A p38 mitogen-activated protein kinase inhibitor protects against renal damage in a non-heart-beating donor model

Carole Doucet,1,* Serge Milin,1* Frederic Favreau,1 Thibault Desurmont,1 Emilie Manguy,1,2 William Hébrard,2 Yuriko Yamamoto,3 Gérard Mauco,1 Michel Eugene,1 Vassilios Papadopoulos,4 Thierry Hauet,1,2* and Jean Michel Goujon1,5*

1Institut National de la Santé et de la Recherche Médicale U927, Université de Poitiers, Poitiers; 2Institut National de la Recherche Agronomique, GEPA, Laboratoire de Transplantation Expérimentale, Domaine Expérimental du Magneraud, Surgères; 3Research Department, Fujisawa Pharmaceutical, Osaka, Japan; 4Department of Biochemistry and Molecular Biology, Georgetown University Medical Center, Washington, District of Columbia; and 5Centre Hospitalier Universitaire de Poitiers, Service d’Anatomie et Cytologie Pathologique et Unité de Pathologie Ultrastructurale et Expérimentale, Poitiers, France

Submitted 30 May 2007; accepted in final form 24 April 2008

Doucet C, Milin S, Favreau F, Desurmont T, Manguy E, Hébrard W, Yamamoto Y, Mauco G, Eugene M, Papadopoulos V, Hauet T, Goujon JM. A p38 mitogen-activated protein kinase inhibitor protects against renal damage in a non-heart-beating donor model. Am J Physiol Renal Physiol 295: F179–F191, 2008. First published April 30, 2008; doi:10.1152/ajprenal.00252.2007.—Ischemia-reperfusion injury is one of the central nonimmunologic processes involved in renal allograft dysfunction. Kidneys from non-heart beating donors (NHBD) exhibit higher rates of delayed graft function (DGF) than those from other donors. Primary nonfunction and DGF are the main barriers to the use of kidneys from NHBD. Using a pig model of NHBD transplantation, we studied the effect of FR167653 (a p38 MAP kinase inhibitor) on the recovery and reparation of kidneys exposed to both warm (WI: 1 h) and cold ischemia (24 h). Our results demonstrate that the addition of FR167653 increases the kinetics of proximal tubule cell regeneration after 60 min of WI. Hypoxia-inducible factor and vascular endothelial growth factor expression was also more important in FR167653-treated kidneys compared with those in nontreated groups. Also, expression of peripheral-type benzodiazepine receptor, involved in tissue repair, was increased in the FR167653-treated groups. At 3 mo, the protective effects of FR167653 were accompanied by a reduction of long-term inflammation process and tubulointerstitial fibrosis development associated with a limitation of ischemia-induced remodeling. This study suggests that such treatment may be useful in protocols aimed at improving the quality of renal transplants from NHBD. In addition, the beneficial role of FR167653 in limiting early injury is associated with secondary reduction in development of tubular atrophy and interstitial fibrosis which are together the hallmark of failing renal transplants. The more efficient effect was observed when FR167653 was added in combination before WI, during cold storage and reperfusion.

Proinflammatory cytokines, tissue repair; renal fibrosis, p38-MAPK pathway plays an important role in cellular stress responses such as proliferation, apoptosis, differentiation, and production of proinflammatory cytokines (14, 18). The p38-MAPK is a member of the serine-threonine kinases that control complex programs such as embryogenesis, differentiation, proliferation, and cell survival. Ischemia triggers activation of p38-MAPK, which may contribute to the pathogenesis of IRI (8, 29). In addition, ischemia-induced alterations in tubule cell metabolism lead to the generation of reactive oxygen species, resulting in cell damage and protein nitrosylation (5). In a study of renal fibrosis, p38-MAPK pathway activation was associated with production and secretion of transforming growth factor (TGF)-β and extracellular matrix proteins (39). Collectively, these data suggest that p38-MAPK could be a target of paramount importance in the transplantation field, particularly with regard to limitation of renal injury associated with procurement from NHBD.

FR167653 (1-[7-(4-fluorophenyl)-1,2,3,4-tetrahydro-8-(4-rydyl)-pyrazolo[5,1c][1,2,4]triazin-2-yl]-2-phenylethanediene sulfamate monohydrate), a synthesized organic compound, was originally discovered to be a selective inhibitor of IL1-β and TNF-α production mediated by p38-MAPK inhibition (Fig. 1) (42–47). FR167653 belongs to a family of compounds that are able to competitively bind to the ATP-binding pocket of p38 kinase and inhibit the phosphorylation of p38 and/or downstream transcription factors (43, 44). In addition, a recent study revealed that FR167653 is a p38-MAPK-selective inhibitor without affecting the activities of other proteins, such as ERK-1, JK-2, protein kinase A, protein kinase C, protein kinase G, or delayed graft function (DGF) compared with conventional heart-beating cadaveric donors (4, 37).

The pathophysiology of ischemia-reperfusion (IR)-induced acute renal failure involves a complex interplay between vascular hemodynamic and tubular injury (2, 3). Kidneys from NHBD are exposed to warm ischemia (WI) followed by cold ischemia (CI), and free radical release from organ tissue is enhanced after reperfusion (4, 7). Inflammatory response to renal IR injury (IRI) results in endothelial cell activation and injury, enhanced leukocyte adhesion, leukocyte entrapment, and a compromise in microvascular blood flow (3, 27). The p38 mitogen-activated protein kinase (MAPK) signaling pathway plays an important role in cellular stress responses such as proliferation, apoptosis, differentiation, and production of proinflammatory cytokines (14, 18). The p38-MAPK is a member of the serine-threonine kinases that control complex programs such as embryogenesis, differentiation, proliferation, and cell survival. Ischemia triggers activation of p38-MAPK, which may contribute to the pathogenesis of IRI (8, 29). In addition, ischemia-induced alterations in tubule cell metabolism lead to the generation of reactive oxygen species, resulting in cell damage and protein nitrosylation (5). In a study of renal fibrosis, p38-MAPK pathway activation was associated with production and secretion of transforming growth factor (TGF)-β and extracellular matrix proteins (39). Collectively, these data suggest that p38-MAPK could be a target of paramount importance in the transplantation field, particularly with regard to limitation of renal injury associated with procurement from NHBD.

The growing demand for organs for renal transplantation makes it necessary to explore alternative routes for kidney donation. The use of non-heart-beating donors (NHBD) has been proposed to help curtail the universal shortage of transplantable organs, especially kidneys. Reluctance to accept NHBD as a source of kidneys is due to the higher rates of primary failure

* C. Doucet, S. Milin, T. Hauet, and J. M. Goujon contributed equally to this work.

Address for reprint requests and other correspondence: T. Hauet, Institut National de la Santé et de la Recherche Médicale U927, CHU de Poitiers, Rue de la Milétrie, B.P. 577, 86021 Poitiers Cedex, France (e-mail: t.hauet@chu-poitiers.fr).
epidermal growth factor receptor kinase (42). FR167653 has protective effects on different organs under various experimental conditions (6, 8, 16, 19, 20, 22, 30, 42, 48–50). FR167653 was shown also to be effective in reducing renal fibrosis (30, 42) and in non-heart beating model, and FR167653 was shown to be protective for heart and liver models (21, 33). Recently, the anti-inflammatory effects of p38-MAPK inhibitors in local inflammation models have been investigated (44). Also, p38-MAPK inhibition through the addition of FR167653 to visiopan (UW) solution has been shown to improve liver graft viability and animal survival rates (50). However, FR167653 has not been evaluated in kidney exposed to NHBD situation with combination of WI and cold preservation. We developed a NHBD model of kidney autotransplantation in pig and evaluated the effect of FR167653 on renal recovery, tissue regeneration, and inflammation.

MATERIALS AND METHODS

Animal Experiments

Large white male pigs (body wt between 30 and 35 kg; Institut National de la Recherche Agronomique, Le Magneraud, Surgères, France) were prepared as previously described (9). The surgical and experimental protocols were performed in accordance with the policy and guidelines of the Ethical Committee for Human and Animal Studies of our institution and the guidelines of the French Agricultural Office and the legislation governing animal studies. The Institutional Animal Care and Use Committee (INRA) and the independent Committee for Ethics (Région Poitou Charentes) approved all of the experimental procedures. The surgical procedures were performed under sterile conditions. Animals were randomly divided into six experimental groups (Table 1). The control group (sham operated), or group A, was comprised of 15 age- and weight-matched animals. Group B was made up of 15 age-, weight-, and nephron mass-matched animals that had undergone nephrectomy of the left kidney. In group C, the nephrectomized left kidney was exposed to 60 min of WI (cross clamping of the renal pedicle), followed by 24 h of cold storage in UW preservation solution (1 liter was used for flushing and preservation). Following a contralateral nephrectomy, autotransplantation of the injured kidney was performed (n = 18). Group D was identical to group C, except that animals received 1 mg/kg iv injections of FR167653 30 min before WI, 30 min before autotransplantation, and continuously, for 2 h after reperfusion (n = 18). In group E, the experimental protocol was identical to group D, but FR167653 was also included in the preservation solution at a concentration of 60 mg/l (n = 18). In group F (n = 16), FR1677653 was added in UW solution. The doses and rational protocols were based on previous studies (16, 45, 46) and from preliminary studies from our laboratory taking account the aqueous solubility of the compound (Hauet T and Jayle C, unpublished observations).

Table 1. Experimental groups

<table>
<thead>
<tr>
<th>Groups</th>
<th>Cold storage</th>
<th>Autotransplantation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group B</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group C</td>
<td>Cold storage</td>
<td>Auto transplantation</td>
</tr>
<tr>
<td>Group D</td>
<td>FR 30 min</td>
<td>15 min</td>
</tr>
<tr>
<td>Group E</td>
<td>FR 30 min</td>
<td>15 min</td>
</tr>
<tr>
<td>Group F</td>
<td>FR 30 min</td>
<td>15 min</td>
</tr>
</tbody>
</table>

WI, warm ischemia; FR, FR167653.

Functional Parameters

Endogenous creatinine clearance (Ccr; ml/min) and urinary proteins were measured at 1, 3, 5, 7, 11, and 14 days and at 1 and 3 mo after reperfusion as previously described (10). Animals were placed in metabolic cages for 24 h before urine and blood sample collections.

Assessment of Inflammatory Cytokines

The effects of FR167653 on the release of cytokines TNF-α and IL-1β were determined in serum before WI, 1, 3, and 24 h after reperfusion and in urine, using commercial enzyme-linked immunoabsorbent assay (Biosource Immunoassay kit, Biosource International, Camarillo, CA). All assays were performed in triplicate.

Morphological Studies

Biopsies were processed for conventional histology. Cortical and medullary kidney tissue was collected at 3 h, 1 day, 7 days, and 3 mo after reperfusion. Samples were randomly collected from echo-guided biopsies. Biopsy samples from the deep cortex outer medulla region of the kidney were fixed in Dubosq-Brazil and 10% formalin in PBS, embedded in paraffin, and stained with hematoxylin and eosin and periodic acid-Schiff. Two histological patterns typical of proximal tubule cell injury (brush-border lesion and cell detachment) were assessed in the following semiquantitative 5-point scale: 0 - no abnormality; 1 - mild lesions affecting less than 25% of kidney samples; 2 - lesions affecting 25–50% of kidney samples; 3 - lesions affecting 50–75% of kidney samples; and 4 - lesions affecting more than 75% of kidney samples (10). Also, the degree of mitochondrial injury (i.e., mitochondrial swelling, rupture of the inner and outer membrane, and leakage of mitochondrial matrix into the cytoplasm) was determined using transmission electron microscopy at high magnification (×15,000) on biopsies performed during the first week.

Immunohistochemical Studies

Alternatively, kidney biopsies were immediately snap-frozen in liquid nitrogen after scission and stored at −70°C until further use. Frozen and paraffin-embedded kidney biopsy sections (5 μm) were...
subjected to indirect immunohistochemistry using standardized avidin-biotin peroxidase methodology. Before immunohistochemical labeling, paraffin sections were deparaffinized, rehydrated, and boiled in a pressure cooker containing citrate buffer (pH 6) for 2 min. The sections were then cooled, rinsed in PBS, and processed for immunohistochemistry. All sections were examined and photographed under blind conditions. Paraffin sections were labeled with a polyclonal antibody to PCNA (1:100, Lab Vision-Neomarkers) to evaluate tubulointerstitial fibrosis. All sections were examined and photographed using the ×40 objective. Image analysis to determine the amount of interstitial fibrosis was performed by either a technician or a pathologist blinded to the experimental conditions of the sample. A computer-aided image analysis program determined the percentage of PS-stained surface. In each representative slide, staining in renal tissue was semiautomatically quantified in 15 fields by the computer program in each experimental condition and expressed as percent of the total surface area examined.

**Western Blotting**

Western blot analysis was performed for protein immunodetection. Protein determination was performed 3 mo after reperfusion in survival animals. Minced tissue was placed in extraction buffer [1 × TEx buffer; 60 mM Tris base (pH 6.8), 10% glycerol, and 3% SDS] containing 5% β-mercaptoethanol and the protease inhibitor Antago-sonan (Hoechst, Paris, France), which were added just before use. Tissues were immediately disrupted with an Ultra Turrax homogenizer at maximum speed for 1 min on ice, and homogenates were centrifuged at 26,500 g for 15 min at 4°C. Supernatant aliquots were stored at −20°C for no longer than 1 wk before use. Protein concentrations were measured according to Lowry et al. (28). Equal amounts of proteins (50 to 100 μg) were separated on SDS-PAGE (12 or 4–20%) under reducing conditions and electrophoretically transferred onto polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA). Membranes were blocked for 1 h in TBST/5% nonfat milk and incubated overnight at 4°C with anti-hypoxia-inducible factor-1α (HIF-1α) antibody (1:500; Novus Biologicals, Littleton, CO). Anti-p38-MAPK and anti-phospho-p38-MAPK (Cell Signaling Technology, Danvers, MN) were used for their cross-reactivity against pig tissue. After being washed in TBST, the membranes were incubated for 1 h with horseradish peroxidase-linked anti-rabbit antibody (1:5,000, Amersham Pharmacia Biotechnology, Piscataway, NJ) in TBST/5% milk, and proteins were visualized by electrochemiluminescence. β-Actin (1:1,000, Sigma, Saint Quentin Fallavier, France) was used as the loading control. Densitometric analysis of the immunoreactive protein bands was performed using the OptiQuant-image analysis software (Packard Bioscience, Meriden, CT). All values were expressed as a percentage of the β-actin band density detected in the same gel.

**VEGF Immunoabsorbent Assay**

Three months after reperfusion, renal tissue concentrations of VEGF were measured by colorimetric ELISA, which employed a monoclonal antibody specific to human VEGF-165 (Quantikine, R&D Systems, Minneapolis, MN). The assay procedure was performed according to the manufacturer’s instructions. The optical density at 450 nm was determined with a microplate reader.

**Evaluation of Tubulointerstitial Fibrosis**

To estimate the level of tubulointerstitial fibrosis, tissue isolated 3 mo postreperfusion was labeled with picro Sirius (PS) for collagen identification (collagen I and III) as recommended (12). The slides were examined with a light microscope, and the images were acquired using the ×40 objective. Image analysis to determine the amount of interstitial fibrosis was performed by either a technician or a pathologist blinded to the experimental conditions of the sample. A computer-aided image analysis program determined the percentage of PS-stained surface. In each representative slide, staining in renal tissue was semiautomatically quantified in 15 fields by the computer program in each experimental condition and expressed as percent of the total surface area examined. Inflammation was determined by evaluation of CD3-positive T lymphocytes (1:300; Cell Marque) and ED1 (1:50; RDI, Paterson, NJ)-positive macrophage infiltration in renal tissue.

**Statistical Methods and Data Analysis**

Quantitative parameters are presented as means ± SE. Comparisons within groups were performed by use of paired Student’s t-test, and among groups by ANOVA with the Bonferroni correction for multiple group comparisons followed by a Student-Newman-Keuls test. To detect changes in renal function and cytokine excretion among the groups and time influence, a two-way ANOVA was performed followed by Newman-Keuls analysis when comparisons were significant. For histological data, the Kruskall-Wallis test for multiple comparison analyses was performed. A P value <0.05 was considered to be significant.

**RESULTS**

**FR167653 Improves Animal Survival and Recovery of Renal Function After IR**

All animals survived in groups A (n = 15), B (n = 15), and E (n = 18). Six animals survived in group C (6/18), 10 animals survived in group D (10/18), and 13 survived in group F (n = 16). All deaths were due to primary nonfunction (PNF), and animals were autopsied to check the absence of vascular anastomosis problem. Creatinine level was over than 1,500 μmol/l in no survival animals at day 5 (Fig. 2A). As shown in Fig. 2A, FR167653-treated animals (groups D, E, and F) exhibited decreased creatinine levels compared with their untreated counterparts. In addition, group differences were consistent over time for all experimental groups after day 1 except for group E. Group E also exhibited decreased proteinuria excretion compared with groups C and D (P < 0.05; Fig. 2B). Proteinuria excretion in urine at 3 mo was also diminished in group F.

**FR167653 Decreases Cytokine Release and p38-MAPK Phosphorylation**

Plasma. As expected, the elevation of TNF-α and IL-1β was significantly attenuated in FR167653-treated animals particularly in groups E and F (P < 0.05 at 1 h and P < 0.05 at 3 h;
Fig. 2. FR167653 improves animal survival and recovery of renal function after ischemia-reperfusion (IR). A: plasma creatinine levels were measured in the indicated experimental group; group A, control group; group B, uninephrectomized group; group C, IR injured; group D, IR injured + FR167653 before warm ischemia and after transplantation; and group E, IR injured + FR167653 before warm ischemia and after transplantation and FR167653 during static cold storage. Group C, *P < 0.05 group C vs. group D; †P < 0.05 group C vs. group E; ‡P < 0.05 group C vs. group F; §P < 0.05 group C (day 7) vs. group C (day 1); group C (day 14) vs. group C (day 1) or group C (month 3) vs. group C (day 1). Group D; *P < 0.05 group D vs. group F; ‡P < 0.05 group D vs. group E; †P < 0.05 group D (day 7) vs. group D (day 1); group D (day 14) vs. group D (day 1) or group D (month 3) vs. group D (day 1); group F; *P < 0.05 group F vs. group E; ‡P < 0.05 group F (day 7) vs. group F (day 1); group F (day 14) vs. group F (day 1). Group F; *P < 0.05 group E vs. groups A and B. B: urine protein excretion was measured 3 mo after reperfusion. Group A, control group; group B, uninephrectomized group; group C, IR injured; group D, IR injured + FR167653 before warm ischemia and after transplantation; group E, IR injured + FR167653 before warm ischemia and after transplantation and FR167653 during static cold storage; and group F, FR167653 during cold storage. Group C, *P < 0.05 group C vs. group D; †P < 0.05 group C vs. group F; §P < 0.05 group C vs. group E; ‡P < 0.05 group C vs. group F; †P < 0.05 group C vs. groups A and B. Group D: *P < 0.05 group D vs. group F; ‡P < 0.05 group D vs. group E; †P < 0.05 group D vs. groups A and B.

Fig. 3, A and B). A significant increase was noted in all experimental groups between the preoperative time and 1 and 3 h postreperfusion (P < 0.05). TNF-α release was undetectable in all groups before surgical procedure. TNF was detected in group C and slightly in group D between 1 and 3 h. Cytokine excretion was reduced in groups E and F.

Urine. In urine, cytokine excretion was observed at day 1 in group E because in five animals, urine production was noted (Fig. 3C). In other experimental groups, urine production was not observed before day 1. However, cytokine excretion was diminished in group E compared with other experimental groups.

Tissue. FR167653 also reduced phospho-p38-MAPK expression in kidney tissue as shown in Fig. 3D at day 1. In addition, observed group differences are also consistent over time. These data confirm the effect of FR167653 on MAPK pathway.

FR167653 Reduces the Degree of Acute Proximal Tubule Epithelial Cell Injury and Influences the Renal Repair Process and Mitochondrial Integrity

Kidney tissue exposed to WI/CI exhibited extensive acute proximal tubule necrosis, particularly in the S3 segment located in the deep cortex-outer medullary areas (Fig. 4, top). One day after reperfusion, disruption of the basement membrane was apparent. Alterations in cell morphology, including loss of brush border, tubular necrosis, and mitochondrial damage, were also observed (Fig. 4, top). Renal sections from FR167653-treated groups, particularly from group E, displayed significantly less proximal tubule cell necrosis (Fig. 4, top), cell detachment, and mitochondrial injury than sections from group C (P < 0.05). Mitochondrial injury was also reduced in groups D and F (Fig. 4, top). Integrity recovery was faster in groups D, E, and F than in group C (Fig. 4, top).

The total number of PCNA-positive tubular cells in the deep cortex-outer medulla areas was increased in FR167653-treated groups, especially in group E (Table 2). At day 1, PCNA expression appeared to be upregulated in the distal tubule, and to a lesser degree, in the swollen proximal tubule (Fig. 4, bottom). Between day 1 and 3 mo after reperfusion, cellular proliferation progressively decreased in both regenerating, partly dedifferentiated, tubules and in normal appearing tubules (Table 2). Consistent with these findings, sections from group E also exhibited numerous vimentin-expressing cells (Fig. 4, bottom, and Table 2). Vimentin staining was moderate 1 day after reperfusion and peaked at day 7. In groups C and D, these increases were delayed (Fig. 4, bottom, and Table 2). In group F, vimentin expression was intermediate between group E and groups C and D.

In the normal kidney, PBR was mainly expressed in the distal portion of nephrons, the collecting ducts and interlobular arteries, and inflammatory cells (Fig. 4, bottom). Immunohistochemical analysis of serially collected tissues from IR-injured kidneys revealed a transient cytoplasmic PBR immunoreactivity in proximal tubule epithelial cells. This immunoreactivity was significantly increased in FR167653-treated groups (P < 0.05). The transient and strong expression of PBR in tubules located in the outer medulla after 1 day of reperfusion is accompanied by the onset of recovery phase.

FR167653 Promotes Cell-Cell Adhesion After IR

IR-injured kidneys exhibited significant cytoplasmatic translocation of N-cadherin, resulting in the disruption of junctional complexes in the damaged proximal tubule (Fig. 5A). In the FR167653-treated groups, the basolateral membrane distribution of N-cadherin along all proximal tubule segments was restored rapidly during the first week after reperfusion. This effect was most notable in group E (Fig. 5A). The specificity of the N-cadherin antibody for pig tissue is shown in Fig. 5, B and C.

FR167653 Increases the Expression of Proangiogenic Factors After IR

In group C, HIF-1α expression was slightly increased at 3 mo after reperfusion compared with groups A and B. In
contrast, HIF-1α expression increased significantly in the FR167653-treated groups (Fig. 6, top).

VEGF expression was barely detectable in group C, whereas it was expressed at much higher levels in all other groups (Fig. 6, middle). VEGF immunohistochemical staining was strongly present as early as day 1 in groups D and E, with it being more intense in the latter group (Fig. 6, middle). VEGF immunoreactivity was most prominent in the outer medullary of all postischemic kidneys. The VEGF-R was also strongly and rapidly expressed in group E compared with the other FR167653-treated groups (Fig. 6, bottom). In FR167653-treated groups, VEGF and VEGF-R staining were strongly and rapidly expressed in the cytoplasm of proximal tubule epithelial cells and peritubular capillaries (Fig. 6, middle and bottom). At 3 mo, VEGF-R remained more expressed particularly in group E. In summary, FR167653 treatment allows angiogenesis and accelerates the recovery phase in proximal tubule epithelial cells.
### Lesions

<table>
<thead>
<tr>
<th>Lesion</th>
<th>Group A</th>
<th>Group B</th>
<th>Group C</th>
<th>Group D</th>
<th>Group E</th>
<th>Group F</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Brush Border Integrity</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 1</td>
<td>4.0 ± 0.2</td>
<td>3.8 ± 0.2</td>
<td>1.1 ± 0.2</td>
<td>2.0 ± 0.2*</td>
<td>3.0 ± 0.2*</td>
<td>3.0 ± 0.2*</td>
</tr>
<tr>
<td>Day 7</td>
<td>3.9 ± 0.3</td>
<td>4.0 ± 0.1</td>
<td>2.1 ± 0.2</td>
<td>2.9 ± 0.2*</td>
<td>3.8 ± 0.2*</td>
<td>3.8 ± 0.2*</td>
</tr>
<tr>
<td><strong>Tubular Necrosis</strong></td>
<td>0</td>
<td>0</td>
<td>3.8 ± 0.2</td>
<td>2.8 ± 0.2*</td>
<td>2.1 ± 0.2*</td>
<td>3.0 ± 0.2*</td>
</tr>
<tr>
<td>Day 1</td>
<td>0</td>
<td>0</td>
<td>2.8 ± 0.2</td>
<td>2.2 ± 0.2*</td>
<td>1.6 ± 0.2*</td>
<td>1.8 ± 0.2*</td>
</tr>
<tr>
<td>Day 7</td>
<td>0</td>
<td>0</td>
<td>2.8 ± 0.2</td>
<td>2.2 ± 0.2*</td>
<td>1.6 ± 0.2*</td>
<td>1.8 ± 0.2*</td>
</tr>
<tr>
<td><strong>Mitochondrial Injury</strong></td>
<td>0.4 ± 0.2</td>
<td>0.4 ± 0.1</td>
<td>4.0 ± 0.2</td>
<td>3.2 ± 0.2*</td>
<td>2.3 ± 0.2*</td>
<td>3.0 ± 0.2*</td>
</tr>
<tr>
<td>Day 1</td>
<td>0</td>
<td>0</td>
<td>3.0 ± 0.3</td>
<td>2.0 ± 0.2*</td>
<td>1.4 ± 0.2*</td>
<td>3.8 ± 0.2*</td>
</tr>
<tr>
<td>Day 7</td>
<td>0</td>
<td>0</td>
<td>3.0 ± 0.3</td>
<td>2.0 ± 0.2*</td>
<td>1.4 ± 0.2*</td>
<td>3.8 ± 0.2*</td>
</tr>
</tbody>
</table>

### Immunohistochemical Expression

**PCNA (P) expression at day 1**

- Group A: +
- Group B: +
- Group C: +
- Group D: ++
- Group E: +++
- Group F: ++

**Vimentin (V) expression at day 1**

- Group A: +
- Group B: +
- Group C: +
- Group D: +
- Group E: +
- Group F: +

**Peripheral benzodiazepine receptor (PBR) expression at day 1**

- Group A: +
- Group B: +
- Group C: +
- Group D: +
- Group E: +
- Group F: +
Table 2. Histological evaluation of PCNA expression and vimentin between 3 h and 3 mo

<table>
<thead>
<tr>
<th>Group Reperfusion Time</th>
<th>Group A (PCNA)</th>
<th>Group B (Vimentin)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 h</td>
<td>&lt;5</td>
<td>50 ±12a</td>
</tr>
<tr>
<td>Day 1</td>
<td>&lt;5</td>
<td>43±5</td>
</tr>
<tr>
<td>Day 7</td>
<td>&lt;5</td>
<td>31±7</td>
</tr>
<tr>
<td>Month 3</td>
<td>&lt;5</td>
<td>&lt;5</td>
</tr>
</tbody>
</table>

Histological evaluation of PCNA expression and vimentin between 3 h and 3 mo.

FR167653 Reduces Renal Fibrosis After IR

Three months after reperfusion, kidneys from group C exhibited a significant increase of interstitial fibrosis and tubule atrophy as determined by PS staining compared with FR167653-treated groups (Fig. 7, top; P < 0.05 at 3 mo). These structural alterations were accompanied by similar increased expression of tubular epithelial to mesenchymal markers, α-SMA and vimentin (Fig. 7, middle). The lowest degree of tissue remodeling was observed in kidneys from group E (P < 0.05). IR-induced injury is also accompanied by severe inflammatory changes. Determination of interstitial CD3+ and ED1+ cell infiltration is shown in Fig. 7, bottom. These changes were significantly reduced in the FR167653-treated groups, particularly in group E and group F (P < 0.05).

Because p38-MAPK in involved in intracellular signals that regulate cellular response to several stresses, such as heat shock, hyperosmolarity, radiation, endotoxin lipopolysaharide, and the proinflammatory cytokines, the protective effect of FR167653 could be involved in different pathways. We summarized in Fig. 8 the different potential targets and/or secondary effects of this drug on the different pathways involved in renal IRI.

DISCUSSION

The members of MAP kinases family mediate a wide variety of cellular behaviors in response to different extracellular stimuli. One of the four main subgroups, the p38 group of MAPK, serves as a nexus for signal transduction and plays a pivotal role in numerous biological processes (51). Our results demonstrate that, in the model of severe ischemia, FR167653, a specific inhibitor of p38-MAPK, is more effective when perfused both before WI, during reperfusion, and added in preservation solution during flushing and cold storage (6, 16, 20, 42–44). In these conditions, FR167653 significantly reduces tubular injury, which is accompanied by secondary reductions of long-term damage. Because FR167653 was not administered continuously during the 3-mo follow-up, the initial protection has long-lasting effects. These changes were associated with improved kidney function, reduced DGF, and a lower rate of PNF. Recent studies showed that renal recovery from NHBD transplants continues for 3 mo after hospital discharge (7). In the pig model, renal recovery was delayed and was associated with increased protein excretion in the urine. The current results suggest that severe ischemic damage may limit the regeneration process and result in a decreased proliferation of renal cells, which could be associated with persistent abnormalities in perfusion. These data are consistent with a recent study which showed that recovery was slower in NHBD transplants than in live donor transplants (7).

The MAPK pathway has been implicated in tight and adherens junction assembly in various cell lines (11, 36). The cadherin-catenin complex plays an important role in cell-cell...

Fig. 4. FR167653 reduces the degree of acute proximal tubule epithelial cell injury and increases renal regeneration and mitochondria integrity. Top: tissue biopsied at 1 day and 7 days after reperfusion was subjected to hematoxylin and eosin and periodic acid-Schiff staining to evaluate the extent of renal damage. Representative images of staining are shown at day 7. Proximal tubule cell injury was determined by grading brush-border integrity, tubular necrosis, and mitochondrial injury. In proximal tubules, FR167653 attenuates the extent of the loss of brush border and cell detachment (ed). bb, Brush border; tn, tubular necrosis. *P < 0.05 group D vs. group C; **P < 0.05 group E vs. group C. Original magnification ×200 (scale bar 200 μm). Mitochondrial injury evaluation: electron microscopy, original magnification ×15,000, scale bar 200 μm. Groups A and B: numerous elongated mitochondria with intact inner and outer membranes (white arrow); group C: severe mitochondrial swelling and rupture of inner mitochondrial membrane; group D: significant mitochondrial swelling and focal rupture of inner mitochondrial membrane; group E: mild mitochondrial swelling and well-preserved membrane integrity; and group F: moderate mitochondrial swelling and mild rupture of inner mitochondrial membrane. Renal injury determination at day 1 and day 7 after reperfusion. *P < 0.05 group D and F vs. group C; and group E vs. group C. BP < 0.05 group D vs. groups D or F and group F vs. group D. Bottom: representative images of PCNA (P) staining on tissues collected 1 day after reperfusion. Scoring of PCNA immunoreactivity in tissues collected from 24 h to 3 mo after reperfusion revealed that this protein was strongly and rapidly expressed after IR injury in the presence of FR167653 (groups D, E, and F compared with C). Inclusion of FR167653 in the organ preservation solution augmented this response (group E). Original magnification ×200 (scale bar 200 μm). Representative images of vimentin (V) staining on tissues collected 1 day after reperfusion are shown. Scoring of vimentin immunoreactivity in tissues collected from day 1 to 3 mo after reperfusion revealed that FR167653 increased the number of dedifferentiated, vimentin-expressing cells in proximal tubule epithelial cells. Original magnification ×200 (scale bar 700 μm) and ×400 for vimentin expression in groups A and B (scale bar 200 μm). Effect of FR167653 on the immunolocalization and expression of the mitochondrial protein, peripheral-type benzodiazepine receptor (PBR), on proximal tubules, after IR injury, are shown. Evolution of PBR staining during the 3-mo follow-up period is presented. Immunostaining for PBR, which was transiently detected in proximal tubule epithelial cells at day 1, was significantly greater in kidneys from FR167653-treated groups soon after reperfusion. In normal kidneys, PBR is mainly detected in distal tubules. Original magnification ×200 (scale bar 200 μm).
adhesion, signal transduction, and the initiation and maintenance of structural and functional organization of cells (36). In pig thymocytes, the activation of MAPK by EGF and TGF-β results in the loss of junctional complexes, and MAPK inhibition via a mitogen-activated protein/extracellular signal-regulated kinase kinase (MEK) inhibitor prevents this change (11). The cadherin-catenin system initiates coordinated changes in the structure of cytocortex, polarization of cytoplasmic organelles, and asymmetrical patterning of the cytoskeleton, ultimately converting cells to a proto-epithelial phenotype. The present study suggests that FR167653 favors the reestablishment of cell-cell interactions and cell polarity, as assessed by N-cadherin distribution. This may have clinically important implications during recovery from ischemic injury.

Tubulointerstitial injury is a hallmark of progressive renal disease, and chronic hypoxia mainly contributes to its development (1). The hallmark of failing renal transplants is tubular atrophy and interstitial fibrosis. Tubular epithelial cells can switch to myofibroblast phenotype via epithelial to mesenchymal transition, characterized by the loss of epithelial markers and a corresponding increase in mesenchymal markers such as vimentin and α-SMA. A growing body of evidence indicates that epithelial-mesenchymal transition of tubular epithelial cells may play an important role in the development and progression of renal fibrosis. In vivo, vimentin is expressed differentially in embryonic and postnatal life, in mesenchymal and endodermic cells in embryos but only in mesenchyme-derived in adults. Consequently, vimentin could play a role in cellular migration and stability in response to ischemic injury. In the current study, FR167653 treatment was associated with a reduction of inflammation, interstitial fibrosis, and of vimentin and α-SMA expressing cells. In the absence of FR167653, reduced or delayed expression of HIF-1α was associated with poor functional recovery and high levels of tissue damage. IRI
HIF-1α expression in renal tissue

Groups: A B C D E F

Month 3

3 months follow-up

<table>
<thead>
<tr>
<th>Relative ratio to actin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Groups</td>
</tr>
<tr>
<td>A</td>
</tr>
<tr>
<td>Groups A</td>
</tr>
<tr>
<td>0.2</td>
</tr>
<tr>
<td>0.4</td>
</tr>
<tr>
<td>0.6</td>
</tr>
<tr>
<td>0.8</td>
</tr>
<tr>
<td>1.0</td>
</tr>
<tr>
<td>1.2</td>
</tr>
<tr>
<td>1.4</td>
</tr>
<tr>
<td>1.6</td>
</tr>
<tr>
<td>1.8</td>
</tr>
<tr>
<td>2.0</td>
</tr>
</tbody>
</table>

VEGF expression in renal tissue

<table>
<thead>
<tr>
<th>Groups</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Month 3</td>
<td>VEGF protein in tissue (pg/ml)</td>
<td>1.6 ± 0.4</td>
<td>1.8 ± 0.3</td>
<td>0.5 ± 0.1*</td>
<td>0.9 ± 0.3*</td>
<td>1.84 ± 0.3#</td>
</tr>
</tbody>
</table>

Day 1

Month 3

VEGF receptor expression in renal tissue

Day 1

Month 3
Fig. 7. Effect of FR167653 on renal fibrosis after IR injury. Top: representative images of picro Sirius staining of tissue collected 3 mo after reperfusion and evolution of the picro Sirius staining during the 3-mo follow-up period. Picro Sirius determination expressed in percentage of the observed area in the different experimental groups. Picro Sirius expression in groups A and B was less than 10%. *P < 0.05 group C vs. groups D, E, and F; †P < 0.05 group D vs. group E and ‡P < 0.05 group F vs. group E. Original magnification ×200 (scale bar 200 μm). Middle: vascular and peritubular expression of actin and immunostaining of vimentin within renal tissue in the different experimental groups at 3 mo. *P < 0.05 group C vs. groups D, E, and F; †P < 0.05 group D vs. group E and ‡P < 0.05 group F vs. group E. tl, Tubular lumen. Original magnification ×200 (scale bar 200 μm). Bottom: representative images of ED1⁺ and CD3⁺ staining of tissue collected 3 mo after reperfusion and time course of ED1⁺ and CD3⁺ cell counts during the 3-mo follow-up period. *P < 0.05 group C vs. group D, †P < 0.01 group C vs. group E; ‡P < 0.05 group C vs. group F; §P < 0.05 group D vs. group F; ¶P < 0.05 group D vs. group E. Original magnification ×400 (scale bar 200 μm).
is associated with loss of microvasculature, which is responsible for chronic hypoxia (1). As recently reported by Rosenberg et al. (38) under experimental conditions associated with severe hypoxic stress, such as those used here, HIF-1α-related adaptive cell responses diminish or disappear. It was also previously suggested that HIF-1α protein may be degraded by reactive oxygen species and consequently induced an inadequate regulation of the expression of angiogenic factors (54). In contrast, HIF-1α protein was substantially increased in FR167653-treated groups. This suggests that FR167653 influences the expression of HIF-1α, which has been shown to upregulate factors that are cytotoxic in hypoxic renal injury (15, 42, 48). This is linked to the renal repair process that is more effective in FR167653-treated groups and suggests that FR167653-treated kidneys, particularly in group E, are able to develop an adapted response to IRI. From a pathophysiological point of view, the marked histological alterations resulting from severe injury are a strength of this model, as the effect of various protective manipulations becomes more evident.

The protective effect of FR167653 was accompanied by a promotion of the VEGF expression. The finding that VEGF levels are low in the IR-injured group not receiving FR167653 has potential relevance to the upregulation in HIF-1α expression that could occur very late after reperfusion in this group. In turn, it is also possible that the reduction of peritubular capillaries may, in fact, result in a decreased local production of VEGF, as suggested by VEGF immunoabsorbent assay data. In support of this hypothesis, VEGF receptor levels were also diminished, accompanying the reduction of renal tubule density and fibrosis development. These HIF-1α and VEGF expression patterns are consistent with fibrosis development. FR167653 could activate or protect essential mechanisms involved in the maintenance of the normally extensive capillary network within the kidney. These data are consistent with previously described attenuation of microcircular disturbance in a model of liver ischemia (44).

According to a previous study, FR167653 also had marked anti-inflammatory effects after IRI, as measured by lymphocyte and monocyte/macrophage infiltration (6). This is not unexpected since FR167653 was originally discovered to have anti-inflammatory effects (6). In addition, tubular atrophy and interstitial fibrosis were strongly attenuated and α-SMA expression was also strongly reduced. These findings suggest that FR167653 protects the tubular epithelial cells and limits loss of epithelial features and expression of fibroblast features. Previous studies demonstrated that the blockade of p38-MAPK suppresses the proliferation of vascular smooth muscle cells in different experimental conditions suggesting a promising role of p38-MAPK inhibitors for the limitation of chronic allograft nephropathy which is marked by vascular injury and interstitial fibrosis (1, 15, 31, 32). All together, these data supported the protective effect could influence some of the pivotal factors of epithelial-mesenchymal transition proteome such as vimentin, α-SMA, and collagen I and III (detected by PS staining) which are increased in the long term.

The last finding is PBR modulation during IRI and influence of FR167653 on its expression. PBR is a mitochondrial outer membrane protein involved in steroidogenic synthesis (34). Recent studies, including ours, showed that PBR modulates necrotic and apoptotic death induced by renal IRI (17, 52, 53). In the current study, PBR expression was related to the degree of protection afforded by FR167653. This suggests that FR167653 has indirect, protective effects on the mitochondria and consequently, organ viability. PBR is a critical component of the so-called mitochondrial permeability transition pore, a multimeric complex located in the outer mitochondrial membrane which seems to be a pivotal element during IR. These results support the pivotal role for mitochondria and the importance of PBR in IRI. The involvement of PBR and its ligands in G1/S arrest is well-documented (40, 41). Recently, PBR antagonist ligands were shown to interact with p38-MAPK to mediate apoptosis and cell cycle arrest (23). The current study suggests that functional preservation of PBR is important during the regeneration process as previously suggested (52, 53). Taken together, these data suggest that PBR expression might be part of the mitochondrial membrane biogenesis process involved in increased cell proliferation and tissue repair. In addition, because PBR expression is essentially expressed within the distal tubules, the transient expression of PBR within proximal tubular cells is related to a phenotypic change of these cells, which parallel the repair process. The modulation of this protein involved in other processes such as cell cycle regulation, apoptosis, or mitochondrial import could be related to the potential role of distal tubular epithelial cells of the kidney to support the proximal tubular cell survival after injury.

This study provides evidence that the addition of FR167653 to the UW solution during cold preservation along with a single perfusion before WI and during reperfusion is protective against renal dysfunction. The model used in the study was chosen to study the effect of WI, mimicking clinical situation of NHBD. The effects of FR167653 in other preservation solutions remain to be assessed. Because this compound has been shown to selectively inhibit p38-MAPK without affecting ERK-1, JNK-2, or cyclooxygenase-1 and -2 in vitro (42), it is reasonable to assume that the results obtained here would be due to the specific inhibition of p38-MAPK. This drug could be useful for controlled NHBD, Maastricht class II uncontrolled...
p38 MAP KINASE INHIBITOR PROTECTS NHBD KIDNEYS

NHBD, and during machine perfusion preservation. The current study provides evidence that p38-MAPK is a novel, appealing therapeutic target for combating DGF, fibrosis development, epithelial-mesenchymal transition, and inflammatory reactions (25). In addition, such drugs could be used in a single injection during this process. The early protective effect of FR167653 is associated to secondary effects on different long-term injury. However, further studies are requested to clarify the mechanism of action of FR167653 on these processes and other pathways. Such strategies would expand the donor pool by improving acceptance criteria and organ preservation and by protecting the nephron mass provided to the recipient.

ACKNOWLEDGMENTS

We thank C. Henry, N. Quellard, B. Fernandez, B. Mannière, S. Joffrin, A. Guettier, S. André, P. Decourt, D. Lochon, and F. Ribardie for technical assistance. We are in debt with J. C. David, SENAH INRA Agrocampus de Rennes, St. Gilles, France, for precious help for Western blot.

GRANTS

This work was supported by the Conseil Regional Poitou-Charente, the University Hospital of Poitiers, and by Groupe Banque Tarneaud.

REFERENCES

32. Pillebout E, Burtin M, Yuan HT, Briand P, Woolf AS, Friedlander G, Terzi F. Proliferation and remodeling of the peritubular microcirculation,


