Injury (Plasma AST) in Wild-Type Mice Treated with Zileuton and in $5\text{-LOX}^{-/-}$ Mice. Compared with sham-operated mice, I/R caused a significant increase in the plasma level of AST (from 135 ± 33 to 2200 ± 226 IU/l; $P < 0.05$) in wild-type mice, suggesting significant reperfusion-injury (Fig. 3). Administration of zileuton significantly attenuated the reperfusion-injury (520 ± 80 IU/l; $P < 0.05$) caused by I/R in wild-type mice (Fig. 3). Compared with wild-type mice subjected to I/R, the plasma level of AST (214 ± 21 ; $P < 0.05$) and, therefore, reperfusion-injury, was significantly lower in $5\text{-LOX}^{-/-}$ mice subjected to I/R (Fig. 3), similar to values obtained from sham-operated mice (Fig. 3).

Renal Injury (Histological Evaluation) in Wild-Type Mice Treated with Zileuton and $5\text{-LOX}^{-/-}$ Mice. Compared with sham-operated mice (Fig. 4A), histological examination of kidneys obtained from wild-type mice subjected to I/R demonstrated a significant degree of renal injury (Fig. 4B). In particular, kidneys obtained from these animals exhibited degeneration of tubular structure, tubular dilatation, swelling and necrosis, luminal congestion, and eosinophilia. In contrast, renal sections obtained from mice treated with zileuton (Fig. 4C) and from $5\text{-LOX}^{-/-}$ mice that underwent I/R (Fig. 4D) demonstrated a marked reduction in the severity of these histological features of renal injury compared with kidneys obtained from wild-type mice subjected to I/R only (Fig. 4B).

On comparison with the histology score measured from kidneys obtained from sham-operated mice, renal I/R produced a significant increase in histology score (from 12 ± 3 to 210 ± 6 ; $P < 0.05$), suggesting significant renal injury (Fig.

4E). Administration of zileuton significantly attenuated the histology score (100 ± 5 ; $P < 0.05$) caused by I/R in wild-type mice (Fig. 4E), indicating a reduction in renal injury. Compared with wild-type mice subjected to I/R, the histology score (89 ± 8 ; $P < 0.05$) and, therefore, renal injury was significantly lower in $5\text{-LOX}^{-/-}$ mice subjected to I/R (Fig. 4E).

Renal Inflammation (ICAM-1 Expression) in Wild-Type Mice Treated with Zileuton and in $5\text{-LOX}^{-/-}$ Mice. Compared with kidneys obtained from sham-operated mice ($1.0 \pm 0.1\%$ of total tissue area) (Fig. 5, A and E), kidneys obtained from wild-type mice demonstrated marked staining for ICAM-1 ($7.1 \pm 0.2\%$ of total tissue area; $P < 0.05$) (Fig. 5, B and E), suggesting adhesion molecule expression during reperfusion. A marked reduction in the staining for ICAM-1 was observed in kidneys obtained from mice subjected to I/R treated with zileuton ($5.0 \pm 0.1\%$ of total tissue area; $P < 0.05$) (Fig. 5, C and E) and from $5\text{-LOX}^{-/-}$ mice ($4.0 \pm 0.1\%$ of total tissue area; $P < 0.05$) subjected to I/R (Fig. 5, D and E) compared with kidneys from wild-type mice subjected to I/R only.

Renal Inflammation (MPO Activity) in Wild-Type Mice Treated with Zileuton and in $5\text{-LOX}^{-/-}$ Mice. Compared with sham-operated mice, the kidneys obtained from wild-type mice subjected to I/R demonstrated a significant increase in MPO activity (from 15 ± 0.3 to 87 ± 2.0 units/g of wet tissue; $P < 0.05$), suggesting increased PMN infiltration into renal tissues (Fig. 6A). The increase in the tissue level of MPO (17 ± 1.0 units/g of wet tissue; $P < 0.05$) observed in mice administered zileuton was significantly smaller than those seen in wild-type mice subjected to I/R (Fig. 6A). In addition, the increase in the tissue level of MPO (24 ± 1.8 units/g of wet tissue; $P < 0.05$) seen in $5\text{-LOX}^{-/-}$ mice subjected to I/R was significantly smaller than those seen in their wild-type littermates (Fig. 6A).

Renal Inflammation (Polymorphonuclear Cell Infiltration) in Wild-Type Mice Treated with Zileuton and in $5\text{-LOX}^{-/-}$ Mice. Quantitation of infiltrating PMNs into renal tissues showed that there was only a minimal number of PMNs ($3.2 \pm 0.1/\text{mm}^2$) in nonischemic kidneys obtained from sham-operated mice (Fig. 6B). However, a large number of infiltrating PMNs ($10.5 \pm 0.2/\text{mm}^2$; $P < 0.05$) were observed in the renal cortex of mice subjected to I/R of the kidney (Fig. 6B). The number of PMNs infiltrating into renal tissues of mice treated with zileuton ($6.1 \pm 0.2/\text{mm}^2$; $P < 0.05$) and in $5\text{-LOX}^{-/-}$ mice ($5.3 \pm 0.2/\text{mm}^2$; $P < 0.05$) was significantly attenuated by approximately 40 and 50%, respectively (Fig. 6B). There were no differences in any of the above biochemical parameters measured between sham-operated mice treated with 3 mg/kg, sham operated $5\text{-LOX}^{-/-}$ mice, or sham-operated wild-type littermates (Figs. 2, 3, 4E, 5E, and 6).

Discussion

Mice subjected to renal I/R demonstrated characteristic signs of renal dysfunction, injury, and inflammation. In particular, renal I/R caused 1) renal dysfunction (increased plasma creatinine and urea levels); 2) reperfusion injury (increased plasma AST levels); 3) characteristic histological signs of marked tubular injury, which were in keeping with the observation that the S_3 segment of the proximal tubule is

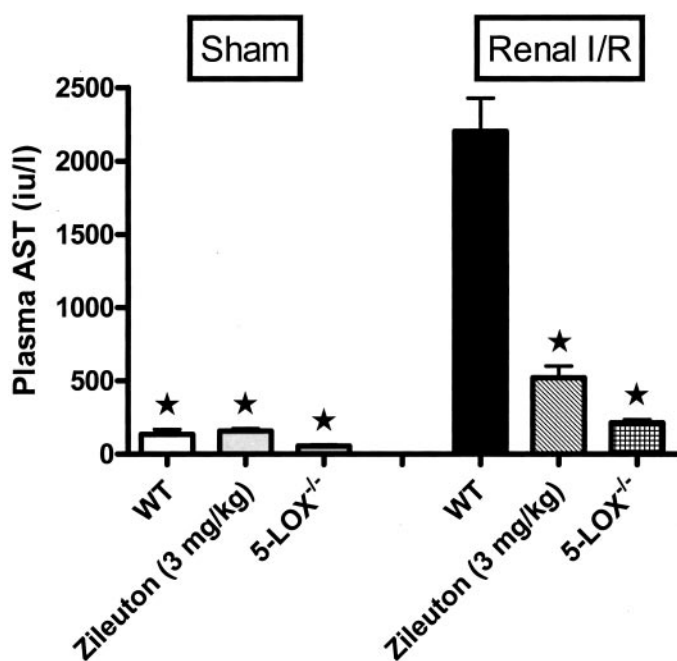


Fig. 3. Reperfusion-injury in wild-type mice treated with zileuton and in $5\text{-LOX}^{-/-}$ mice. Plasma AST levels were measured as a biochemical marker of reperfusion-injury subsequent to sham-operation [sham wild-type (WT), $n = 9$; sham zileuton (3 mg/kg), $n = 4$; sham $5\text{-LOX}^{-/-}$, $n = 4$] or renal I/R [I/R WT, $n = 13$; I/R zileuton (3 mg/kg), $n = 7$; I/R $5\text{-LOX}^{-/-}$, $n = 8$]. Data represent mean \pm S.E.M. for n observations; *, $P < 0.05$ versus renal I/R WT group.

particularly susceptible to renal I/R injury (Venkatachalam et al., 1978); 4) PMN accumulation in renal tissues (PMN counts and MPO activity); and 5) expression of the adhesion molecule, ICAM-1 (immunohistochemistry). All this data confirmed a well known pattern of renal dysfunction and injury caused by I/R of the kidney (Paller, 1994b; Kribben et al., 1999; Sheridan and Bonventre, 2001) and that that renal I/R causes both renal and tubular dysfunction (Paller, 1994a) as well as secondary inflammation (Dragun et al., 2000).

Zileuton is a selective and potent inhibitor of 5-LOX, which is currently being used in the therapy of patients suffering from asthma (1997). In this study, we demonstrated for the first time that zileuton also reduced the renal dysfunction and injury caused by bilateral occlusion (30 min) and reperfusion (24 h) of the kidneys of mice. What, then, is the mechanism(s) by which 5-LOX or its products amplify the

inflammatory response and ultimately the tissue injury and dysfunction caused by renal I/R? Bilateral renal ischemia and reperfusion in the mouse resulted in a significant increase in the plasma levels of LTB₄. This increase in 5-LOX activity caused by I/R was almost abolished by treatment of wild-type mice with zileuton. To confirm that the reported beneficial effects of zileuton were indeed caused by inhibition of 5-LOX (rather than a nonspecific effect), we have subsequently compared the effects of bilateral renal artery occlusion and reperfusion in 5-LOX^{-/-} (no detectable levels of LTB₄ in either sham-operated mice or 5-LOX^{-/-} mice subjected to I/R) with those obtained using their wild-type littermates. We report here for the first time that the degree of renal injury, dysfunction and inflammation are reduced in 5-LOX^{-/-} mice. Thus, we propose that 1) metabolites of 5-LOX contribute to the pathophysiology of renal I/R injury,

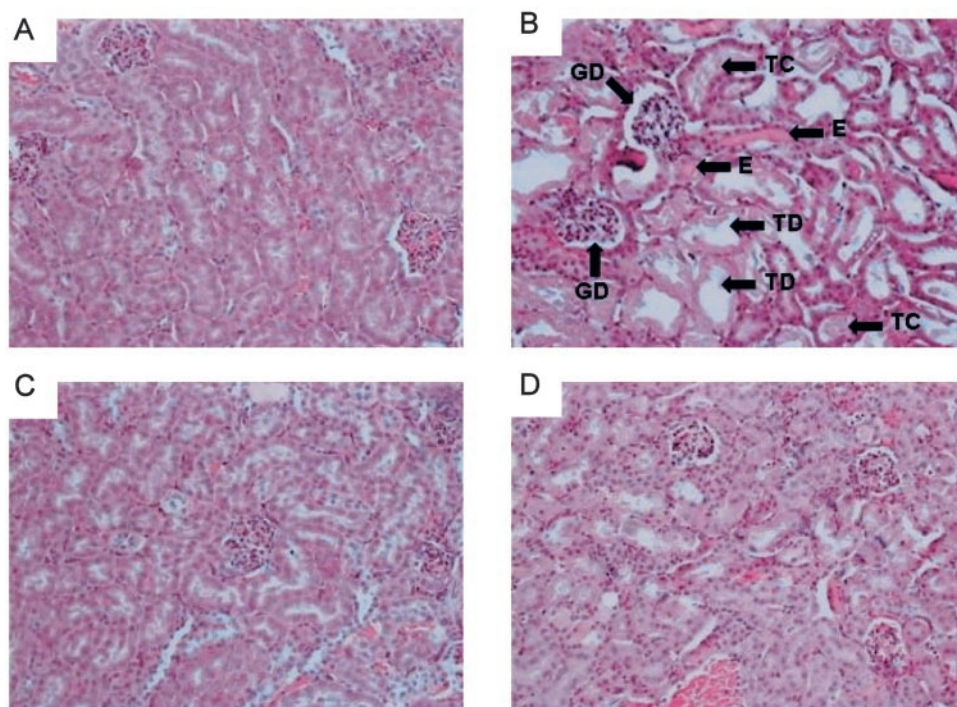
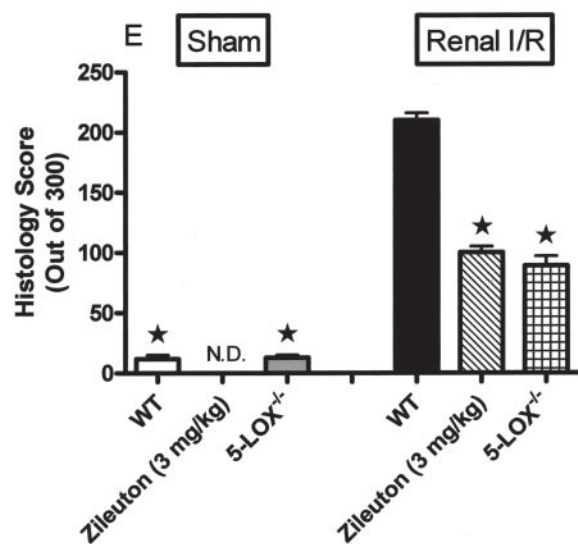


Fig. 4. Renal injury in wild-type mice treated with zileuton and in 5-LOX^{-/-} mice: Histological examination; A renal section taken from a sham-operated mouse (A) is compared with that of kidney sections prepared from mice subjected to ischemia followed by reperfusion for 24 h demonstrated recognized features of renal injury (B), including glomerular degeneration (GD), tubular dilation (TD), tubular congestion (TC), and the presence of eosinophilia (E). Wild-type mice subjected to I/R that were administered 3 mg/kg zileuton (C) displayed a reduction in renal injury. In addition, a renal section taken from a 5-LOX^{-/-} mouse subjected to renal I/R is also represented (D). Hematoxylin and eosin; original magnification, 150 \times . Figures are representative of at least three experiments performed on different days. E, renal sections were scored for characteristic histologic signs of renal injury out of a total of 300 (see *Materials and Methods* section) after sham-operation [sham wild-type (WT), $n = 9$; sham 5-LOX^{-/-}, $n = 4$] or renal I/R [I/R WT, $n = 13$; I/R zileuton (3 mg/kg), $n = 7$; I/R 5-LOX^{-/-}, $n = 8$]. N.D., not determined. Data represent mean \pm S.E.M. for n observations; \star , $P < 0.05$ versus renal I/R WT group; \blacksquare , $P < 0.05$ versus renal I/R zileuton group.



and 2) that inhibition of 5-LOX activity with zileuton, for example, reduces the renal injury, dysfunction, and inflammation associated with I/R of the kidney. We report here that zileuton reduced the expression of ICAM-1 caused by I/R of the kidney in wild-type mice, with a similar reduction in ICAM-1 expression in *5-LOX*^{-/-} mice. There is some evidence that the expression of ICAM-1, in addition to the expression of P- and E-selectin, is also reduced in *5-LOX*^{-/-} mice subjected to either cerulein-induced pancreatitis (Cuzzocrea et al., 2003a) or carrageenan-induced lung injury (Cuzzocrea et al., 2003b). In models of I/R of the skin, zileuton reduces the expression of CD18 and $\beta 2$ integrins (Dolan et al., 1998a,b), but it is not known whether this effect is indeed caused by inhibition of 5-LOX.

We report here that the accumulation of PMNs was re-

duced in kidneys from *5-LOX*^{-/-} mice or in kidneys from wild-type mice treated with zileuton, which had been subjected to I/R. Thus, inhibition of 5-LOX activity does reduce PMN accumulation after I/R of the kidney. This is not entirely surprising given that the 5-LOX metabolite LTB₄ is a potent chemokine. LTA₄ hydrolase, which converts LTA₄ to LTB₄, is expressed in the rat nephron (mRNA expression: inner medulla > outer medulla > cortical homogenates) (Nakao et al., 1999). Most notably, LTB₄ plays a pivotal role in the recruitment of PMNs in kidneys subjected to I/R, and LTB₄ receptor-antagonists abolish the PMN accumulation after I/R of the kidney (Noiri et al., 2000).

It should be noted that inhibition of 5-LOX activity also reduces the formation of lipoxins. Lipoxin-A₄ (or its analogs) reduces the degree of inflammation (Takano et al., 1997; Goh

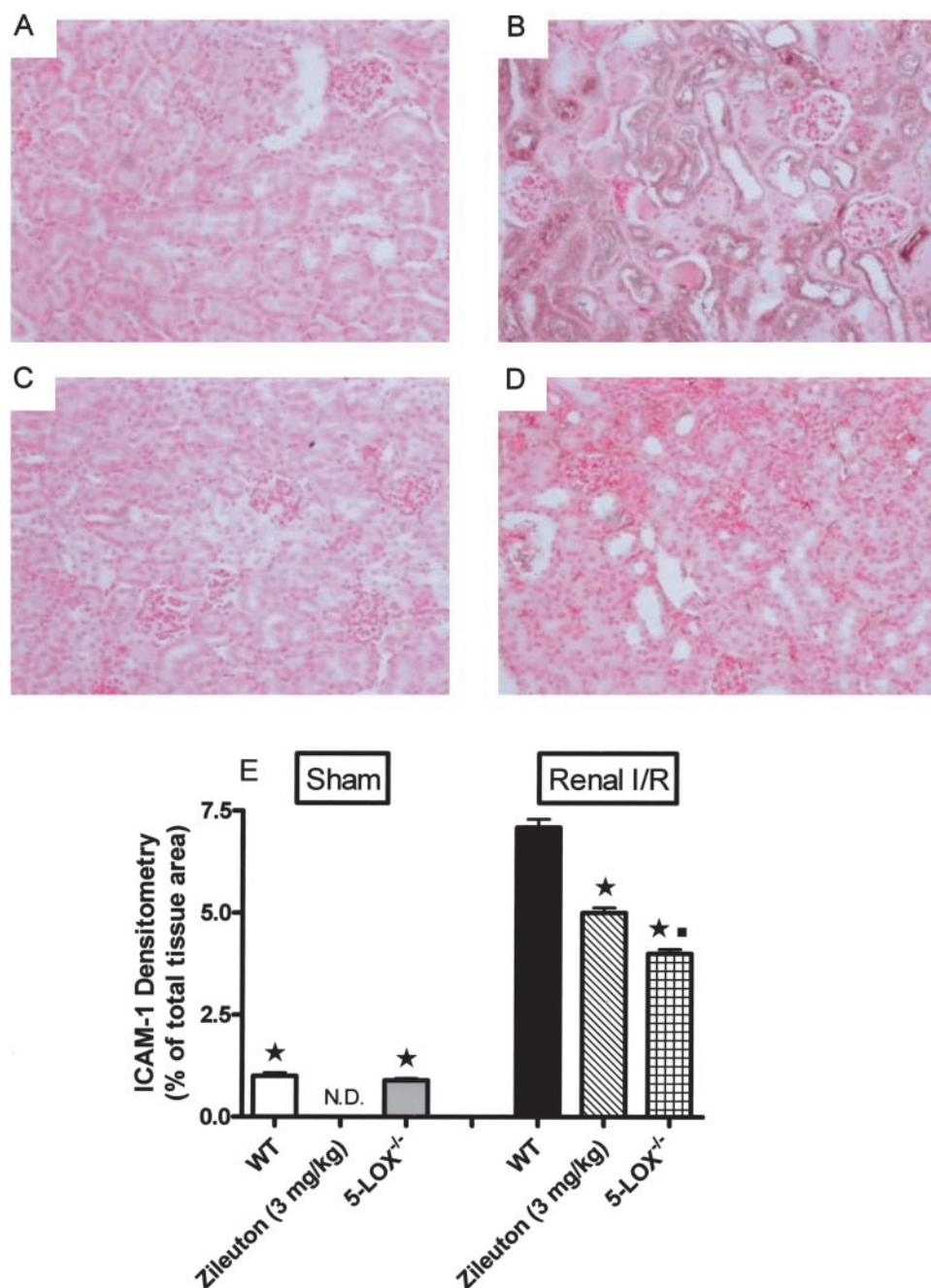


Fig. 5. Renal inflammation in wild-type mice treated with zileuton and in *5-LOX*^{-/-} mice. ICAM-1 expression; sham-operated group (A), wild-type group subjected to renal I/R (B), zileuton treated group subjected to renal I/R (C), and *5-LOX*^{-/-} mice group subjected to renal I/R (D). Anti-ICAM-1 antibody, original magnification, 125 \times ; figures are representative of at least three experiments performed on different experimental days ($n = 4-13$ for all groups). E, typical densitometry evaluation. Densitometry analysis of immunohistochemistry photographs ($n = 5$) for ICAM-1 was assessed. Data represent mean \pm S.E.M. for n observations; \star , $P < 0.05$ versus renal I/R WT group; \blacksquare , $P < 0.05$ versus renal I/R zileuton group.

et al., 2001) as well as the renal injury and dysfunction caused by I/R (Leonard et al., 2002). Thus, it has been suggested that LXA₄ may play an important role in the resolution of inflammation after renal injury. Our findings indicate that the pro-inflammatory effects of LTB₄ (and other LTs)

are more important in the regulation of the acute inflammatory response associated with I/R of the kidney than LXA₄ (and other endogenous lipoxins). However, the contribution of 5-LOX metabolites to the regulation of the inflammatory response in more chronic settings of inflammation cannot be discounted. Indeed, there is evidence that the rejection of renal allografts is accelerated in 5-LOX^{-/-} mice (Goulet et al., 2001).

It should be noted that the renal injury and dysfunction observed in 5-LOX^{-/-} or wild-type mice treated with zileuton was not entirely abolished. In addition, the degree of inhibition of plasma urea and creatinine were not as complete as that of plasma AST. This is not surprising, given that many other pathophysiological mechanisms, which are independent of LTs and/or an enhanced inflammatory response, will contribute to the observed injury during ischemia and/or reperfusion. These mechanisms may include (but are not limited to) the generation of reactive oxygen and nitrogen species (Chatterjee et al., 2000a), an enhanced formation of nitric oxide (Chatterjee et al., 2003b), modification of endogenous lipoxin generation (Leonard et al., 2002), or the activation of the nuclear enzyme poly(ADP-ribose) polymerase (Chatterjee et al., 2000b).

In conclusion, our results support the view that metabolites of 5-LOX contribute to the renal injury, dysfunction, and inflammation caused by I/R of the kidney. We propose that 5-LOX inhibitors may be useful in the treatment of conditions associated with I/R and/or inflammation of the kidney and possibly other organs. This view is supported by recent clinical data demonstrating that zileuton reduces the formation of LTB₄ and prostaglandin E₂ in rectal dialysates as well as the associated inflammatory response (histology score) in patients with inflammatory bowel syndrome (Rask-Madsen et al., 1992).

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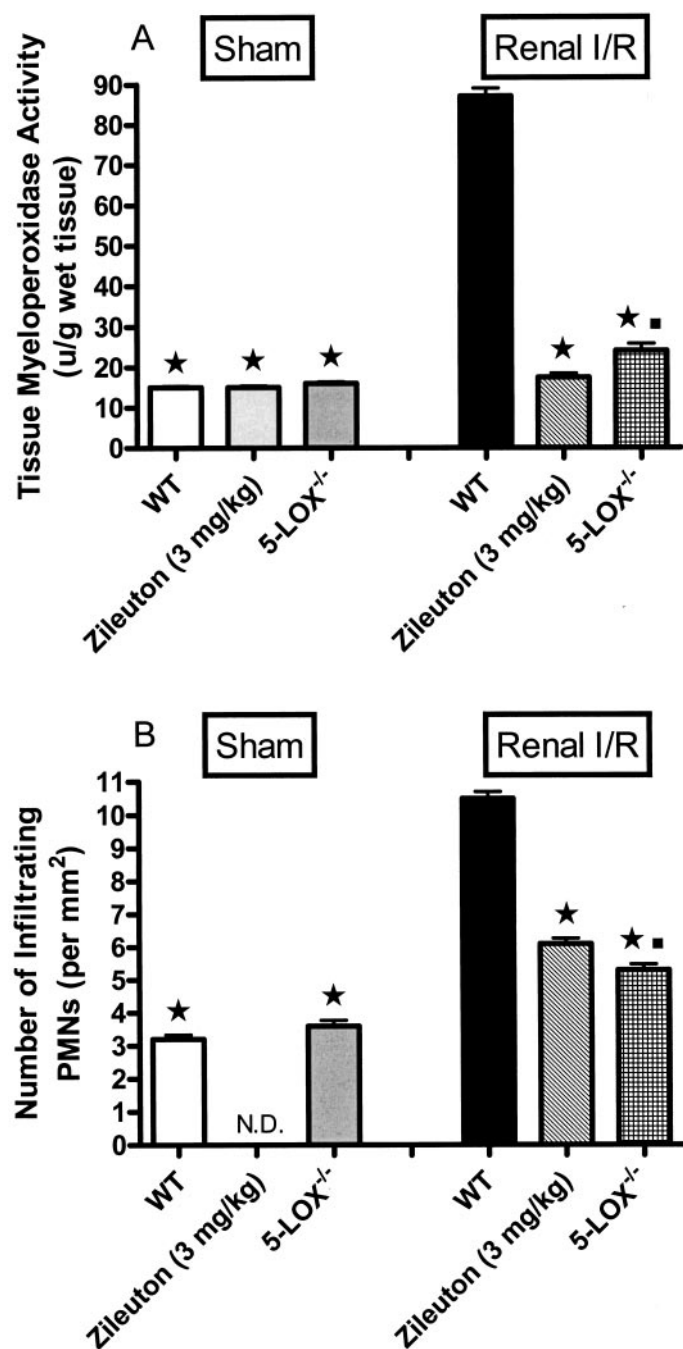


Fig. 6. Renal inflammation in wild-type mice treated with zileuton and in 5-LOX^{-/-} mice. MPO activity and PMN infiltration. A, kidney MPO activity. B, the total number of infiltrating leukocytes (e.g., neutrophils and mononuclear cells) in cortical interstitial spaces was assessed quantitatively by counting the number of PMNs in 20 high-power fields. Measurements were performed after sham-operation [sham wild-type (WT), $n = 9$ for both determinations; sham 5-LOX^{-/-}, $n = 4$ for both determinations; I/R zileuton (3 mg/kg), $n = 7$ for both determinations; I/R 5-LOX^{-/-}, $n = 8$ for both determinations]. N.D., not determined. Data represent mean \pm S.E.M. for n observations; *, $P < 0.05$ versus renal I/R WT group; ■, $P < 0.05$ versus renal I/R zileuton group.

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