Lysophosphatidic Acid Prevents Renal Ischemia-Reperfusion Injury by Inhibition of Apoptosis and Complement Activation

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Renal ischemia-reperfusion (I/R) injury is an important cause of acute renal failure as observed after renal transplantation, major surgery, trauma, and septic as well as hemorrhagic shock. We previously showed that the inhibition of apoptosis is protective against renal I/R injury, indicating that apoptotic cell-death is an important feature of I/R injury. Lysophosphatidic acid (LPA) is an endogenous phospholipid growth factor with anti-apoptotic properties. This tempted us to investigate the effects of exogenous LPA in a murine model of renal I/R injury. LPA administered at the time of reperfusion dose dependently inhibited renal apoptosis as evaluated by the presence of internucleosomal DNA cleavage. I/R-induced renal apoptosis was only present in tubular epithelial cells with evident disruption of brush border as assessed by immunohistochemistry for active caspase-7 and filamentous actin, respectively. LPA treatment specifically prevented tubular epithelial cell apoptosis but also reduced the I/R-induced loss of brush-border integrity. Besides, LPA showed strong anti-inflammatory effects, inhibiting the renal expression of tumor necrosis factor- α and abrogating the influx of neutrophils. Next, LPA dose dependently inhibited activation of the complement system. Moreover, treatment with LPA abrogated the loss of renal function in the course of renal I/R. This study is the first to show that administration of the phospholipid LPA prevents I/R injury, abrogating apoptosis and inflammation. Moreover, exogenous LPA is capable of preventing organ failure because of an ischemic insult and thus may provide new means to treat clinical conditions associated with I/R injury in the kidney and potentially also in other organs. (*Am J Pathol* 2003, 163:47–56)

medicine. Acute renal failure because of ischemia-reperfusion (I/R) injury is observed after transplantation and as a major complication in cardiac and vascular surgery, septic as well as hypovolemic shock, and trauma.¹

Ischemia directly induces cellular injury, which is aggravated by reperfusion.² In the setting of I/R injury cellular damage will, if severe enough, lead to cell death that may be apoptotic as well as necrotic.³ Both apoptosis and necrosis have been implicated in the induction of inflammation in the course of I/R, an important cause of tissue injury induced by renal I/R.⁴ This inflammatory response is characterized by activation of the complement system, induction of cytokines and chemokines, and subsequent influx of neutrophils.^{2,4} Previously, we showed that early apoptosis is essentially involved in the initiation of this inflammatory reaction and moreover that inhibition of apoptosis protects against I/R injury.^{5,6} On the other hand, inflammation has been shown to be involved in the regulation of apoptotic cell-death in several models of renal inflammation.^{7–11}

Growth and survival factors, such as insulin-like growth factor-1, fibroblast growth factor, and hepatocyte growth factor have been shown to prevent apoptosis, inflammation, and to protect against renal I/R injury.^{5,12} Lysophosphatidic acid (LPA) is a member of the phospholipid growth factor family, having pleiotropic effects such as enhancement of cell survival and cell proliferation. LPA is normally present in serum, binding with high affinity to albumin, in concentrations ranging between 5 to 20 $\mu\text{mol/L}$.¹³ LPA signals via the G-protein-coupled receptors LPA₁, LPA₂, and LPA₃.¹⁴ All LPA receptors have been found to be localized among others in renal tissue.^{14,15} *In vitro*, LPA has been shown to function as a growth and survival factor for renal proximal tubular cells, inhibiting apoptosis induced by growth factor deprivation.¹⁶

The aim of this study was to characterize the *in vivo* effects of exogenous LPA in a renal I/R model, with special emphasis on the influence of LPA on I/R-induced apoptosis. Furthermore, we aimed to study the effects of LPA on the inflammatory response in the course of renal I/R, in particular the activation of the complement system,

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Postischemic organ failure of organs such as the heart, brain, and kidneys represents a major problem in clinical

the induction of chemokines, cytokines, and the influx of neutrophils.

Materials and Methods

Antibodies and Reagents

Lysophosphatidic acid (1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphate) was purchased from Avanti Polar Lipids (Alabaster, AL), polyclonal rabbit anti-active caspase-7 antibody was from Biovision (Mountain View, CA), and Texas Red-phalloidin was from Molecular Probes (Eugene, OR). Rabbit anti-mouse C6 was kindly provided by Dr. N. R. Cooper (Scripps Research Institute, La Jolla, CA), rabbit anti-rat C9 was a kind gift from Dr. B. P. Morgan (University of Wales College of Medicine, Cardiff, UK), and goat anti-mouse C3 was purchased from Cap-pel (ICN Biomedicals, Aurora, OH). NIMP-R14 (rat anti-mouse neutrophil monoclonal antibody) was kindly provided by Dr. M. Strath (National Institute for Medical Research, London, UK). Secondary antibodies, peroxidase-conjugated rabbit anti-goat, goat anti-rabbit, and goat anti-rat IgG as well as fluorescein isothiocyanate-labeled goat anti-rabbit IgG were purchased from Jackson (West Grove, PA). All other reagents were purchased from Sigma (St. Louis, MO).

Experimental Protocol

Male Swiss mice weighing 25 to 30 g were obtained from Charles River Breeding Laboratories (Heidelberg, Germany). Animals were housed individually in standard laboratory cages and were allowed free access to food and water. The studies were performed under a protocol approved by the Institutional Animal Care Committee of the University of Maastricht. At the start of the experiments, mice were anesthetized with sodium pentobarbital (100 mg/kg i.p.). Body temperature was maintained at 38°C by a heating pad until animals recovered from anesthesia. Under aseptic conditions a 1.0-cm-long midline abdominal incision was made and ischemia was induced by applying a nontraumatic vascular clamp to the left renal pedicle for 45 minutes. After removal of the clamp the contralateral kidney was removed and the wound was closed in two layers. The animals were sacrificed 24 hours after reperfusion. At the time of sacrifice, blood was collected and the left kidney was harvested for analysis.

During ischemia, just before reperfusion, mice were administered 0.01, 0.1, 1, or 2 mg of LPA (intraperitoneally) dissolved in 1.0 ml of phosphate-buffered saline (PBS) ($n = 6$ per group). The control group was treated with vehicle consisting of 1.0 ml of PBS intraperitoneally ($n = 6$).

Renal Histology

Cryostat sections (5 μ m) of frozen tissue were cut and double-stained for active caspase-7 and filamentous actin (F-actin). Briefly, slides were dried, fixed in acetone for 10 minutes, and air-dried. Slides were immersed in PBS

for 5 minutes and subsequently in 5% normal goat serum in PBS to block aspecific antibody binding. Slides were stained for 1 hour at room temperature with the anti-active caspase-7 primary antibody in PBS with 0.1% bovine serum albumin. After three washes in PBS with 0.1% Tween for 5 minutes each, slides were incubated for 30 minutes with the fluorescein isothiocyanate-labeled secondary antibody diluted in the same buffer with the addition of Texas Red-phalloidin that specifically binds to F-actin. After three washes in PBS-Tween, the slides were mounted using glycerol-PBS with 1,4-diazabicyclo (2,2,2)octane and 4,6-diamidino(2)phenylindole, and viewed with an immunofluorescence microscope. No significant staining was detected in slides incubated with control serum instead of the primary antibody indicating the absence of significant background staining. The number of caspase-7-positive tubules was quantified by counting 20 fields of vision per kidney section (three sections per kidney, four kidneys per group) at $\times 200$ magnification in a blinded manner.

Cryostat sections (5 μ m) of frozen tissue were stained for complement factors C3, C6, and C9. Briefly, slides were dried and fixed in acetone. Slides were immersed in Tris-buffered saline (TBS) for 5 minutes, in 0.03% H_2O_2 in TBS for 30 minutes at room temperature to eliminate endogenous peroxidase activity, and in 5% normal goat or rabbit serum (depending on the antibodies used) in TBS to block aspecific antibody binding. Subsequently, slides were stained for 1 hour at room temperature with primary antibodies in TBS with 0.1% bovine serum albumin. After three washes in TBS with 0.1% Tween (TTBS) for 5 minutes each, slides were incubated for 30 minutes with appropriate peroxidase-labeled secondary antibodies diluted in the same buffer. After three washes in TTBS staining was visualized by 3-amino-9-ethylcarbazole followed by hematoxylin counterstain. Finally, the slides were coverslipped and viewed with a light microscope. No significant staining was detected in slides incubated with control sera instead of the primary antibody indicating the absence of significant background staining.

Staining for neutrophils was performed with monoclonal antibody NIMP-R14 as described above. Neutrophils were counted by examining 10 fields of vision per kidney section (three to four sections per kidney, four kidneys per group) at $\times 200$ magnification in a blinded manner.

Apoptosis Assay

Presence of internucleosomal DNA cleavage in kidneys was investigated with a commercial ligase-mediated polymerase chain reaction (PCR) assay kit (Apoalert; Clontech, Palo Alto, CA) enabling semiquantitative measurement of the extent of apoptosis. In brief, DNA was isolated from tissue samples using a commercially available DNA purification kit (Promega, Madison, WI). DNA purity and concentration were determined by electrophoresis through a 0.8% agarose gel containing ethidium bromide followed by visualization under ultraviolet illumination as well as by measuring absorbance at 260/280 nm. Dephosphorylated adaptors were ligated to 5'-phos-

phorylated blunt ends with T4 DNA ligase (during 16 hours at 16°C) and served as primers in a ligase-mediated PCR. Amplified DNA was subjected to gel electrophoresis on a 1.2% agarose gel containing ethidium bromide.

Measurement of Renal KC, MIP-2, and Tumor Necrosis Factor (TNF)- α mRNA Levels by Reverse Transcriptase-PCR

For reverse transcriptase-PCR total RNA was extracted from kidneys using the SV Total RNA isolation system (Promega) and treated with RQ1 RNase-Free DNase (Promega). Total RNA was reverse-transcribed using oligo (dT) primer and Moloney murine leukemia virus reverse transcriptase (Life Technologies, Paisley, UK).

For semiquantitative PCR analysis cDNA samples were standardized based on the content of β -actin cDNA as housekeeping gene. β -actin cDNA was evaluated by performance of a β -actin PCR on multiple dilutions of each cDNA sample. The amount of amplified product was estimated by densitometry of ethidium bromide-stained 1.2% agarose gels using a charge-coupled device camera and Imagemaster VDS software (Pharmacia, Uppsala, Sweden). To determine renal KC and MIP-2 mRNA expression, PCR reactions with specific primers were performed using appropriate dilutions of the cDNA. Primers used for β -actin, KC, and MIP-2 PCR have been described in detail previously.⁶

Real-time quantitative reverse transcriptase-PCR for TNF- α was performed on a TaqMan ABI 7700 sequence detection system (Applied Biosystems, Foster City, CA). β -Actin was used as reference gene. The following oligonucleotide primers and probes were used: β -actin, 5'-GAC AGG ATG CAG AAG GAG ATT ACT G-3' (sense) and 5'-CCA CCG ATC CAC ACA GAG TAC TT-3' (anti-sense) both at a concentration of 300 nmol/L, and internal fluorescence-labeled probe (JOE) 5'-ATC AAG ATC ATT GCT CCT CCT GAG CGC-3' at a concentration of 200

nmol/L; TNF- α , 5'-CAT CTT CTC AAA ATT CGA GTG ACA A-3' (sense) and 5'-TGG GAG TAG ACA AGG TAC AAC CC-3' (anti-sense) both at a concentration of 200 nmol/L, and internal fluorescence-labeled probe (FAM) 5'-CAC GTC GTA GCA AAC CAC CAA GTG GA-3' at a concentration of 100 nmol/L. All primers and probes were obtained from Applied Biosystems.

Renal Function

Blood urea nitrogen (BUN) was measured in serum obtained at the time of sacrifice using a Urea 25 Kit (ABX Diagnostics, Eindhoven, Holland) in a Cobas Fara auto-analyzer (Roche, Basle, Switzerland).

Statistical Analysis

Data are expressed as the mean $\pm$ SEM, and statistical analysis was performed by Mann-Whitney *U*-test. *P* < 0.05 was taken to denote statistical significance.

Results

LPA Protects against Renal Dysfunction after Renal I/R

Renal I/R injury is characterized by loss of renal function. Renal function loss was measured by serum BUN. Mice treated with LPA showed a dose-dependent reduction in BUN values compared to control-treated animals. A dose of 0.01 mg of LPA already significantly reduced BUN levels [25.86 mmol/L for LPA (0.01 mg) *versus* 43.27 mmol/L for control PBS treatment, *P* < 0.05], whereas higher doses of LPA dose dependently protected against renal function loss [15.69 mmol/L for LPA (0.1 mg) and 10.38 mmol/L for LPA (1 mg), *P* < 0.01]. Higher doses of LPA (2 mg) had no additional protective effects, thus at 1 mg of LPA a plateau was reached regarding prevention

Figure 1. LPA dose dependently reduces loss of renal function after renal I/R. Compared to control-treated animals LPA significantly reduced BUN values as measured 24 hours after renal I/R. Statistical significance as compared to control-treated animals was denoted at *P* < 0.05 (*) or *P* < 0.01 (*). The data shown are means $\pm$ SEM.

Figure 2. LPA treatment prevents I/R-induced apoptosis. The extent of renal apoptosis was reflected by fragmented DNA amplified by ligase-mediated PCR and visualized on ethidium-bromide-stained gel. In PBS-treated animals internucleosomal DNA cleavage was evident. LPA dose dependently reduced renal apoptosis. Data shown are representative for three independent assays on different renal samples (*n* = 3 per group). M, 100-bp molecular weight marker.

Figure 3. LPA significantly reduces I/R-induced caspase-7 activation and brush-border disruption. Kidneys of healthy animals (**A** and **B**) showed no activation of caspase-7 (green) and intact brush borders as determined by filamentous actin staining (red). Renal I/R (**C** and **D**) induced caspase-7 activation localized to tubular epithelial cells. Caspase-7 activation was only present in tubular cells that lost brush-border integrity (**C** and **D**). LPA treatment (1 mg) evidently reduced the I/R-induced activation of caspase-7 that is accompanied with the preservation of brush-border integrity (**E** and **F**). To quantify caspase-7 activity positive tubules were counted after immunohistochemical staining (three sections per kidney, four kidneys per group). These data (**G**) are expressed as the mean of number of positive tubules per field of vision $\pm$ SEM. Statistical significance as compared to PBS-treated animals was denoted at $P < 0.05$ (*). Red staining, filamentous actin (Texas Red); green staining, active caspase-7 (fluorescein isothiocyanate); blue staining, nuclei (4,6-diamidino(2)phenylindole). Original magnifications: $\times 400$ (**A**, **C**, **E**); $\times 600$ (**B**, **D**, **F**).

of I/R-induced renal function loss (Figure 1). These data show that LPA is strongly protective against renal failure in the course of renal I/R injury.

LPA Prevents I/R-Induced Apoptosis in the Course of Renal I/R

In vitro, LPA is known to have anti-apoptotic properties. This tempted us to study the effects of LPA *in vivo* on I/R-induced apoptosis, especially because apoptotic cell death has been shown to play a major role in renal I/R injury. Here, we show that LPA is capable of inhibiting apoptosis *in vivo* (Figure 2). Compared to control-treated animals, kidneys of animals treated with LPA, showed a dose-dependent reduction in internucleosomal DNA cleavage, a specific hallmark of apoptosis. To determine the apoptotic cell type in the course of renal I/R we used immunofluorescence staining for active caspase-7. Here we show that in control kidneys no activation of caspase-7 is detectable (Figure 3, A and B). Ischemia followed by reperfusion, however, induced a significant activation of caspase-7 that localized specifically to tubular epithelial cells (Figure 3, C and D). Interestingly, apoptotic cells often co-localized within one tubule (Figure 3, C and D).

An important hallmark of cellular injury in the course of renal I/R injury is the disruption of brush border.¹⁷ We therefore stained renal tissue for filamentous actin (F-actin), which is normally present in the brush border of tubular epithelial cells but is broken down during renal I/R injury under influence of actin-depolymerizing factor.¹⁸ Indeed, in normal kidney sections F-actin is abundantly expressed in the brush border of tubular epithelial cells (Figure 3, A and B). Renal I/R induced a significant disruption of the brush border visible as the disappearance of tubular F-actin staining (Figure 3, C and D). Moreover, the data show that the activation of caspase-7 is present in tubular cells that also showed a severe disruption of brush border (Figure 3, C and D).

In animals treated with LPA (1 mg), I/R-induced caspase-7 activation is significantly reduced as shown in Figure 3G. This reduction of tubular apoptosis by LPA is accompanied by a reduced loss of brush-border integrity (Figure 3, E and F).

LPA Prevents Inflammation in the Course of Renal I/R

A prominent feature of I/R-induced inflammation is the influx of neutrophils. Previously, we showed that the inhibition of apoptosis by caspase-inhibition or growth factor administration prevents massive neutrophil infiltration.⁵ To estimate the effects of LPA on inflammation we measured the influx of neutrophils by immunohistochemistry. To quantify the neutrophil infiltration NIMP-R14-positive cells were counted in tissue sections of kidneys in control and experimental groups at 24 hours of reperfusion. The data show that LPA treatment significantly reduced neu-

Figure 4. LPA significantly reduces I/R-induced neutrophil influx. Neutrophils were counted after immunohistochemical staining (three to four sections per kidney, four kidneys per group). Neutrophils were scarcely present in kidneys obtained from healthy mice, whereas ischemia followed by 24 hours of reperfusion led to a strong influx of neutrophils. Treatment with LPA dose dependently reduced the influx of neutrophils at 24 hours of reperfusion. Data are expressed as the mean of number of neutrophils per field of vision $\pm$ SEM. Statistical significance as compared to PBS-treated animals was denoted at $P < 0.05$ (*) and $P < 0.01$ (+).

trophil influx in a dose-dependent manner at 24 hours of reperfusion (Figure 4).

Next, we investigated whether LPA reduces the induction of proinflammatory chemokines and cytokines involved in recruitment of inflammatory cells. We determined renal mRNA levels of TNF- α as well as the mouse CXC-chemokines KC and MIP-2. Here, we show that the induction of TNF- α is strongly inhibited after LPA treatment (1 mg LPA) (Figure 5A). Interestingly, the I/R induced up-regulation of the chemokines KC and MIP-2 was unaffected by LPA (Figure 5B).

LPA Abrogates Complement Activation after Renal I/R Injury

The data show that LPA significantly inhibits apoptosis as well as inflammation in the course of renal I/R injury. An important system that regulates inflammatory responses as well as apoptosis after renal I/R injury is the complement system.⁷ To investigate the effects of LPA on the activation of the complement system we stained renal tissue for early as well as late complement deposition. Here, we show that LPA strongly reduces C3 deposition. Compared to healthy animals, in control-treated animals renal I/R induces severe depositions of C3 (Figure 6, A and B). LPA dose dependently inhibits C3 deposition as shown for animals treated with 0.1 and 1 mg of LPA (Figure 6, C and D, respectively). Moreover, LPA dose dependently reduces I/R-induced membrane attack complex (MAC) formation. Whereas the deposition of complement factor C6 (indicating MAC formation) is induced by renal I/R (Figure 7, A and B), administration of LPA clearly prevented C6 depositions at dosages of 0.1 mg (Figure 7C) and 1 mg (Figure 7D) of LPA. Staining for C9, also indicative for MAC formation, showed a similar inhibition of I/R-induced MAC deposition by LPA treat-

Figure 5. LPA treatment inhibits I/R-induced up-regulation of TNF- α whereas KC and MIP-2 mRNA levels are unaffected. Renal I/R induced a significant up-regulation of TNF- α , as well as KC and MIP-2, at 24 hours (**A** and **B**, respectively). Treatment with LPA (1 mg) strongly inhibited expression of TNF- α at 24 hours of reperfusion (**A**). The induction of KC and MIP-2 was not effected by LPA treatment (**B**). Shown are representative samples ($n = 4$ per group) calibrated against equal amounts of β -actin mRNA.

ment (data not shown). In conclusion, this study provides evidence that the administration of LPA dose dependently abrogates renal failure after I/R by inhibition of apoptosis and inflammation.

Discussion

LPA is a naturally circulating phospholipid with numerous biological actions. LPA signals via three different G protein-coupled receptors, until recently called Edg-2, Edg-4, and Edg-7; however, recently the nomenclature was changed to LPA₁, LPA₂, and LPA₃, respectively.^{14,19} All three LPA receptors have been localized in murine renal tissue.^{14,15} Also in human kidney LPA receptors are present, although human and murine tissues have a different expression pattern of the LPA receptors.¹⁴ The present study is based on reported *in vitro* properties of LPA: enhancement of cell survival and cell proliferation, and in particular its function as a growth and survival factor for renal proximal tubular cells.¹⁶ This study is the first to show that LPA functions as a renal survival factor *in vivo*, abrogating ischemia-induced apoptosis, and moreover that LPA prevents function loss in the course of renal I/R injury. Earlier we showed that survival factors such as insulin-like growth factor-1 prevent apoptosis *in*

vivo in the course of renal I/R injury.⁵ These data suggest that administration of LPA could have beneficial effects *in vivo* in diseases that are mediated by apoptosis such as I/R injury. Besides the potent growth factor and anti-apoptotic properties of LPA *in vitro*, this compound has been reported to have vasoactive effects *in vivo*.^{20,21} Interestingly, in rodents LPA appears to have vasoconstrictive effects, whereas in rabbits and cats LPA has vasodilatory properties.^{20,21} The extent to which the vasoactive properties *in vivo* add to the growth factor and anti-apoptotic properties of LPA still needs to be elucidated.

In vitro, LPA also stimulates cytoskeletal activation and remodeling.²² This cytoskeletal activation also seems to be of importance in ischemic injury, because disruption of the actin cytoskeleton has been implicated in the pathophysiology of renal I/R injury.^{17,18,23} Indeed, this study shows that F-actin, which is a major constituent of the epithelial brush border, is disrupted in the course of renal I/R injury. LPA treatment evidently prevents this disruption of brush border. *In vitro* studies have shown a clear relationship between caspase activation and changes in cell structure.^{24–26} Here, we show for the first time that caspase activation induced by renal I/R is localized to tubular epithelial cells. Moreover, this study shows that caspase-7 activation is only present in tubular epithelial cells that have lost their brush border. Whether the protective effects of LPA in this study are primarily mediated by protection of cellular integrity or by direct inhibition of apoptosis remains to be established.

In vitro studies have shown proinflammatory functions of LPA, mainly mediated by the activation of nuclear factor- κ B.^{27–30} The activation of nuclear factor- κ B and the subsequent induction of proinflammatory genes could potentially deteriorate I/R injury by enhancing the I/R-induced, tissue-damaging, inflammatory response.^{31,32} Thus, besides potential protective effects, LPA could also aggravate renal I/R injury. These *in vitro* data on LPA tempted us to study the role of LPA in I/R-induced inflammation *in vivo*. The present study demonstrates that LPA treatment has strong anti-inflammatory effects. We previously showed that the inhibition of apoptosis prevents the induction of chemokines and subsequently the influx of neutrophils after renal I/R.^{5,6} Our present data demonstrate that LPA prevents the induction of TNF- α and subsequent neutrophil influx. Interestingly, LPA did not affect the induction of chemokines, indicating that the abrogation of neutrophil influx by LPA is primarily mediated by a reduction of both TNF- α production and complement activation. The observation that LPA does not inhibit the induction of chemokines is in contrast to the effects of apoptosis inhibition by the caspase inhibitor ZVAD-fmk on chemokine induction in the course of renal I/R injury.⁶ This induction of chemokines during ischemia has been attributed to hypoxia and to the activation of caspase-7 that can lead to the generation and release of the mature chemoattractant endothelial monocyte-activating polypeptide (EMAP-II).^{5,6,33,34}

Figure 6. Treatment with LPA strongly reduces C3 deposition induced by renal ischemia reperfusion (I/R). Healthy animals (A) showed no staining for C3, whereas I/R induced an intense staining for C3 localized to injured tubular epithelium and tubular debris as shown at 24 hours of reperfusion (B). LPA treatment dose dependently reduced the I/R-induced deposition of C3 (C, 0.1 mg; D, 1 mg). Original magnifications, $\times 200$.

Recent work indicates that activation of the complement system plays an important role in I/R injury in the kidney as well as in other organs.^{35–41} We recently reported that inhibition of complement activation protects against renal I/R injury by inhibition of chemokine induction, neutrophil influx as well as the inhibition of apoptosis.⁷ The present study is the first to show that LPA abrogates the renal deposition of early as well as late complement factors. LPA dose dependently reduced the deposition of C3, C6, as well as C9, the latter two indicating formation of the MAC. The MAC has been implicated in renal I/R injury, by inducing proinflammatory cytokines, chemokines, promoting neutrophil influx, and moreover the induction of apoptotic as well as necrotic cell death.^{35,36,42,43} Recently, we showed that C5a also plays a major role in I/R injury of the kidney.⁴⁴ Inhibition of C5a activity, using a C5a receptor (C5aR) antagonist, significantly reduced the renal induction of chemokines and the influx of neutrophils. Moreover, C5a/C5aR interaction was shown to be primarily responsible for renal function loss after I/R. Also cardiac and intestinal I/R injury are mediated by activation of

the complement system and in particular by generation of C5a.^{37–41} The reduced deposition of C3 and MAC after LPA administration indicates that C5 activation is also prevented, and thus that C5a-mediated phenomena are inhibited by LPA. The mechanism that underlies the reduced complement activation in LPA-treated animals is as yet obscure. The activation of complement after ischemic injury has been attributed to mannose-binding lectin, to the classical pathway for the heart and also to the alternative pathway for the kidney.^{35,45,46} LPA may inhibit complement activation directly by inhibition of apoptosis because reoxygenation after hypoxia has been shown to induce complement activation by apoptotic cells *in vitro*.⁴⁷ Necrotic cells are also recognized as an important inducer of complement activation. The LPA-mediated inhibition of apoptosis might also prevent secondary necrosis that occurs when apoptotic cells are not timely phagocytosed, which could also contribute to the abrogation of complement activation.^{48,49}

Taken together, this study shows that LPA dose dependently inhibits inflammation by abrogation of comple-

Figure 7. Ischemia followed by reperfusion leads to strong intrarenal deposition of C6, which is totally abrogated by LPA treatment. Healthy animals showed a faint linear staining for C6 at the basal aspect of tubular epithelium (A). I/R induced intense additional staining of C6 that was localized to damaged tubular epithelium (B). LPA treatment totally prevented I/R-induced deposition of C6 as shown at 24 hours of reperfusion in a dose-dependent manner (C, 0.1 mg; D, 1 mg). Original magnifications, $\times 400$.

ment activation, cytokine induction, and neutrophil infiltration in the course of renal I/R. The exact mechanism of this anti-inflammatory capacity of LPA *in vivo* needs further investigation.

The aim of the present study was to characterize the *in vivo* effects of exogenous LPA in an experimental model for renal I/R injury. Local application of LPA has been shown to have beneficial effects on intestinal and cutaneous wound healing in rat models.^{50,51} Recently, oral administration of LPA also has been shown to have protective effects against radiation-induced apoptosis of intestinal epithelial cells in mice.⁵² These studies demonstrate the therapeutic potential of the naturally circulating phospholipid growth factor LPA in a wide variety of diseases in which apoptotic cell death as well as overwhelming inflammatory reactions play a pathophysiological role. Further *in vivo* studies are needed to delineate the LPA receptors responsible for the protective effects of LPA. Specific receptor agonists could be advantageous by having specific beneficial effects while abrogating potential side effects of LPA.⁵³

In summary, this study is the first to show the protective effects of exogenous LPA against renal I/R injury. LPA abrogates renal apoptosis, TNF- α production, complement activation, and neutrophil influx. Moreover, exogenous LPA is capable of preventing organ failure because of I/R and thus may provide new means to treat clinical conditions mediated by I/R injury.

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