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p21 and mTERT are novel markers for determining different ischemic time periods in renal ischemia-reperfusion injury

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Hochegger K, Koppelstaetter C, Tagwerker A, Huber JM, Heininger D, Mayer G, Rosenkranz AR. p21 and mTERT are novel markers for determining different ischemic time periods in renal ischemia-reperfusion injury. *Am J Physiol Renal Physiol* 292: F762–F768, 2007. First published September 12, 2006; doi:10.1152/ajprenal.00084.2006.—In many clinical settings, the duration of renal ischemia and therefore the outcome of acute renal failure cannot be determined adequately. Renal ischemia reperfusion injury is known to shorten telomeres and upregulate stress-induced genes, such as the cyclin-dependent kinase (CDK) inhibitor p21. So far, the expression and role of CDK inhibitors, as well as mouse telomerase reverse transcriptase (mTERT), has not been investigated in a model with variable lasting ischemic periods. Male C57Bl/6 mice were subjected to renal ischemia reperfusion injury by clamping both renal pedicles for 10, 20, 30, and 45 min, and the kidneys were allowed to be reperfused for 3, 24, and 48 h. Expression of different CDK inhibitors and mTERT was evaluated. Mice developed signs of acute renal failure linear to the duration of the ischemic period. Real-time PCR revealed that mTERT was only significantly upregulated in kidneys after short ischemic periods (20 min). In contrast, p21 was constantly upregulated in kidneys after long ischemic intervals (30 and 45 min), but not in kidneys, which were clamped for shorter periods. Mainly, tubular cells contributed to the observed increase in p21 expression. Targeting p21 via the selective p53 inhibitor pifithrin-α was able to prevent acute renal failure when administered immediately before ischemia. The expression of another CDK inhibitor, namely p16, was differentially regulated, depending on the time of reperfusion. Taken together, we detected mTERT and p21 as "indicator" genes for short and long ischemic intervals, respectively. These two proteins might also be possible new therapeutic targets in the treatment and prevention of acute renal failure.

cyclin-dependent kinase inhibitors; acute renal failure; cell cycle

ACUTE RENAL FAILURE IS A COMMON clinical event that is followed by decreased allograft survival in the setting of kidney transplantation and is associated with mortality rates of 30–40% in patients with native kidneys (9, 13, 31). There is growing evidence that the family of cyclin-dependent kinase (CDK) inhibitors plays a major role in renal ischemia reperfusion injury (IRI) (5, 17, 33).

Progression through each phase of the cell cycle requires the formation of a specific complex, comprising a cyclin and a CDK. In addition, the cell cycle is also coordinated by endogenous negative cell cycle regulatory proteins called CDK inhibitors, such as $p15^{INK4b}$ (p15), $p16^{INK4a}$ (p16), $p21^{WAF1/CIP1}$ (p21), and $p27^{Kip1}$ (p27). These bind to and inhibit the activity of cyclin/CDK complexes (22, 23). CDK inhibitors can get activated by certain types of stress, such as telomere shortening, DNA damage, or oxidative stress, which is followed by permanent and irreversible proliferation arrest (20).

CDK inhibitors are divided into two major families: the Cip/Kip family, which is represented by the prototypes p21, p27, and p57 (16, 24, 32), and the INK4 family, including p15, p16, p18, and p19 (29). In several types of acute renal failure, an increase in p21 transcription and protein expression has been demonstrated (18, 19). The expression of p21 is mainly regulated by p53, which is activated by several stress-induced mechanisms, such as telomere shortening or DNA strand breaks.

Recently, Chkhotua and coworkers (5) showed that the cell cycle inhibitor p16 is increased in an experimental pig model of ex vivo renal hemoperfusion. Additionally, p16 was stably increased in a model of chronic renal allograft rejection (10), but to date there are no existing data about a regulation of p16 in renal IRI.

Renal IRI is associated with telomere shortening (6), which is followed by p21 activation (20). The ribonucleoprotein telomerase can inhibit the shortening of telomeres and contains two essential components: the telomerase reverse transcriptase (TERT) and the telomerase RNA, which provides the template for the reverse transcription of new telomere DNA by TERT (reviewed in Ref. 3). In cells with forced expression of TERT, the progressive shortening of telomeres is prevented, and these cells continue to proliferate, even at numbers of cell divisions at which they would normally go into cell cycle arrest (4). However, limited experience with mouse TERT (mTERT) exists. In different stress-induced disease models, such as hindlimb ischemia and ischemic brain injury, mTERT played an important role in tissue regeneration (1, 35). Interestingly, gene knockout experiments revealed that $mTERT^{-/-}$ mice had no obvious abnormalities in early generations, although significant telomere shortening was observed in successive generations (27).

In our present study, we detected mTERT and p21 as stable markers for short and long periods of renal ischemia, respectively. By targeting p21 via the p53 inhibitor pifithrin-α (PIF), we demonstrated the in vivo relevance of p21 as a mediator of acute renal failure.

MATERIAL AND METHODS

Animals and renal ischemia model. Experiments were performed on male C57BL/6 mice weighing 18–22 g. Mice were maintained on standard diet, and water was freely available. Mice were anesthetized,

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and an incision was made on the central abdomen. Avoiding intestines and bowel, microvascular clamps were applied to bilateral renal pedicles. After 10, 20, 30, and 45 min of renal ischemia, clamps were removed, and the incision was closed. During the procedure, mice were well hydrated, and their body temperature was controlled at ~37°C using an adjustable heating pad. Animals were killed after 3, 24, and 48 h. Sham-operated animals underwent the same surgical procedure without clamping the renal pedicles. In one set of experiments, 2 mg/kg PIF (Calbiochem, Merck KG, Darmstadt, Germany) was applied intraperitoneally immediately before or 1 h after inducing ischemia. All animal experiments were approved by Austrian veterinary authorities.

Assessment of renal function. Blood samples were obtained from mice 3, 24, and 48 h after ischemia, and serum creatinine levels were measured as markers of renal function. Serum creatinine was measured using a creatinine autoanalyzer (Beckman Coulter, Fullerton, CA).

Assessment of histological injury. Kidney tissue was fixed in buffered 4% formalin overnight and then embedded in paraffin wax. The kidneys were sectioned at 5 μm and stained with periodic acid-Schiff or hematoxylin and eosin using a standard protocol. Tubular injury was assessed in periodic acid-Schiff-stained sections using a semiquantitative scale, as recently described (25, 26). Briefly, the percentage of corticale tubules showing epithelial necrosis was scored: 0 = no tubular necrosis; 1 = <10%; 2 = 10–25%; 3 = 26–75%; 4 = >75% tubular necrosis. The scoring was performed by one blinded reviewer.

Isolation of tubular, glomerular, and interstitial cells from kidneys. Mice were subjected to renal IRI by clamping both renal pedicles for 30 min and reperfusion for 24 and 48 h. Tubules, glomeruli, and interstitial cells (ICs) were isolated using three sieves (150, 90, and 45 μm), as has been described earlier (28). Shortly, minced murine kidneys were passed through the top 150-μm sieve (Newark Wire Cloth, Clifton, NJ). From the top of the middle 90-μm sieve (Newark Wire Cloth), tubuli were collected. From the top of the third 45-μm sieve (Newark Wire Cloth), the glomeruli were collected. The flow through was referred to as ICs. The isolation of the different compartments was checked by phase-contrast microscopy (data not shown). Isolated compartments were washed with phosphate-buffered saline and subjected to isolation of total RNA.

Quantitation of mRNA by real-time PCR. Total RNA was isolated from whole kidneys and isolated compartments of the kidney using TRIzol reagent (Sigma), according to the standard protocol. Thereafter, 1 μg of total RNA was reverse transcribed using Omniscript Reverse Transcription Kit (Qiagen, Hilden, Germany) and random primers (Roche, Basel, Switzerland). Real-time PCR was performed on an ABI Prism 7700 (Applied Biosystems, Foster City, CA) using the TaqMan gene expression assays (Applied Biosystems) for detection of p16 (Mm00494449m1), p21 (Mm00432448m1), p27 (Mm00438168m1), and mTERT (Mm004369231m1), according to the manufacturer's protocol. As reference gene 18S was detected by using a TaqMan gene expression assay. 18S revealed to be stably expressed after induction of renal IRI (data not shown).

Immunohistochemistry of p16 and p21. p21 was determined on 5-μm paraffin-embedded tissue sections, which were deparaffinized with xylene and rehydrated with graded ethanol. Slides were treated with antigen unmasking solution (Vector Laboratories, Burlingame, CA), according to the manufacturer's protocol. Endogenous peroxidase was blocked with fresh-prepared 0.3% H_2O_2-methanol for 30 min. Slides were blocked with 10% goat serum, 20% FCS for 20 min. p21 was detected using a rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA), diluted 1:100. Biotinylated goat anti-rabbit or goat anti-mouse antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA), diluted 1:200, were used as secondary antibodies. p21 was visualized using the ABC Elite Vectastain Kit (Vector Laboratories).

p16 was also determined on paraffin-embedded tissue sections. After deparaffinization, rehydration, antigen unmasking, and blockage of the endogenous peroxidase, slides were blocked and stained with the help of the M.O.M. staining kit (Vector Laboratories) and the mouse anti-mouse p16 monoclonal antibody (Santa Cruz Biotechnology).

Statistical analysis. Statistical significance was assessed by ANOVA and the Newman-Keuls post hoc test for multiple comparisons. In case of groups with $n = 4$, Kruskal-Wallis test was performed, and then each group was compared with the sham-operated controls by Mann-Whitney *U*-test. The level of significance was corrected to the number of groups ($P < 0.0125$ was considered significant). When only two groups were compared, Mann-Whitney *U*-test was performed, and differences were considered significant at $P < 0.05$.

RESULTS

Increase in kidney injury is dependent on the ischemic and reperfusion time period. In the clinical setting such as vascular surgery or transplantation, ischemia of the kidneys for different periods of time can result from various complications. To evaluate the influence of variable lasting ischemic periods on the outcome of kidney injury, mice were subjected to renal ischemia for 10, 20, 30, and 45 min and followed for 3, 24, and 48 h of reperfusion. Serum creatinine (Fig. 1) and tubular injury (data not shown) linearly increased with the duration of ischemia and reperfusion.

This model reflects the clinical setting of acute renal failure, in which serum creatinine and histological changes increase linearly to the length of ischemia and reperfusion.

Expression of the CDK inhibitor p21$^{WAF1/CIP1}$ in renal IRI. So far, there exists no marker to detect the duration of ischemia in kidneys. Therefore, we used our model of different ischemic periods for the evaluation of CDK inhibitors, such as p21, which has been described to be regulated in renal IRI (12, 17). The CDK inhibitor p21 gene expression was significantly increased in all kidneys, which underwent extended intervals

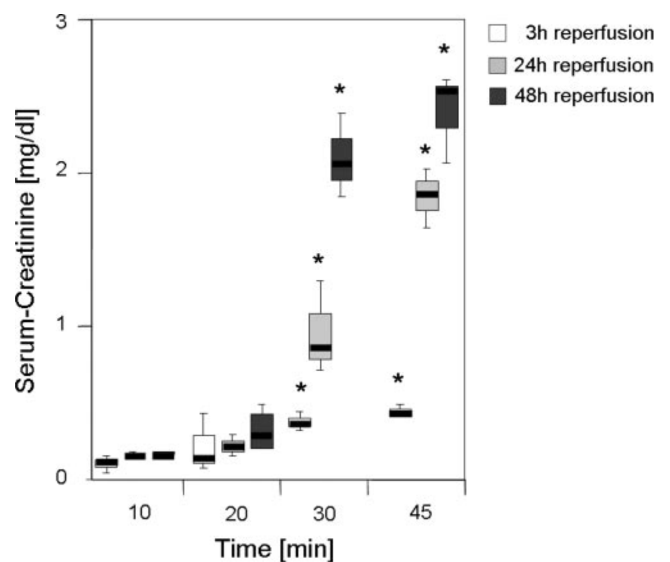

Fig. 1. Serum creatinine. Both renal pedicles were clamped for the indicated time periods and were reperfused for 3 (open box blot; $n = 4$ mice per group), 24 (shaded box blot; $n = 4$ mice per group), and 48 h (solid box blot; $n = 8$ mice in the 10-, 20-, and 30-min group, $n = 4$ in the 45-min group). Serum creatinine was evaluated. *$P < 0.0125$ compared with sham-operated animals.

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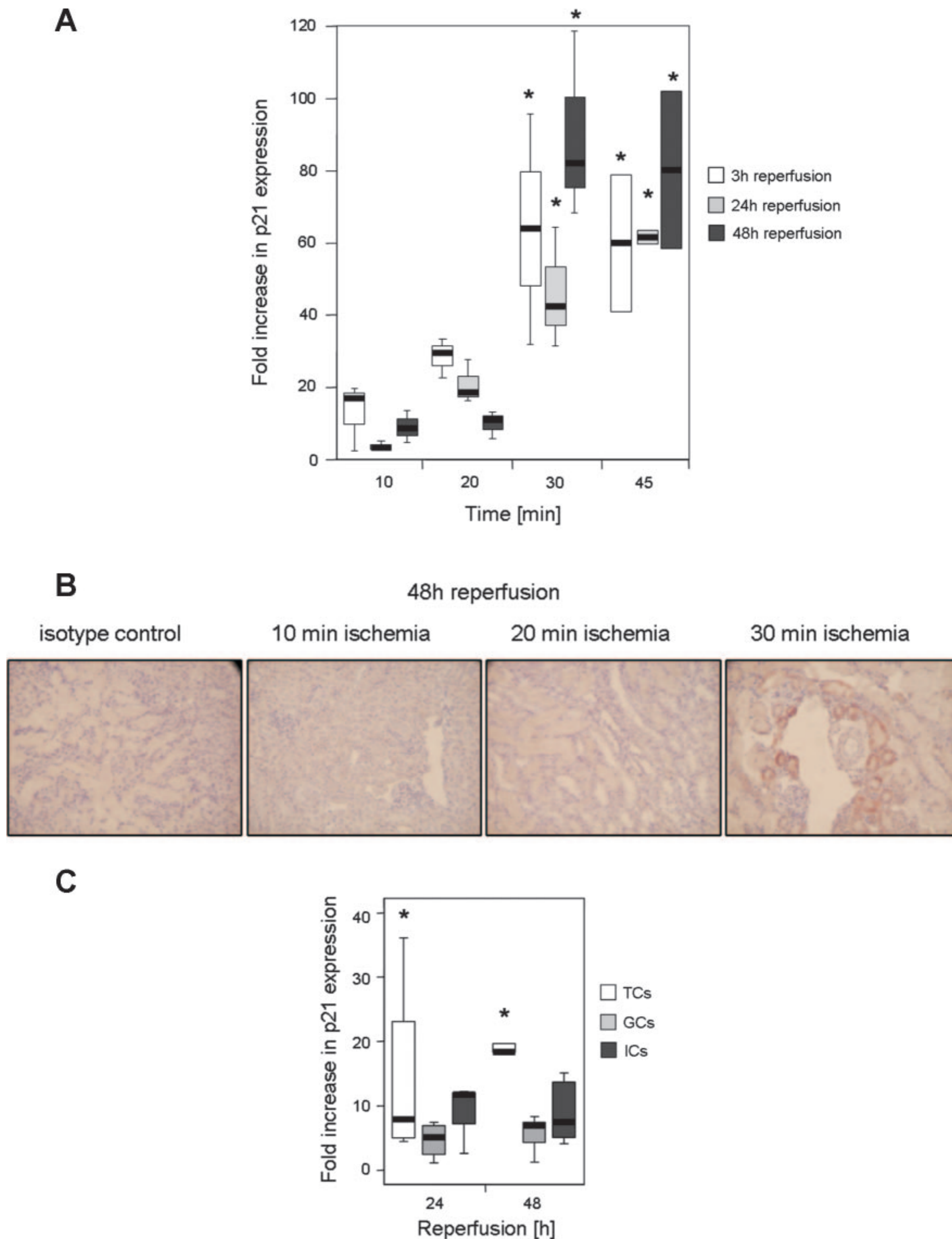

Fig. 2. Gene expression of p21. *A*: quantitative PCR for the detection of p21 was performed in ischemic kidneys after a reperfusion interval of 3 (open box blot; n = 4 mice in each group), 24 (shaded box blot; n = 4 mice in each group), and 48 h (solid box blot; n = 8 mice in the 10-, 20-, and 30-min group, n = 4 in the 45-min group). $^*P < 0.0125$ compared with sham-operated animals. *B*: p21 staining was performed in sham-operated and ischemic kidneys 48 h after ischemia. Representative pictures are presented. Magnification ×200. *C*: quantitative PCR for the detection of p21 was performed in isolated tubular cells (TCs; open box blot), glomerular cells (GCs; shaded box blot), and interstitial cells (ICs; solid box blot) of kidneys, which were clamped for 30 min and reperfused for 24 h (n = 6) and 48 h (n = 7). Data are provided as fold increase of p21 expression in TCs, GCs, and ICs of diseased kidneys compared with respective cells of sham-operated controls. $^*P < 0.05$.

of ischemia, namely 30 and 45 min, independent of the reperfusion time. Even 3 h after the longer ischemic events, p21 transcription was significantly increased in the kidneys (Fig. 2*A*). In line with these data and the findings of Megyesi and coworkers (17), we also observed increased expression of p21 on protein level in kidneys, which underwent 30 min of ischemia. In detail, upregulation of the p21 protein after 30 min of ischemia was mainly detectable in tubular cells (TCs), as

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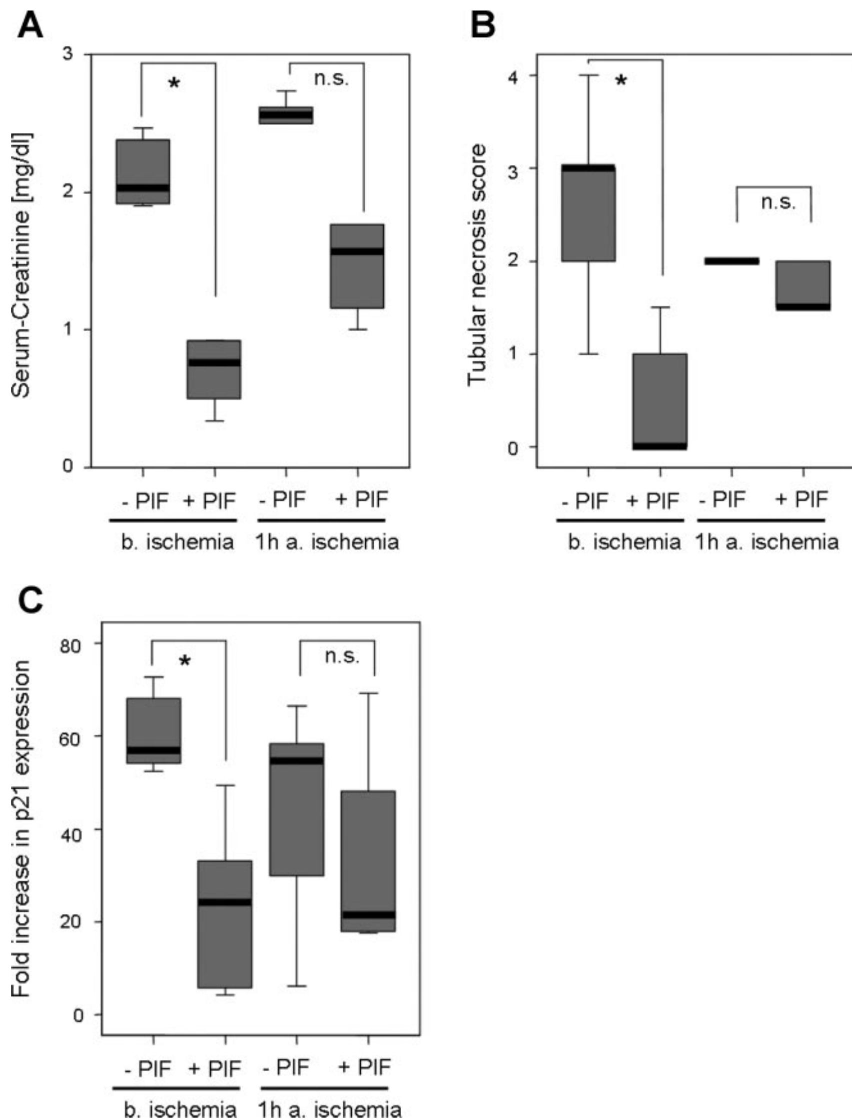

Fig. 3. Effect of pifithrin-α (PIF) on renal ischemia reperfusion injury (IRI). PIF or vehicle was injected intraperitoneally directly before or 1 h after clamping both renal pedicles for 30 min (n = 5 in each group). Serum creatinine (*A*), tubular necrosis score (*B*), and p21 transcription (*C*) were analyzed after 48 h of reperfusion. n.s., Not significant. $^*P < 0.05$.

demonstrated by immunohistochemistry (Fig. 2*B*). To further test which compartment of the kidney mainly contributes to the observed increase in p21 expression in long ischemic kidneys, we isolated mRNA from different compartments of kidneys, which were clamped for 30 min and reperfused for 24 and 48 h. Interestingly, we found significantly increased p21 mRNA only in TCs of ischemic kidneys compared with TCs of sham-operated controls after 24 and 48 h. No significantly increased p21 transcription was found in glomerular cells (GCs) and ICs of ischemic kidneys compared with GCs and ICs of sham-operated controls (Fig. 2*C*). In contrast, no significant changes in gene expression of the family member of p21, p27, were found in the murine model of renal IRI used throughout the study (data not shown).

To test whether the observed regulation of p21 is important for the outcome of renal IRI and since there exists no inhibitor for p21, we targeted the upstream protein p53 by using the selective inhibitor for p53 PIF in our model of renal IRI (11). When PIF was administered at a concentration of 2 mg/kg intraperitoneally directly before clamping the renal pedicles for 30 min, serum creatinine (Fig. 3*A*) and tubular necrosis score (Fig. 3*B*) were found to be significantly decrease after 48 h of reperfusion compared with mice that received the vehicle. To test whether PIF is also able to protect the mice when applied after renal ischemia, we clamped the renal pedicles for 30 min, injected PIF after 1 h, and reperfused for 48 h. Interestingly, we found no significant differences in serum creatinine (Fig. 3*A*) and tubular necrosis score (Fig. 3*B*) between mice receiving PIF and vehicle, respectively. The p21 mRNA expression in the kidney was found to be significantly decreased when PIF was applied directly before inducing renal IRI, whereas no significant changes in p21 mRNA transcription were seen in mice that received PIF 1 h after renal IRI was induced (Fig. 3*C*).

p21 mRNA transcription is stably upregulated in kidneys, more precisely in TCs, which underwent 30- or 45-min ischemia, starting already at 3 h of reperfusion. In contrast, no regulations of p21 expression were observed in kidneys, which were clamped for 10 or 20 min. Additionally, mice can be protected from renal IRI by indirectly targeting p21 via the p53 inhibitor PIF.

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Expression of mTERT in renal IRI. Renal IRI is associated with telomere shortening (6), which is followed by subsequent p53 and p21 activation (20). Telomerase has been shown to inhibit the shortening of telomeres and partly consists of mTERT (reviewed in Ref. 3). Therefore, the gene expression of mTERT was tested in our model of renal IRI. In contrast to p21 expression, mTERT mRNA expression was significantly increased in kidneys, which were clamped for 20 min and reperfused for 3, 24, and 48 h compared with sham-operated controls. Additionally, mTERT expression was significantly increased in kidneys, which were ischemic for 10 min and reperfused for 48 h (Fig. 4). In contrast, no regulation at all was seen in kidneys after 30 and 45 min of ischemia at the different reperfusion times. Taken together, we found a new and stable marker for short ischemic intervals (10 and 20 min), which is already upregulated 3 h after ischemia. Therefore, mTERT can be considered as a marker for a positive outcome of renal IRI, as was reflected by the absent increase of serum creatinine.

Expression of the CDK inhibitor $p16^{INK4a}$ in renal IRI. The CDK inhibitor p16 has been shown to be activated by stress factors, such as renal IRI, independent of the p53/p21 pathway (20), and has been shown to be regulated in a model of chronic renal allograft rejection (10) and of ex vivo renal hemoperfusion (5). Interestingly, p16 revealed to be differentially regulated in the kidneys, depending on the ischemic and reperfusion time. After 3 h of reperfusion, p16 was not differentially regulated compared with the sham-operated controls. In contrast, p16 was significantly increased in kidneys, which underwent ischemia for 20, 30, and 45 min and reperfusion of 24 h. The observed increased transcription was linear to the serum creatinine. Interestingly, 48 h after ischemia, p16 transcription was only significantly increased in kidneys, which were clamped for 20 min (Fig. 5*A*). Protein expression of p16 in kidneys after 48-h reperfusion detected by immunohistochemistry revealed to be linear to the gene expression profiles. In detail, increased p16 protein expression in kidneys after 20 min of ischemia and 48 h of reperfusion was mainly detected in TCs. In contrast, significantly decreased numbers of p16 expressing TCs were found in kidneys, which underwent 30 min of ischemia (Fig. 5*B*).

Thus p16 gene and protein expression in our model of renal IRI is strongly dependent on the reperfusion time and can therefore not be used to differentiate between long and short periods of ischemia.

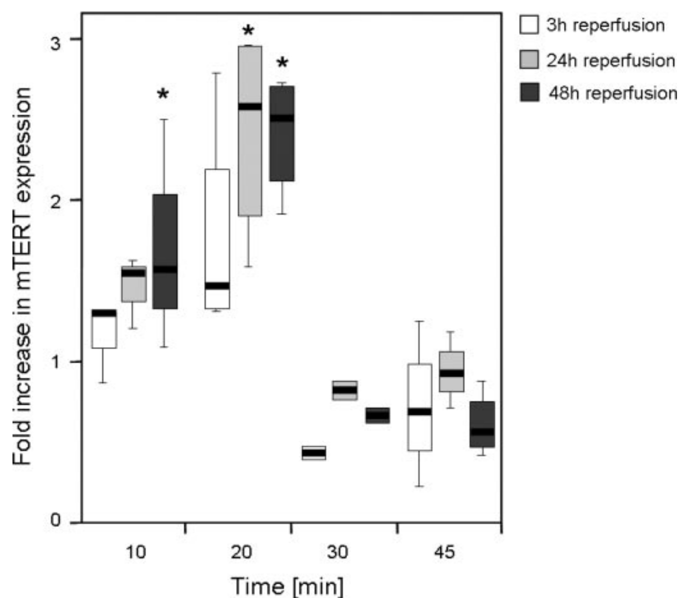

Fig. 4. Gene expression of mouse telomerase reverse transcriptase (mTERT). Quantitative PCR for the detection of mTERT was performed in kidneys, which were clamped for the indicated time intervals and reperfused for 3 (open box blot; $n = 4$ mice in each group), 24 (shaded box blot; $n = 4$ mice in each group), and 48 h (solid box blot; $n = 8$ mice in the 10-, 20-, and 30-min group, $n = 4$ in the 45-min group). $*P < 0.0125$ compared with sham-operated animals.

DISCUSSION

Renal IRI is associated with a cascade of cellular events, leading to tissue damage and subsequent increased clinical morbidity in the setting of acute renal failure, or decreased allograft survival in the setting of kidney transplantation (9, 31). In vascular or abdominal surgery, acute renal failure can occur because of sustained ischemic complications, but, to date, no studies exist investigating the clinical setting of different ischemic and reperfusion intervals. Therefore, we clamped the renal pedicles for different time periods and reperfusing for 3, 24, and 48 h. In line with the process of acute renal failure, serum creatinine and tubular injury increased linear to the time of ischemia and reperfusion. Here we present evidence that one part of telomerase, namely mTERT, and the CDK inhibitor p21 are stable and reproducible markers for short or long ischemic intervals, respectively. This finding was independent of the reperfusion time. The detection of markers such as these two proteins might help to explain the performance of a kidney under stress. Targeting p21 via the p53 inhibitor PIF abolished renal failure and can therefore be seen as a possible prophylactic substance in acute renal failure.

Since Megyesi and coworkers (17) have convincingly demonstrated that p21 plays a major role in renal IRI, we focused on the regulation of CDK inhibitors in our model. The CDK inhibitor p21 has been shown to be increased in cells of the thick ascending loop and distal convoluted tubules (19). In line with this study, we found p21 transcription in murine kidneys to be significantly increased after long, but not after short, renal ischemic intervals. More precisely, mainly TCs contributed to the observed increase in p21 transcription. Interestingly, the observed significant increase of p21 transcripts was independent of the reperfusion time, suggesting that p21 is activated very rapidly and has to play an important role in long-time ischemic kidneys. Contrary to recent findings of Chkhotua and coworkers (5), who investigated the expression of CDK inhibitors in a pig model of ex vivo hemoperfusion, we did not find any increased transcription of the other prominent member of the Cip/Kip family, namely p27, in murine kidneys, which underwent renal IRI. Although several lines of evidence exist that p21 and p27 exert similar effects on cyclin-CDK complexes and the cell cycle, there are other observations that both CDK inhibitors have non-overlapping functions. Furthermore, it has been demonstrated that p21 and p27 are often differentially expressed, e.g., in fibroblasts (30), and that p21 and p27 knock-out mice display different phenotypes (7, 8, 14).

Murine telomerase, which consists partly of mTERT, has been shown to be an important factor in angiogenesis and tissue regeneration after hindlimb ischemia (35) and to be

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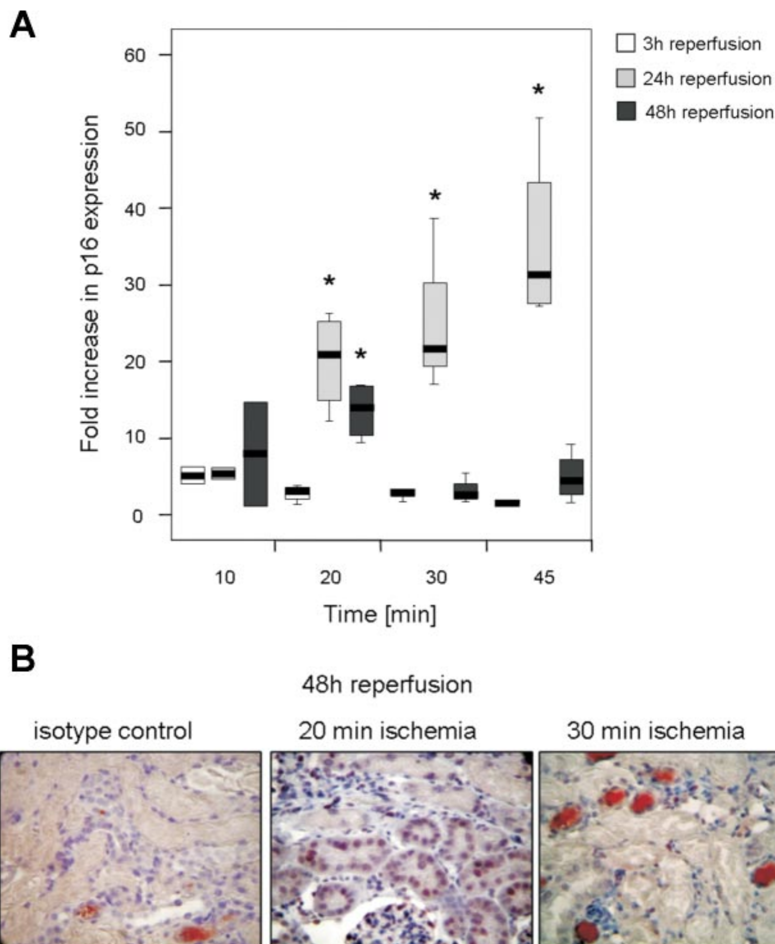

Fig. 5. Gene expression of p16. *A*: quantitative PCR for the detection of p16 was performed in ischemic kidneys after 3 (open box blot; $n = 4$ mice in each group), 24 (shaded box blot; $n = 4$ mice in each group), and 48 h (solid box blot; $n = 8$ mice in the 10-, 20-, and 30-min group, $n = 4$ in the 45-min group). $^{*}P < 0.0125$ compared with sham-operated animals. *B*: p16 staining was performed in 20- and 30-min ischemic kidneys 48 h after ischemia. Representative pictures of the isotype control and the p16-stained kidney sections are presented. Magnification ×400.

upregulated in astrocytes after ischemic brain injury (1). The expression of mTERT mRNA has been described to be ubiquitous in all mice tissues (15). We detected constitutive expression of mRNA of mTERT in the mouse kidneys and further found significant increased expression in mice, which underwent short periods of ischemia, namely 10 and 20 min. Furthermore, transcription levels of mTERT increased linearly to the reperfusion time until a threefold increase was reached after 48 h of reperfusion. In contrast, after long ischemic time periods, mTERT expression did not exceed baseline expression levels. Together with the finding that p21 is not upregulated after short times of ischemia, it can be suggested that repair mechanisms, such as restoration of telomeres, are only possible after short times of ischemia. After long intervals of ischemia, no restoration processes can take place, p21 is activated, and TCs undergo necrosis.

The prototype of the other CDK inhibitor family INK4, namely p16, has been shown to be stably increased in a model of chronic renal allograft rejection (10) and in an experimental pig model of ex vivo renal hemoperfusion (5). Melk and coworkers (21) provided compelling evidence that an increased p16 expression is directly correlated with increased tubular atrophy, interstitial fibrosis, and impaired function in renal allografts. In our murine model of renal IRI, we found no significant changes in the p16 transcription and translation after 3 h of reperfusion. But after 24 h of reperfusion, p16 was found to be significantly upregulated in parallel to serum creatinine and tubular necrosis score. After 48 h of reperfusion p16 transcription was only significantly increased in 20-min ischemic kidneys. In the light of the findings of Beausejour and coworkers (2), who describe p16 in contrast to p21 to induce an irreversible cell death, the loss of p16 expression after 48-h reperfusion could be explained by the fact that the cells, which have expressed p16 after 24-h reperfusion, have undergone cell death. Nevertheless, our data indicate that p16 plays a role in the pathogenesis of renal IRI, but further studies are necessary to evaluate the exact function of p16 in renal IRI.

In the clinical setting, targeting one of above described players in the cell cycle could offer new therapeutic options in acute renal failure. Since there exists no p21 or mTERT inhibitor or activator, we targeted the upstream molecule of p21, namely p53, by using the selective p53 inhibitor PIF, which has already been shown to protect from renal IRI in a rat model (11). In line with the data of Kelly and coworkers (11), PIF injected before inducing renal IRI prevented an increase in serum creatinine and kidney damage in mice that underwent 30 min of ischemia and 48 h of reperfusion. This effect was mediated by p21 inhibition, since p21 mRNA transcription was found to be significantly decreased in mice that underwent PIF treatment. It has to be emphasized that PIF only worked in a prophylactic setting, since PIF did not restore kidney function when administered 1 h after ischemia. These data are contrary to the findings of Megyesi and coworkers (17), who found p21 activation in renal IRI independent from p53 activation and

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knock-out mice to be protected from renal IRI. The same group underlined their data by performing in vitro experiments. By using an adenovirus-transfection system, they found specific domains of p21 to protect from cisplatin-induced cell death in vitro (34). Thus further in vivo studies without using of genetically modified animals are necessary to evaluate the exact role and function of p21 in renal IRI.

In the presented study, we detected new "indicator" genes for short and long ischemic periods. mTERT was only significantly upregulated after short ischemic periods, possibly reflecting a rescue mechanism, since mice did not develop signs of acute renal failure. In contrast, the CDK inhibitor p21 was constantly upregulated after long ischemic intervals, leading to acute renal failure. Targeting p21 via the selective p53 inhibitor PIF blunted p21 upregulation and prevented acute renal failure. Therefore, p21 and mTERT are not only indicators, but also offer new therapeutic possibilities in acute renal failure.

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