Ischemia-Reperfusion Induces Glomerular and Tubular Activation of Proinflammatory and Antiapoptotic Pathways: Differential Modulation by Rapamycin

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Abstract. Ischemia-reperfusion (I-R) injury in transplanted kidney, a key pathogenic event of delayed graft function (DGF), is characterized by tubular cell apoptosis and interstitial inflammation. Akt–mammalian target of rapamycin–S6k and NF-κB–inducing kinase (NIK)–NF-κB axis are the two main signaling pathways regulating cell survival and inflammation. Rapamycin, an immunosuppressive drug inhibiting the Akt axis, is associated with a prolonged DGF. The aim of this study was to evaluate Akt and NF-κB axis activation in patients who had DGF and received or not rapamycin and in a pig model of I-R and the role of coagulation priming in this setting. In graft biopsies from patients who were not receiving rapamycin, phosphorylated Akt increased in proximal tubular, interstitial, and mesangial cells with a clear nuclear translocation. The same pattern of activation was observed for S6k and NIK. However, in rapamycin-treated patients, a significant reduction of S6k but not Akt and NIK activation was observed. A time-dependent activation of phosphatidylinositol 3-kinase, Akt, S6k, and NIK was observed in the experimental model with the same pattern reported for transplant recipients who did not receive rapamycin. Extensive interstitial and glomerular fibrin deposition was observed both in pig kidneys upon reperfusion and in DGF human biopsies. It is interesting that the activation of both Akt and NIK–NF-κB pathways was induced by thrombin in cultured proximal tubular cells. In conclusion, the data suggest that (1) coagulation may play a pathogenic role in I-R injury; (2) the Akt axis is activated after I-R, and its inhibition may explain the prolonged DGF observed in rapamycin-treated patients; and (3) NIK activation in I-R and DGF represents a proinflammatory, rapamycin-insensitive signal, potentially leading to progressive graft injury.

Clinical and experimental evidence suggests that an initial insult to kidney graft may influence both early and late transplant function (1). Ischemia followed by reperfusion has key implications in the pathogenesis of early graft damage. Overall, ischemia of a kidney graft is the sum of a possible transient warm ischemic interval before or during removal from the donor and cold ischemia associated with preservation and storage (2). Reperfusion, critical to the viability of the organ, may amplify the ischemic damage (3). Prolonged exposure of donor kidney to warm and/or cold ischemia increases the incidence of delayed graft function (DGF) upon transplantation and primes a programmed process within the kidney, which may lead to chronic and progressive lesions (4). Several studies report clear evidence that DGF, the main clinical manifestation of the initial ischemia-reperfusion (I-R) injury, may significantly affect graft outcome and suggest that long-term graft function might be improved by addressing the early graft injury induced by I-R (5).

I-R injury at the renal level is characterized by two main features: Apoptosis of tubular cells and interstitial inflammation. Renal ischemia induces tubular cell injury with decreased levels of ATP, increased levels of calcium, and alteration in membrane lipid and enzyme activity (6). Reperfusion of the ischemic organ exacerbates ischemic injury by producing cytotoxic oxygen species and free radicals (7). In addition, the deleterious role of the inflammatory response in I-R–induced organ damage is suggested by an enhanced expression of adhesion molecules and proinflammatory mediators (cytokines, chemokines), activation of the complement system, priming of the coagulation cascade, and subsequent leukocyte infiltration (8–10). In this setting, the role of the coagulation
cascade in the pathogenesis of interstitial inflammation and in the modulation of tubular cell functions is still largely unclear. Coagulation factors may influence renal resident cell activation interacting with specific cell-surface receptors that belong to the protease-activated receptor (PAR) family (11,12).

Among the signaling pathways that regulate cell survival and inflammation, the phosphatidylinositol 3-kinase (PI 3-K)–Akt–mammalian target of rapamycin (mTOR)–p70S6k and NF-κB–inducing kinase (NIK)–NF-κB axis could play a pivotal role in the postischemic phenomena. Akt or protein kinase B is a 60-kD serine/threonine kinase at the crossroad of multiple signaling pathways modulating several cell functions (13). Akt is recruited by its pleckstrin homology domain to membrane-localized PI 3,4,5-triphosphate and PI-3,4-P2, the second messenger products of PI 3-K. Membrane localization of Akt leads to its activation via phosphorylation at serine 473 in the C-terminal regulatory domain and at threonine 308 in the catalytic domain mediated by phosphoinositide-dependent protein kinases (13). Activated Akt dissociates from the plasma membrane and phosphorylates a variety of substrates in the cytoplasm and nucleus. Growing evidence indicates that Akt is a critical mediator of survival signals that protect cells from apoptosis (14,15). The particular clinical relevance in transplantation of the Akt–mTOR–p70S6k pathway is due to its specific inhibition by rapamycin. This potent immunosuppressive agent interacts with the mTOR and induces a cell-cycle arrest in the early G1 phase and apoptosis of tubular cells (16). Rapamycin has been shown to impair the recovery of renal function in a rabbit model of I-R injury (17). This observation was confirmed in clinical settings. Indeed, we recently reported about the mechanism underlying its activation in this setting (20). This transcription factor is composed of homo- or heterodimers of rel family members, which are held in the cytoplasm by the inhibitor IκB proteins. NF-κB activation results from the phosphorylation of IκB and the subsequent ubiquitination and degradation by the 26S proteasome (21). After destruction of IκB, NF-κB can then translocate to the nucleus to activate target genes (22). The signal-induced IκB phosphorylation is carried out by an IκB kinase (IKK) complex that is phosphorylated and then activated in response to several stimuli by the NIK. This serine-threonine kinase plays a pivotal role in the modulation of NF-κB activity because kinase-dead NIK blocks NF-κB activation by IL-1, TNF-α, and fas (21). Ozes et al. (23) recently demonstrated that Akt is necessary for NF-κB activation, thus suggesting a link between the ant apoptotic and proinflammatory pathways.

In the present study, we investigated the activation of PI 3-K, Akt, p70S6k, and NIK in a pig model of warm and short I-R. In addition, to investigate the role of rapamycin in the modulation of these two pathways during DGF, we studied Akt, p70S6k, and NIK activation in graft biopsies from patients with DGF and biopsy-proven acute tubular injury with no evidence of acute rejection treated with or without rapamycin. Finally, we evaluated the potential role of the coagulation cascade in these early events that lead to I-R-induced renal lesions.

Materials and Methods

Patients

Fifteen patients who received a kidney transplant from a cadaveric donor and presented with DGF, having given their informed consent, were included in the study. Seven patients were treated with cyclosporin A (CsA; group 1), and eight were treated with rapamycin (group 2). The two groups were matched for age, gender, cold ischemia time, and main donor’s features (Table 1).

All patients were given corticosteroids (500 mg methylprednisolone intraoperatively and then 250 mg prednisone daily, tapered to 25 mg by day 8), a chimeric anti-CD25 mAb (Simulect, two doses of 20 mg intravenously at day 0 and day 4), and mycophenolate mofetil (1 g twice a day). Patients of group 1 received a reduced CsA dose (5 to 8 mg/kg per d aiming at C2 blood levels of 800 to 1000 ng/ml). Group 2 patients received rapamycin (15 mg as loading dose, then 5 mg/d, aiming to blood through levels of 8 to 12 mg/ml). In all patients, a graft biopsy was performed after 7 to 10 d of DGF according to our clinical protocol. As control, we used seemingly normal tissue fragments from kidneys that were removed for renal cell carcinoma. The study was carried out according to Declaration of Helsinki principles and was approved by our institutional review board.

Renal I-R Injury Model

Small Landrace pigs (three female and three male) that weighed 12 to 18 kg were used. The animals were allowed to become accustomed to their surroundings for at least 5 d before surgery. All operative procedures were performed under general anesthesia (azaperone 2 mg/kg and atropine 0.04 mg/kg), and the animals were cared for by our standard protocol. The left renal vascular pedicle was atraumatically isolated by laparoscopy, and a vessel loop was positioned around the renal artery with a right-angle clamp. A renal biopsy was performed after 15 min, a second fragment of renal tissue was obtained, and thereafter the vessel loop was released. Multiple biopsies were then performed 15, 30, 45, and 60 min after reperfusion. A portion of each biopsy specimen was immediately snap-frozen in OCT (Tissuetek) medium and stored in liquid nitrogen.

<table>
<thead>
<tr>
<th>Patients (n)</th>
<th>Group 1</th>
<th>Group 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>47.3 ± 10.2</td>
<td>49.3 ± 9.7</td>
</tr>
<tr>
<td>Donor age (y)</td>
<td>57.3 ± 9.5</td>
<td>59.4 ± 13.5</td>
</tr>
<tr>
<td>Donor hypertension (Y/N)</td>
<td>4/4</td>
<td>4/4</td>
</tr>
<tr>
<td>Cold ischemia time (h)</td>
<td>14.5 ± 4.3</td>
<td>15.8 ± 1.8</td>
</tr>
<tr>
<td>Mismatches</td>
<td>2.8 ± 0.8</td>
<td>3.3 ± 0.7</td>
</tr>
<tr>
<td>DGF (d)</td>
<td>13.1 ± 6.6</td>
<td>23.4 ± 7.0^{a}</td>
</tr>
</tbody>
</table>

^{a} DGF, delayed graft function.
^{b} P < 0.001 versus group 1.
and another portion was fixed in buffered formalin (4%) for 12 h and embedded in paraffin using standard procedures.

**Microscopy Study**

Paraffin-embedded renal specimens from the animal model and from renal biopsies were used for conventional histologic staining (hematoxylin-eosin, periodic acid-Schiff, silver methenamine, and Masson’s trichrome). Tubulointerstitial and glomerular lesions were evaluated using a semiquantitative scoring system by two observers (L.G. and G.G.) who were unaware of the origin of the slides.

**Tissue Immunofluorescence and Confocal Laser Scanning Microscopy**

The activation of PI 3-K, Akt, p70S6k, and NIK in the animal model and in human biopsies was evaluated by indirect immunofluorescence and confocal microscopy analysis using specific antibodies that recognize the phosphorylated (p) and thus active form of the enzymes. For each enzyme, we performed a double-fluorescence immunolabeling to evaluate on the same tissue section the expression of the enzyme and the levels of its activated form. Anti–PI 3-K p85α mouse monoclonal antibody was raised against a peptide corresponding to amino acids 333 to 430 and mapping within the amino-terminal SH2 of the 85 subunit of PI 3-K (Santa Cruz Biotechnologies, Santa Cruz, CA). Anti–p–PI 3-K p85α goat polyclonal antibody was raised against a peptide corresponding to a short amino acid sequence that contained phosphorylated Tyr 508 (Santa Cruz Biotechnologies). Anti-Akt1 mouse mAb recognizes the sequence 345 to 480 of human and pig Akt1 (Santa Cruz Biotechnologies). Anti–p–Akt 1 was a rabbit polyclonal antibody raised against a short amino acid sequence that contained phosphorylated Ser 473 of human origin (Santa Cruz Biotechnologies). Anti-p70S6 kinase mouse mAb was raised against a peptide mapping at the carboxy terminus of rat p70S6 kinase (Santa Cruz Biotechnologies). Anti–p–p70S6 kinase mouse mAb was raised against a peptide that contained the phosphorylated Ser-411 (Santa Cruz Biotechnologies). Anti–p–NIK rabbit polyclonal antibody was raised against a peptide corresponding to a short amino acid sequence that contained phosphorylated Thr-559 of human NIK (Santa Cruz Biotechnologies).

**Cell Isolation and Culture**

HK2, an immortalized PTEC line from normal adult human kidney (24), was obtained from American Type Culture Collection (Rockville, MD). Cells were grown to confluence in DMEM/F12 medium supplemented with 10% FBS, 100 μg/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml sodium selenite, 5 pg/ml T3, 5 ng/ml hydrocortisone, and 5 pg/ml prostaglandin E1. For passage, confluent cells were washed with PBS, removed with 0.05% trypsin/0.02% EDTA in PBS, and fixed with paraformaldehyde 4%. Then, the cells were incubated for 5 min with Triton X-100 0.2% in PBS, washed, and incubated for 1 h with a blocking solution of BSA 3% in PBS. Subsequently, the cells were incubated with two primary antibodies: A monoclonal anti-Akt (1:500 dilution in BSA 3%/PBS; Santa Cruz Biotechnologies) for 2 h and a polyclonal anti-pAkt (1:200 dilution in BSA 3%/PBS; Santa Cruz Biotechnologies) antibodies for 1 h at room temperature in a humidified container. After washing, the cells were incubated in a mixture of two secondary antibodies for 1 h at room temperature: Alexa Fluor 488 goat anti-mouse IgG-FITC conjugate (1:200 dilution; Molecular Probes, Eugene, OR) and Alexa Fluor 543 goat anti-rabbit IgG-TRITC conjugate (1:600 dilution; Molecular Probes). The slides were then mounted in Gel/MB (Biomed) and sealed.

**Statistical Analyses**

Data are expressed as the mean ± SD and compared by ANOVA. P < 0.05 was considered statistically significant.

**Results**

**Akt Phosphorylation in Graft Biopsies from Patients with DGF**

We first evaluated the activation of Akt-p70S6k and NIK in the setting of DGF and their modulation by rapamycin. To this purpose, we considered two group of patients with DGF and biopsy-proven acute tubular damage treated with or without rapamycin. The main clinical features of the patients enrolled in the study are summarized in Table 1. No significant differences between the groups could be observed for donors’ features, cold ischemia time, and number of mismatches, whereas the length of DGF was strikingly longer in rapamycin-treated patients. We first investigated the Akt phosphorylation in this patient population. Confocal microscopy analysis of frozen renal tissue immunostained with specific anti-pAkt showed that contained 40 μg of proteins from each lysate were subjected to SDS/PAGE on a 10% gel under reducing conditions and then electrotransferred onto nitrocellulose membrane (Hybond C; Amersham, UK). The filter was blocked for 2 d at room temperature with 2% BSA in PBS that contained 0.1% tween-20 (TBS) and incubated with polyclonal anti–phospho-NIK antibody (Santa Cruz Biotechnologies) at 1:500 dilution in TBS at 37°C for 2 h. The membranes were washed twice in TBS and incubated for 1 h at room temperature with horseradish peroxidase–conjugated sheep anti-mouse IgG at 1:1500 dilution in TBS. The membranes were washed three times at room temperature in TBS and then once with 0.1% SDS in PBS. The ECL enhanced chemiluminescence system (Amersham) was used for detection. The same membranes were then stripped and immunoblotted again with anti-human NIK mAb at 1:1000 dilution in TBS (Santa Cruz Biotechnologies) at 37°C for 2 h. The ECL enhanced chemiluminescence system (Amersham) was used for detection.

**Cell Immunofluorescence and Confocal Laser Scanning Microscopy**

The activation of Akt was estimated also in vitro stimulating confluent and quiescent HK2 cells with thrombin (5 U/ml) for 5, 15, 30, and 60 min. We performed a double-fluorescence immunolabeling to evaluate the expression of the enzyme and the levels of its activated form. The cells were analyzed by confocal laser scanning microscopy using the Leica TCS SP2 (Leica, Wetzlar, Germany). To this purpose, HK2 cells (5 × 10⁴/slide) were seeded on cover glass, washed with PBS, and fixed with paraformaldehyde 4%. Then, the cells were incubated for 5 min with Triton X-100 0.2% in PBS, washed, and incubated for 1 h with a blocking solution of BSA 3% in PBS. Subsequently, the cells were incubated with two primary antibodies: A monoclonal anti-Akt (1:500 dilution in BSA 3%/PBS; Santa Cruz Biotechnologies) for 2 h and a polyclonal anti-pAkt (1:200 dilution in BSA 3%/PBS; Santa Cruz Biotechnologies) antibodies for 1 h at room temperature in a humidified container. After washing, the cells were incubated in a mixture of two secondary antibodies for 1 h at room temperature: Alexa Fluor 488 goat anti-mouse IgG-FITC conjugate (1:200 dilution; Molecular Probes, Eugene, OR) and Alexa Fluor 543 goat anti-rabbit IgG-TRITC conjugate (1:600 dilution; Molecular Probes). The slides were then mounted in Gel/MB (Biomed) and sealed.
that in normal conditions, at the tubular level, the phosphorylation level of this serine-kinase was low (Figure 1A). In group 1 patients, we detected in the proximal tubular and infiltrating cells a striking increase of phosphorylated Akt, predominantly located in the cytoplasm (Figure 1B). In group 2 patients, we observed a significant activation of the enzyme within tubular and infiltrating cells (Figure 1, C and D). It is interesting that nuclear Akt translocation was evident in this group of patients.

**p70S6 Kinase Phosphorylation in Patients with DGF**

We then investigated the activation of p70S6k in the same set of patients and the effects on this enzyme of rapamycin treatment. In normal kidneys, the active form of enzyme was observed at the proximal tubular level with a cytoplasmic distribution (Figure 2A). Group 1 patients presented a striking increase in p70S6k phosphorylation with a clear nuclear translocation of the enzyme (Figure 2, B and C). Conversely, in group 2 patients, p70S6k phosphorylation was almost completely abolished (Figure 2D).

**NIK Phosphorylation in Patients with DGF**

Finally, we investigated NIK phosphorylation in normal conditions and during DGF. A basal activation of NIK was observed only in mesangial cells (Figure 3A). A different situation was observed in all patients with DGF. Our immunohistochemical analysis revealed a striking NIK activation in the proximal tubular cells. In particular, the fluorescence signal showed to be located both in the cytoplasm and in the nucleus, and no difference was evident between groups 1 and 2 patients (Figure 3, B and C).

**Histologic Features of the Experimental Model of I-R**

We then analyzed a pig model of short-term warm ischemia followed by a limited period of reperfusion. The normal renal
architecture (Figure 4, A and B) was completely altered already after 30 min of reperfusion with the presence of extensive tubular damage and the appearance of an inflammatory infiltrate at the glomerular and interstitial levels, primarily constituted by polymorphonuclear leukocytes (PMN) but with a significant percentage of mononuclear cells (Figure 4, C and D). The inflammatory changes were accompanied by glomerular as well as interstitial clotting (Figure 4, E and F). Only after 60 min could we observe the presence of histologic signs of tubular cells apoptosis (Figure 4G). We then used this model to investigate the role of I-R in the activation of the signaling pathways upregulated during DGF in transplant recipients.

**I-R–Induced Glomerular and Tubular PI 3-K Phosphorylation**

We then examined the activation of PI 3-Kα in the I-R model. We observed a slight basal phosphorylation of the enzyme within the cytoplasm of podocytes before ischemia (Figure 5A). After 30 min of reperfusion, PI 3-Kα phosphorylation significantly increased in the mesangial area and reached the peak after 60 min (Figure 5, B and C). At the tubular level, we observed an early activation of PI 3-K p85α (Figure 5E) peaking at 60 min of reperfusion (Figure 5F). The phosphorylated enzyme presented a diffuse cytoplasmic distribution. The quantitative changes in PI 3-K phosphorylation

![Figure 4.](image1.png)

*Figure 4. Histology photomicrograph of pig kidney corticomedullary region during ischemia-reperfusion (I-R) injury. Periodic acid-Schiff (PAS) staining shows a normal structure of glomeruli and tubulointerstitium before clamping the renal artery (T0; A and B, respectively). After 30 min of reperfusion, in the glomeruli and in the proximal tubules, we observed an early cellular infiltrate (C and D). Increased numbers of infiltrating cells were seen 45 min after reperfusion associated with interstitial edema and an evident tubular vacuolization (E and F). After 60 min of reperfusion, proximal tubular cells presented apoptotic changes, including condensed and fragmented nuclear chromatin and shrinkage of cytoplasm (G).*

![Figure 5.](image2.png)

*Figure 5. Time course of glomerular and tubular phosphatidylinositol 3-kinase (PI 3-K) p85α phosphorylation during I-R in pig kidney. The activation of PI 3-K p85α was analyzed by confocal microscopy using antibodies that recognize phosphorylated (red) or nonphosphorylated (green) PI 3-K p85α. In basal conditions (T0), the active form of PI 3-K is detectable only on podocytes (A). After 30 min (T30) of reperfusion, the enzyme phosphorylation significantly increased (B) and reached the peak after 60 min (T60) both in mesangial cells and in podocytes (C). At the tubular level, in the basal condition, there was no detectable pPI 3-K (D). Reperfusion induced an early (15 min) activation of PI 3-K p85α (E), reaching the peak after 60 min with a diffuse cytoplasmic distribution (F). (G) Quantification of PI 3-K p85α phosphorylation during I-R. Results are expressed as mean ± SD of pPI 3-K/PI 3-K from five experiments. *P < 0.05 versus T0.*
reached statistical significance at 45 and 60 min of reperfusion at both glomerular and tubular levels (Figure 5G).

I-R–Induced Glomerular and Tubular Akt Phosphorylation

In the glomeruli, the active form of Akt was occasionally expressed in podocytes before ischemia (Figure 6A). After the induction of reperfusion, pAkt levels dramatically increased in mesangial and infiltrating cells (Figure 6B). At 30 min of reperfusion, we observed a diffuse nuclear Akt translocation peaking at 45 min (Figure 6C). At the tubular level, before the induction of ischemia, we observed a slight and diffuse Akt phosphorylation mainly located within the tubular cell cytoplasm (Figure 6D). After reperfusion, we detected a striking increase in the phosphorylated form of the enzyme at the nuclear level (Figure 6E). Akt phosphorylation and nuclear translocation significantly increased and reached the peak at 60 min in proximal tubular cells and within the cellular infiltrate (Figure 6F). The quantification of Akt phosphorylation levels demonstrated that at the tubular level, the difference with T0 reached statistical significance only after 60 min of reperfusion, whereas at the glomerular level, already at 15 min the difference was statistically significant (Figure 6G).

I-R–Induced Glomerular and Tubular p70S6 Kinase Phosphorylation

We then examined p70S6 kinase phosphorylation. In basal conditions, the active form of the enzyme was observed at the glomerular level in a diffuse pattern (Figure 7A). After 15 min of reperfusion, p70S6 kinase activation significantly increased in the mesangial area and reached the peak at 45 min (Figure 7, B and C). An early enzyme activation was observed at the proximal tubular level, reaching the peak at 45 min (Figure 7D–F). It is interesting that at this time point, there was a significant nuclear translocation (Figure 7F). Quantification of p70S6 kinase phosphorylation demonstrated a statistically significant increase, both at tubular and glomerular, after 45 min of reperfusion (Figure 7G).

I-R–Induced Glomerular and Tubular NIK Phosphorylation

Finally, we investigated NIK activation during reperfusion stress. A basal phosphorylation of NIK was evident within the glomerular tuft in podocytes (Figure 8A). After 15 min of reperfusion, we observed a striking activation of NIK (Figure 8B), reaching the peak after 30 min (Figure 8C). At the tubular level, we observed a basal, although slight, activation of NIK (Figure 8D). Reperfusion induced a striking increase in the levels of pNIK (Figure 8, E and F), reaching statistical significance after 30 and 60 min (Figure 8G).

Coagulation Cascade Priming in Patients with DGF

Coagulation priming was studied in all patients with DGF by immunofluorescence using a specific antifibrin antibody. Fibrin deposition, absent in normal kidney (Figure 9A), was strikingly increased in both groups 1 and 2 patients (Figure 9, B and C). The local priming of the coagulation cascade during I-R was further confirmed in the pig model. A clear upregulation of glomerular and interstitial fibrin deposition compared with basal levels was already evident after 15 min of reperfusion and reached a peak at 45 min (Figure 10).

Thrombin-Induced Akt and NIK Phosphorylation in HK2

To evaluate whether the priming of the coagulation cascade could play a role in the activation of the antiapoptotic pathway at the tubular level, we evaluated in vitro the ability of thrombin to induce the phosphorylation of Akt in cultured proximal

![Figure 6](image_url). Time course of glomerular and tubular Akt phosphorylation during I-R in pig kidney. The activation of Akt was analyzed by confocal microscopy using antibodies that recognize phosphorylated (red) or nonphosphorylated (green) Akt. Nuclei were stained with To-pro (blue). At T0, before the clamping of the renal artery, p-Akt was evident in some podocytes (A). After 15 min (T15) of I-R, pAkt levels dramatically increased in the mesangial area and infiltrating cells (B), reaching the peak after 45 min (T45) with a striking nuclear translocation (C). At T0, there was a slight activation of Akt predominantly located within the cytoplasm (D). Reperfusion induced a significant increase in p-akt levels after 30 min, with a peak at 60 min, when we observed a clear nuclear translocation (F). (G) Quantification of Akt phosphorylation during I-R. Results are expressed as mean ± SD of pAkt/Akt from five experiments. *P < 0.05 versus T0.
tubular cells. The serine protease caused a striking and time-dependent increase in Akt phosphorylation (Figure 11, A through E) that was statistically significant at 30 and 60 min of incubation (Figure 11F). It is interesting that the coagulation factor induced a significant nuclear translocation of the active form of Akt strictly resembling the in vivo pattern of activation.

We then investigated whether thrombin could influence the phosphorylation of NIK. The coagulation factor induced a time-dependent increase of NIK activation (Figure 12A) that reached statistical significance after 15 min of incubation (Figure 12B).

**Discussion**

In the present study, we demonstrated for the first time the early and simultaneous activation of two key signaling pathways in a pig model of I-R–induced renal damage and in the acute tubular damage featuring DGF in kidney transplant. This activation was correlated timely in both cases with a significant priming of the coagulation cascade as demonstrated by extensive fibrin deposition. In addition, we observed in vitro the ability of thrombin to reproduce these events in cultured tubular cells. I-R injury may play a pivotal role in the early phase of renal transplantation, and there is an increasing body of
patients who had DGF and did or did not receive rapamycin (B and C), fibrin deposition, absent in normal kidney (A), was evident in all patients who had DGF and did or did not receive rapamycin (B and C).

Figure 9. Coagulation cascade priming during DGF. Fibrin deposition was analyzed in all patients who had DGF and did or did not receive rapamycin by fluorescence microscopy. Interstitial and glomerular fibrin deposition, absent in normal kidney (A), was evident in all patients who had DGF and did or did not receive rapamycin (B and C).

Figure 10. I-R–induced interstitial coagulation priming. Fibrin deposition was evaluated by immunofluorescence and confocal analysis as described in Materials and Methods. Interstitial fibrin deposition, absent at T0 (A), was significantly upregulated after 15 min of reperfusion (B) and reached a peak at 45 min (C).

evidence suggesting its influence also in the pathogenesis of chronic allograft nephropathy (2,25). The particular clinical relevance in transplantation of the Akt pathway is due to its specific inhibition by rapamycin. This potent immunosuppressive drug, recently introduced in clinical use in renal transplantation, binds mTOR, a downstream target of Akt, inducing cell-cycle arrest in the early G1 phase and apoptosis of tubular cells (16,26,27). It has been shown that rapamycin severely impairs the recovery of renal function in a rabbit model of I-R injury (17). In addition, we and others recently observed a prolonged DGF in patients who received a renal transplant and were treated with rapamycin (18,28). The present observation in human biopsies of the ability of rapamycin to abolish the activity of p70S6k, a key enzyme in cell survival, may provide a molecular basis for this clinical effect.

I-R–induced Akt modulation was first demonstrated in the heart (29,30). Recently, Andreucci et al. (31) reported an increased phosphorylation of Akt in the crude lysate of renal tissue in a rat model of I-R, although they could not define precisely the cells in which this enzyme was activated. The use of confocal microscopy allowed us to identify tubular cells as the main cell type involved, although we also observed a significant upregulation of pAkt within glomerular and interstitial cells. The activation of Akt in different cell populations may suggest different prevalent functions of the signaling enzyme in each cell type: An antiapoptotic effect in tubular cells and a proinflammatory action in interstitial infiltrating cells.

Akt is a key survival signal that protects cells from apoptosis, and there is an increasing body of evidence that this enzyme may represent a nodal point to coordinate growth factor signaling in the early phase of I-R in several organs, including heart and liver (32–34). Akt activation exerts a powerful cardioprotective effect after transient ischemia. Matsui et al. (30) examined the effects of a constitutively active Akt mutant (myr-Akt) in a rat model of cardiac I-R injury. In vivo gene transfer of myr-Akt reduced infarct size by 64% and the number of apoptotic cells by 84%. Preconditioning is a well-known phenomenon by which a brief exposure to ischemia makes a tissue more tolerant to a subsequent I-R–induced insult (35). The observation that I-R may induce the activation of a powerful antiapoptotic enzyme may explain the ability of preconditioning to reduce the deleterious effect of I-R on tubular cell survival (36).

The mechanisms underlying the potential Akt antiapoptotic effects in this particular setting are still largely unclear. It has been shown that Akt promotes cell survival mainly through direct phosphorylation of proapoptotic proteins and regulating the transcription of pro- and antiapoptotic genes (37). The Forkhead (FKH) family of transcription factors NF-κB and p70S6 kinase are among the main cellular targets regulated by Akt potentially involved in its beneficial effects on cell survival (14). Recently, Shimamura et al. (38) demonstrated that the Akt pathway acts as a survival signal in cultured mesangial cells mainly via NF-κB. However, it is well known that the powerful proapoptotic activity of FKH is severely impaired upon serine phosphorylation by Akt (37). Finally, p70S6 kinase is a key enzyme directly activated by mTOR (16,25). This serine kinase may phosphorylate and inactivate BAD, one of the main proapoptotic mediators (37).

Andreucci et al. (31) demonstrated a significant increase of FKH serine phosphorylation in a rat model of I-R. However, several reports demonstrated the early activation of NF-κB after I-R, and this transcription factor may play a key role not only in the proinflammatory but also in the regenerative response (39). In the present study, we observed that Akt phosphorylation after reperfusion was followed by the activation of two downstream signaling enzymes: The antiapoptotic p70S6 kinase and the proinflammatory NIK. p70S6 kinase phosphorylation closely resembles the one observed for Akt including the nuclear translocation, further suggesting a close relationship between Akt and S6 kinase phosphorylation and subsequent activation. Noteworthy, the nuclear translocation of both enzymes is a novel finding in this setting, although already reported in several cell types in vitro. In the nucleus, Akt directly phosphorylates p300, a key co-activator of NF-κB, and is required for stimulating its transactivation effects, thus potentiating NF-κB activity (40).

In addition, Ozes et al. (24) recently demonstrated that Akt is necessary for NF-κB activation, thus supporting the hypothesis of a link between the antiapoptotic and proinflammatory pathways. These authors suggest that the two signaling pathways, PI-3k-Akt and TRAF-NIK, converge on IKK to cause the downstream activation of NF-κB, and they demonstrate that both pathways are necessary to obtain this final effect. In
the present study, we demonstrate for the first time that I-R activates both pathways simultaneously in the same cells. In addition, the glomerular and interstitial increase in Akt phosphorylation, most likely as a result of its activation within infiltrating inflammatory cells, may further suggest a potential and unexpected role for Akt in inflammation. Many studies have established a crucial role of the PI 3-K/Akt signaling pathway in chemotaxis (41,42). Indeed, Akt asymmetric recruitment to the leading edge of cell membrane is absolutely necessary for chemotaxis of several cell lines, although the precise mechanism of how activation of Akt leads to chemotaxis is still largely unclear. In addition, Hirsch et al. (43) suggested a role for Akt in priming the respiratory burst in PMN.

It is well established that Akt is a downstream effector of PI 3-K, and we observed a significant increase in tyrosine phosphorylation of PI 3-Kα after I-R. It is interesting that the spatial pattern of PI 3-Kα activation, limited to resident cells, did not reproduce the one observed for Akt. However, we need to consider that there are several isoforms of PI 3-K: α, β, and γ (44). The last misses the regulatory subunit and does not require tyrosine phosphorylation for its activation. There is an increasing body of evidence that this isoform, mainly activated by G protein–coupled receptors including chemokine receptors, is a key modulator of inflammation. Hirsch et al. (43) demonstrated that PI 3-Kγ–null mice showed a reduced recruitment of macrophages in a septic peritonitis model. Thus,

Figure 11. Thrombin-induced Akt phosphorylation in cultured proximal tubular cells (HK2). The activation of Akt was analyzed by confocal microscopy with antibodies that recognize phosphorylated (red) or nonphosphorylated (green) Akt. Thrombin caused a striking and time-dependent increase in Akt phosphorylation (B compared to control A). We observed a significant nuclear translocation of the active form of Akt after 30 min of stimulation with thrombin (C and D) that reached the peak after 60 min (E). (F) Quantification of Akt phosphorylation in HK2. Results are expressed as mean ± SD of pAkt/Akt from three experiments. *P < 0.05 versus basal condition.

Figure 12. Thrombin-induced activation of NIK in cultured proximal tubular cells (HK2). (A) After exposure to thrombin (5 U/ml) for the indicated time periods, cells were harvested and p-NIK was studied as described in Materials and Methods. (B) Quantification of Akt phosphorylation in HK2. Results are expressed as mean ± SD of pNIK/NIK from three experiments. *P < 0.05 versus basal condition.

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it is conceivable than Akt activation may be due to PI 3-Kα in resident cells and to PI 3-Kγ in infiltrating cells.

The activation of Akt and NIK pathways observed in the experimental model was confirmed in the human counterpart of I-R injury: Acute tubular injury of renal graft leading to DGF. Indeed, in graft biopsies of patients with DGF, we observed for the first time a strikingly increased phosphorylation of Akt, p70S6k, and NIK. The administration of rapamycin completely abolished p70S6k activation. This intracellular event may represent one of the molecular mechanisms underlying the prolonged period of DGF observed in patients who are treated with this drug (18,28). However, rapamycin does not influence NIK activation, and this may represent an escape pathway for tubular cell regeneration. Indeed, Chen et al. (39) reported that inhibition of NF-κB, although reducing the inflammation-mediated tissue damage, irreversibly compromised epithelial regeneration in an experimental model of I-R-induced intestinal damage. However, it is conceivable that NIK activation completely unaffected by rapamycin may represent a proinflammatory signal, potentially leading to progressive renal damage.

Endothelial cell activation was shown recently to be a key event I-R–induced renal injury (45). The observation of a significant priming of the coagulation cascade may strongly support this hypothesis. It is interesting that there is an increasing body of evidence that rapamycin may deeply influence endothelial cell function, reducing cell survival, increasing thrombogenicity, and inducing a proinflammatory status (46,47). Thus, it is conceivable that the deleterious effects of this drug on I-R–induced renal injury may be due, at least partly, to its effects on endothelial cells. The relevance of coagulation priming, often disregarded in I-R–induced renal damage, has been clearly demonstrated in heart injury caused by I-R (48). Indeed, Erlich et al. (48) demonstrated that thrombin inhibition by hirudin infusion significantly reduces the infarct size by 59% in the heart exposed to I-R, decreasing both chemokine expression and inflammatory cell infiltration. There is evidence that thrombin can contribute to local inflammation and tissue damage by activation of three protease-activated receptors (PAR) that stimulate cells to express cytokines and growth factors (49). However, we previously demonstrated that tubular cells express high level of PAR-2, and its activation may cause an increased expression of proinflammatory cytokines, including monocyte chemoattractant protein-1, but also a regenerative response (11). In light of these findings, we investigated in vitro the ability of thrombin to induce the phosphorylation of Akt and NIK in cultured proximal tubular cells. We demonstrated that thrombin can induce a striking and time-dependent activation of Akt and NIK that resembles the time pattern observed in vivo after reperfusion. These data suggest a role for this protease in the simultaneous activation of these two pathways within the kidneys undergoing the I-R insult. A potential role for PAR in this scenario was already suggested by Napoli et al. (50). This group observed that PAR-2 activation could prevent some of the I-R damage in the heart. However, although tubular cells express PAR-2, its activation does not cause either Akt or NIK activation (data not shown).

In conclusion, the activation of the Akt axis in I-R may represent an antiapoptotic mechanism, and its inhibition by rapamycin may be one of the mechanisms of delayed tubular regeneration caused by this immunosuppressive drug. In addition, the contemporary activation of NIK and Akt may suggest these enzymes as the crossroad between inflammation and regeneration in this scenario. Finally, their activation by thrombin may support the hypothesis of a role for the coagulation cascade not only in the inflammatory but also in the regenerative response to I-R.

Acknowledgments
This study was supported by the Centro di Eccellenza Genomica in Campo Biomedico ed Agrario (CEGBA), the MIUR (PRIN 2002 to F.P.S. and L.G.), and the 5th European Framework “Quality of Life and Management of Living Resources (QLG1-2002-01215 to G.G.). A.L. is supported by a grant from the University of Foggia.

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