FR167653 improves renal recovery and decreases inflammation and fibrosis after renal ischemia reperfusion injury

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Objective: **Acute tubular necrosis (ATN) secondary to induced warm ischemia (WI) results in inflammatory and delayed fibrotic processes and remains a common clinical problem with serious consequences. Because tumor necrosis factor-α (TNF-α) is a prominent proinflammatory factor implicated in the pathophysiology of acute renal ischemia reperfusion injury (IRI), we hypothesized that FR167653 (FR), a potent inhibitor of TNF-α and interleukin-1β production, may reduce IRI.**

Methods: **IRI was induced in male pigs by bilateral clamping of the renal pedicle for 90 minutes (WI90), or unilateral renal clamping (90 minutes) after contralateral nephrectomy (1/2N×90), or unilateral renal clamping without contralateral nephrectomy (WIuni90). FR was administered intravenously 60 minutes before WI (1 mg/kg/h), during WI, and continuously for 3 hours (1 mg/kg/h) during reperfusion in treated groups (FRWI90, FR1/2N×90, or FRWIuni90). Blood and urine samples were collected between day 1 and 3 months after reperfusion for assessment of renal function. Kidneys were excised and renal tissues were collected at 3 months for morphologic and inflammation evaluation and protein analysis. Experimental groups were compared with sham operated (control) and heminephrectomized (Unif) groups without renal ischemia.**

Results: **Three WI90 animals (43%) and five 1/2N×90 (70%) were euthanized and necropsied at day 7 because of no urine production or poor conditions. Mortality was significantly improved after FR treatment. Survival was 100% in the control, Unif, WIuni90, and FR groups. In Unif groups, FR significantly reduced renal failure and bilateral renal ischemia ($P < .05$). At 3 months, proteinuria was significantly reduced in FR-treated groups ($P < .01$). Inflammatory cells count was also dramatically diminished in FR-treated pigs ($P < .01$ for CD3-positive cells). The second aspect of transient ischemia is the fibrotic process determined at 3 months. FR treatment was characterized by a reduction of renal fibrosis, particularly in Unif groups. TNF-α protein expression was diminished in FR-treated groups.**

Conclusion: **This is the first evidence that FR reduced the early and long-term effect of WI in the severe ischemia model. This effect was particularly marked against fibrosis and inflammation, which would contribute to deterioration of a patient's renal function. (J Vasc Surg 2009;49:728-40.)**

Clinical Relevance: **Acute ischemia of the kidney is common in the setting of renal artery or aortic surgery. Deterioration in renal function is a common cause of morbidity in patients treated surgically for juxtarenal and suprarenal abdominal aortic aneurysms. FR167653 represents a useful therapeutic approach to prevent renal damage in a planned period of warm ischemia and during suprarenal aortic surgery.**

Despite many advances in vascular surgery, the incidence and attendant mortality and global morbidity of postoperative ischemia and reperfusion injury (IRI) is especially high for patients who undergo major aortic surgery.[1] The repair of complex aneurysms and surgeries requiring temporary total or partial renal ischemia increases IRI.[1-3]

Acute kidney injury, as encountered in suprarenal aortic clamping and reperfusion and shock from any cause, is associated with unacceptability high morbidity that can range from 40% to 60%. The etiology of acute kidney injury is diverse and IRI, due to the microvascular blood flow reduction, is the major cause.[4] Like other pathologic conditions of the kidney, renal ischemia may ultimately progress to chronic advanced kidney disease characterized by tubule and capillary loss as well as regional interstitial fibrosis related to chronic hypoxic stress.[5-8] IRI is associated with tubulointerstitial inflammation, which is characterized by an influx of leukocytes early after reperfusion.[6-12] The ischemic proximal tubular epithelium can generate a number of mediators that potentiates the inflammatory response. These include cytokines such as tumor necrosis factor (TNF) or interleukin-1 (IL-1),

From Inserm, U927, University Poitiers,[a] Service de Chirurgie Vasculaire, Pôle Neuro Vasculaire,[b] and Service d'Anatomo Pathologie, Centre Hospitalier et Universitaire[d] Poitiers; INRA, UE 967, Génétique et Epérimentation en Productions Animales, Surgères[e]; and Clinical and Molecular Pharmacology, City of Hope, National Medical Center, Duarte[c].

This work was supported by Inserm, INRA, University of Poitiers and Conseil Régional Poitou Charentes, and Banque Tarneaud.

Competition of interest: none.

Additional material for this article may be found online at www.jvascsurg.org.

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0741-5214/$36.00

doi:10.1016/j.jvs.2008.09.056

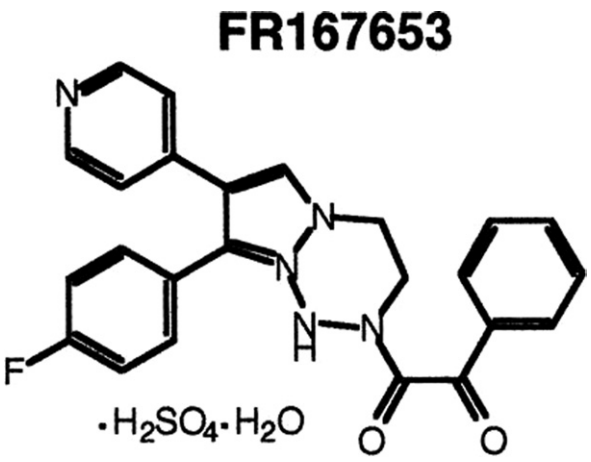


Fig 1. Chemical structure of FR167653: 1-[7-(4-fluorophenyl)-1,2,3,4-tetrahydro-8-(4-pyridyl)pyrazolo[5,1-*c*][1,2,4]triazin-2-yl]-2-phenylethanedione sulfate monohydrate.

which are pivotal factor in the IRI process for native or transplanted kidneys.[13,14]

A strong link has been established between TNF-α production and oxidative stress during the IRI process.[13,14] The underlying mechanisms appear to involve TNF-α production by resident renal cells, and the molecular switch that links ischemia with inflammation involves TNF-α by activating the p38 mitogen-activated protein kinase (p38-MAPK) pathway.[13-16] The MAPK pathway, and particularly p38-MPK, is a key inflammatory mediator that acts by inducing chemokine/cytokine gene expression.[14]

The p38-MAPK inhibitor FR167653 (FR; $C_{24}H_{18}FN_5O_2.H_2SO_4.H_2O$; 1-[7-(4-fluorophenyl)-1,2,3,4-tetrahydro-8-(4-pyridyl)pyrazolo[5,1-*c*][1,2,4]triazin-2-yl]-2-phenylethanedione sulfate monohydrate; Fig 1) was demonstrated to ameliorate IRI of the kidney potentially by reduced production of inflammatory cytokines (TNF-α, IL-1) that may contribute to damage to the ischemic kidney and the distant organs. Recently, the underlying mechanism for FR protection was attributed to the selective inhibition of phosphorylation of p38-MAPK without affecting the activities of other proteins, such as extracellular signal-regulated kinase-1, c-Jun NH2-terminal kinase-2, or protein kinase A, C, or G.[17-19]

Previous studies have described that FR markedly ameliorated renal IRI, possibly by inhibiting cytokine/chemokine expression.[20-22] These preliminary studies evaluated the effect of FR after WI for 60 minutes and contralateral nephrectomy and for a follow-up limited to 7 days after reperfusion. Ischemic acute kidney injury is a self-healing disease, but only to a limited extent, and exceeding this extent results in irreversible loss of renal function.

Nephron mass seems to be a critical long-term prognostic factor. We have developed different models mimicking the early and long-term effect of renal IRI with inflammatory process and fibrosis development, which are pivotal events.[23-26] We initiated the current study to simulate clinical renal ischemia and to focus on the potential use of FR for operations with a planned period of WI, such as renal artery bypass, juxtarenal or suprarenal aortic aneurysm repair, and thoracoabdominal aortic aneurysm repair. We studied the effect of a severe model of WI (90 minutes) in the conditions of bilateral renal ischemia and unilateral renal ischemia, with or without contralateral nephrectomy, which represent different patient populations exposed to renal ischemia and delayed renal function recovery. These models also allow studying the influence of nephron mass in these conditions and the pure effect of ischemia and reperfusion.

METHODS

Surgical procedures and experimental groups. Male pigs (Strain Large white; INRA, Le Magneraud, Surgères, France) were prepared as previously described.[23] Prolonged WI was induced by clamping the renal pedicle for 90 minutes with a vascular nontraumatic clamp. To simulate the planned period of WI and different nephron mass conditions, three experimental groups were studied: pigs with bilateral renal ischemia lasting 90 minutes (WI90, n = 7), pigs with unilateral WI and contralateral nephrectomy (50% of nephron mass reduction, 1/2N×90, n = 7), and pigs with unilateral WI without contralateral nephrectomy (WIuni90, n = 7). These two last groups were designed to assess the effect of nephron mass after WI.

After removal of the renal clamp, the kidneys were inspected for adequate reperfusion. No immediate renal artery thrombosis was observed. When the contralateral kidney was retained in place, it was left undisturbed in situ. FR was administered intravenously before WI (1 mg/kg/h; 60 minutes before WI induction) and continuously during WI and for 3 hours (1 mg/kg/h) immediately after clamp removal in treated groups (FRWI90, FR1/2N×90, and FRWIuni90). The control groups received saline solution. The doses and rational protocols were based on previous studies (Supplementary Figures 1 and 2).

These experimental groups were compared with a uninephrectomy group (Unif; n = 7, matched by age, weight, and nephronic mass) and a control group (control; n = 7, matched by age and weight). All experiments were done in accordance with the Guidelines of the French Agricultural Office and the legislation governing animal studies.

Functional parameters. The pigs were placed in a cage to allow specific 24-hour urine collections as previously described.[23-26] Plasma and urinary creatinine (Cr) was measured enzymatically (Modular, Roche, Meylan, France), and the concentration of urinary proteins was determined using a photometry method (Modular, Roche).

Histopathologic studies. After WI and reperfusion, kidney samples were collected by ultrasound-guided biopsy at day 1, day 7, and day 14. At 12 weeks after reperfusion, survival animals were euthanized and kidneys were removed and sampled for different studies. Samples were fixed with Duboscq-Brasil and 10% formalin in phosphate buffer (0.01 mmol/L; pH 7.42) and embedded in paraffin.

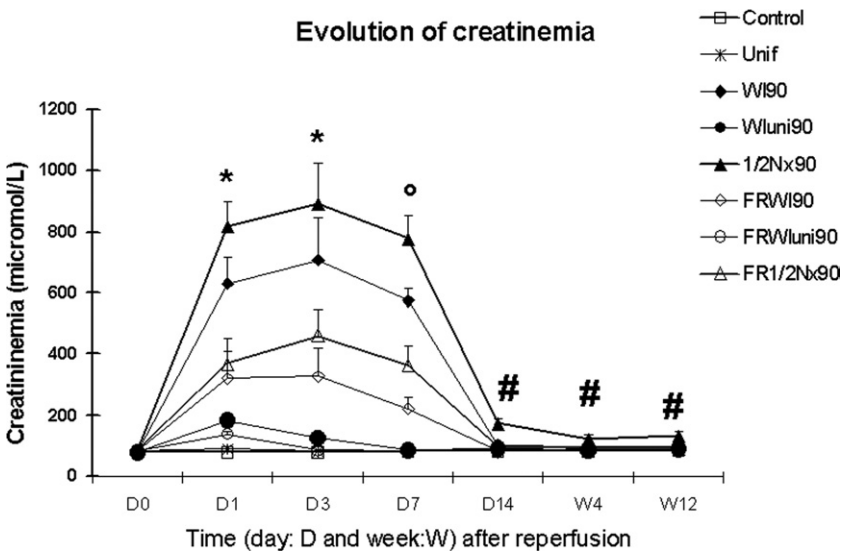


Fig 2. Effect of FR167653 *(FR)* treatment on renal function. Serum creatinine was determined as a marker of renal function in the experimental groups after different warm ischemia *(WI)* and compared with a control group and an uninephrectomized *(Unif)* group. Serum creatinine was measured before and at days 1, 2, 3, 7, and 14 and at week 1 and 12 after WI induction. The values reported are the mean ± standard error of the mean. *1/2N×90*, 90 minutes of unilateral renal clamping after contralateral nephrectomy; *WIuni90,* unilateral WI for 90 minutes. *$P < .05$ FR-treated groups vs no-FR-treated groups, °$P < .05$ FR-treated groups vs no-FR-treated groups, and #FR1/2N×90 vs 1/2N×90.

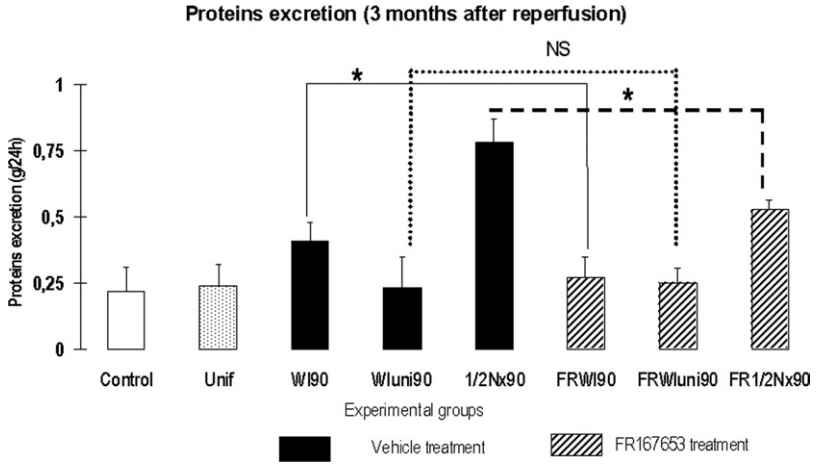


Fig 3. Effect of FR167653 *(FR)* treatment on proteinuria. Proteinuria was determined in urine as a marker of renal injury in the different experimental groups after different warm ischemia *(WI)* and compared with control group and uninephrectomized *(Unif)* group. The values reported are the mean ± standard error of the mean.*$P < .05$, FR-treated groups vs no-FR-treated groups. *1/2N×90*, 90 minutes of unilateral renal clamping after contralateral nephrectomy; *WIuni90,* unilateral warm ischemia for 90 minutes.

Conventional stains were applied (hematoxylin and eosin, periodic acid-Schiff).

The magnitude of tubular epithelial cell loss, necrosis, intratubular debris, and tubular cast formation was scored into six levels on the basis of the percentage of affected tubules in a high-power field under light microscopy, as previously described[27]: 0 (no abnormality), 0.5 (<10%), 1 (10%-25%), 2 (25%-50%), 3 (50%-75%), and 4 (>75%). All sections were examined under blind conditions by a pathologist and a nephrologist, and photographed. Tubular atrophy was scored using the same scale. Another lobe of tissue was removed and shock-frozen in liquid nitrogen and stored at −80°C.

Immunohistochemical staining of inflammatory cells and fibrosis. Frozen and paraffin-embedded kidney biopsy sections (5 μm) were processed for indirect immu-

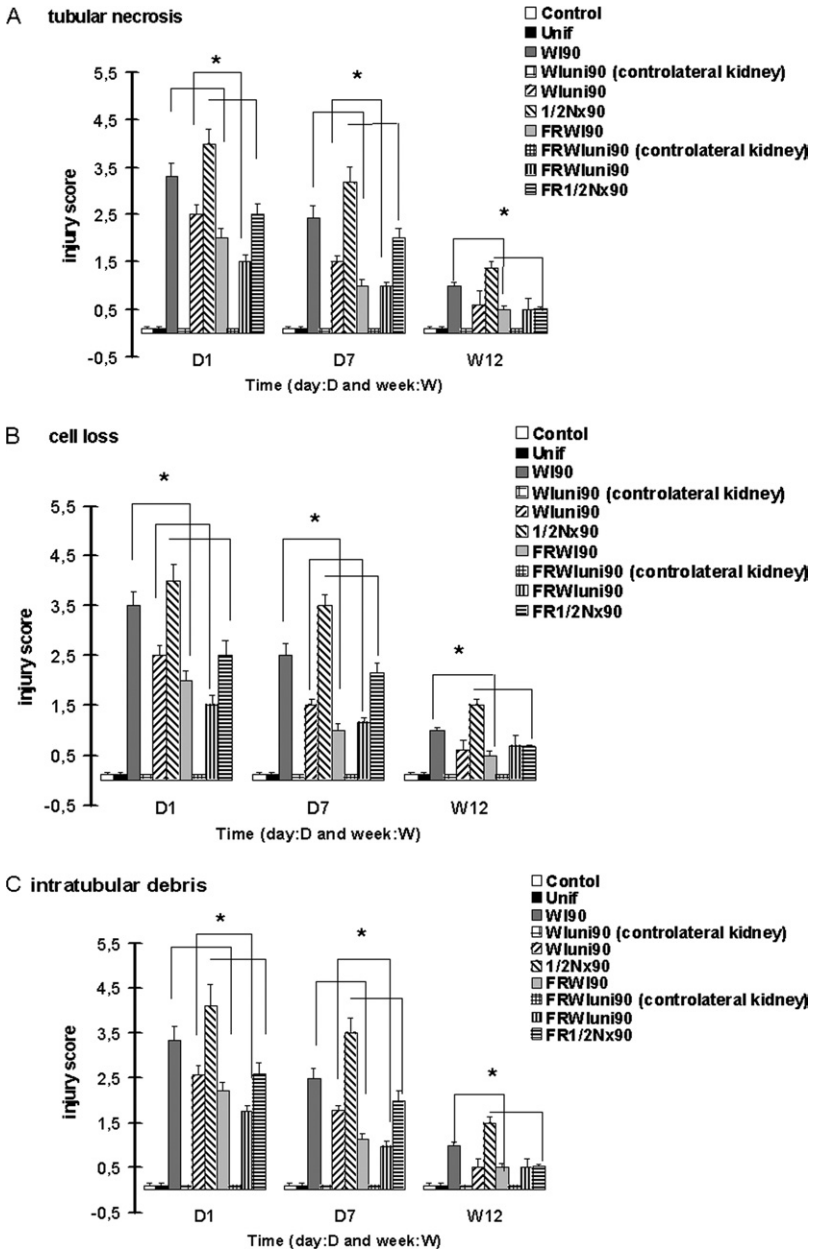


Fig 4. Effect of FR167653 *(FR)* treatment on renal tubular injury. Tubular injury was defined as **(A)** tubular epithelium necrosis, **(B)** intratubular debris, and **(C)** cell loss. The no-FR-treated groups have very severe tubular injury in all categories, whereas the FR-treated groups showed mild injury accompanied with a fast and efficient repair process as shown by evaluation of tubular injury at week 12. The values reported are the mean ± standard error of the mean. *1/2N×90*, 90 minutes of unilateral renal clamping after contralateral nephrectomy; *Unif,* uninephrectomized group; *WIuni90,* unilateral warm ischemia for 90 minutes.* $P < .05$, FR-treated groups vs no-FR-treated groups.

nohistochemistry. For this analysis, renal biopsy was performed at day 7, and a supplementary biopsy was performed at 6 weeks and 3 months. Sections were deparaffined, rehydrated, and heated in a pressure cooker containing citrate buffer (pH 6) the to boiling point for 2 minutes. The sections were then cooled, rinsed in phosphate-buffered saline, and processed for indirect immunohistochemistry, as previously described.[17,18] Inflammation was determined by evaluation of CD3-positive cells (1:150; Dako, Glostrup, Denmark) in renal tissue. For positive controls, antibodies were used in frozen sections of porcine abdominal lymph nodes (data not shown). Negative controls were

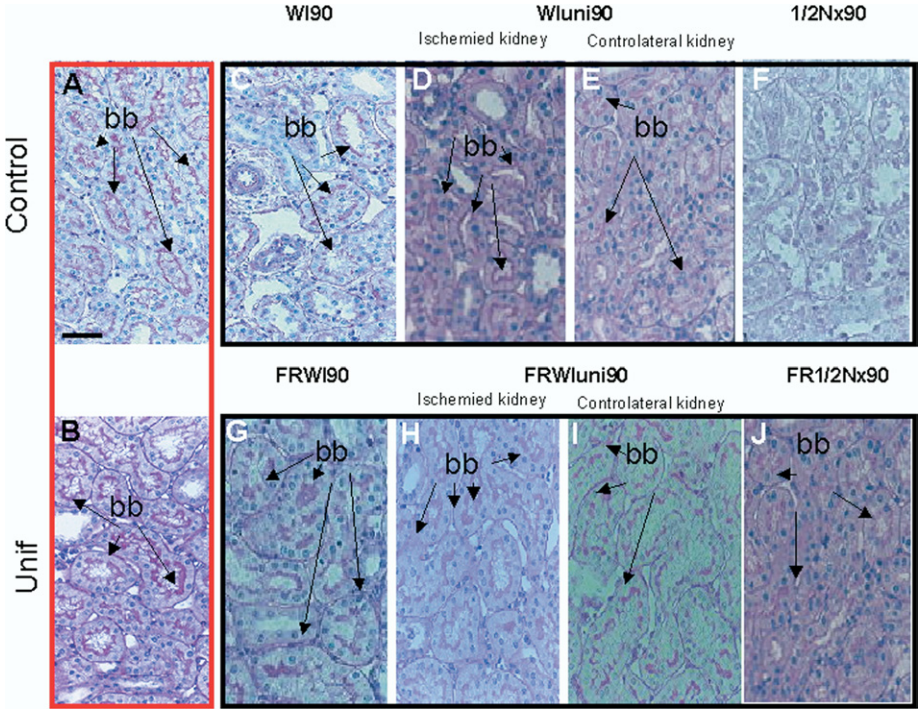


Fig 5. Effect of FR167653 *(FR)* on renal tubular injury after warm ischemia *(WI)* and reperfusion. The *black arrow* points to brush border *(bb)* integrity. **A,** Uninephrectomy *(Unif)* group. **B,** Control group. **C to F**, Groups not treated with FR167653: WI for 90 minutes *(WI90)*, unilateral renal clamping after contralateral nephrectomy *(1/2N×90)*, unilateral WI for 90 minutes *(WIuni90)*, and WIuni90 contralateral kidneys. **G to J**, Groups treated with FR: FRWI90, FR1/2N×90, FRWIuni90, and FRWIuni90 contralateral kidneys (original magnification ×200; scale bar, 200 μm).

obtained by absence of staining after omission of the primary antibody (data not shown). All sections were examined under blind conditions by a pathologist and a nephrologist and photographed. The positive cells were counted in at least 10 corticomedullary fields.

A standard procedure was used as described previously to estimate the level of tubulointerstitial fibrosis using picrosirius staining as recommended.[28,29] Picrosirius red–positive staining has been used for nearly 30 years, and the use of computerized-assisted quantification of fibrosis in the picrosirius red–stained renal allograft biopsies appears to be a surrogate marker to predict long-term allograft function.[28] In the current model, picrosirius red staining is also a useful tool to both analyze and quantify injuries that could predispose to chronic disease.

Western blotting procedure. Western blot analysis was performed for protein immunodetection,. as described previously.[24,25] Protein determination was performed 3 months after reperfusion in survival animals (cortical and outer medulla mainly) at the day of euthanasia. Minced tissue was placed in extraction buffer (1 × tissue extraction buffer; 60mM Tris-base (2-amino-2-hydroxymethyl-propane-1,3-diol; pH 6.8), 10% glycerol, and 3% sodium dodecyl sulfate (SDS) containing 5% β-mercaptoethanol and the protease inhibitor Antagosan (Hoechst, Paris, France), which were added just before use. Tissues were immediately disrupted with an Ultra Turrax (IKA, Staufen, Germany) homogenizer at maximum speed for 1 minute on ice, and homogenates were centrifuged at 26,500*g* for 15 minutes at 4°C. Supernatant aliquots were stored at –20°C for no longer than 1 week before use.

Equal amounts of proteins (50 to 100 μg) were separated on SDS–polyacrylamide gels (12% or 4%-20%) under reducing conditions and electrophoretically transferred onto polyvinylidene difluoride membranes (Bio-Rad, Hercules, Calif). Membranes were blocked for 1 hour in Tris-buffered saline Tween-20/5% nonfat milk and incubated overnight at 4°C with antibodies against TNF-α, (1:200, Santa Cruz Biotechnology, Santa Cruz, Calif). Anti-p38-MAPK and antiphospho p38-MAPK (Cell Signaling Technology, Danvers, Mass) were used for their cross reactivity against pig tissue and the ratio was determined. Total proteins in the extract in each condition were measured by the BCA protein assay kit with bovine serum albumin as a standard. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 1:10,000; Trevigen Inc) or actin (1:1,000; neoMARKERS Inc, Fremont, Calif) was used as a loading control. Intensities of the protein bands (duplicate for each animal) were determined using densitometry, quantified, and averaged.

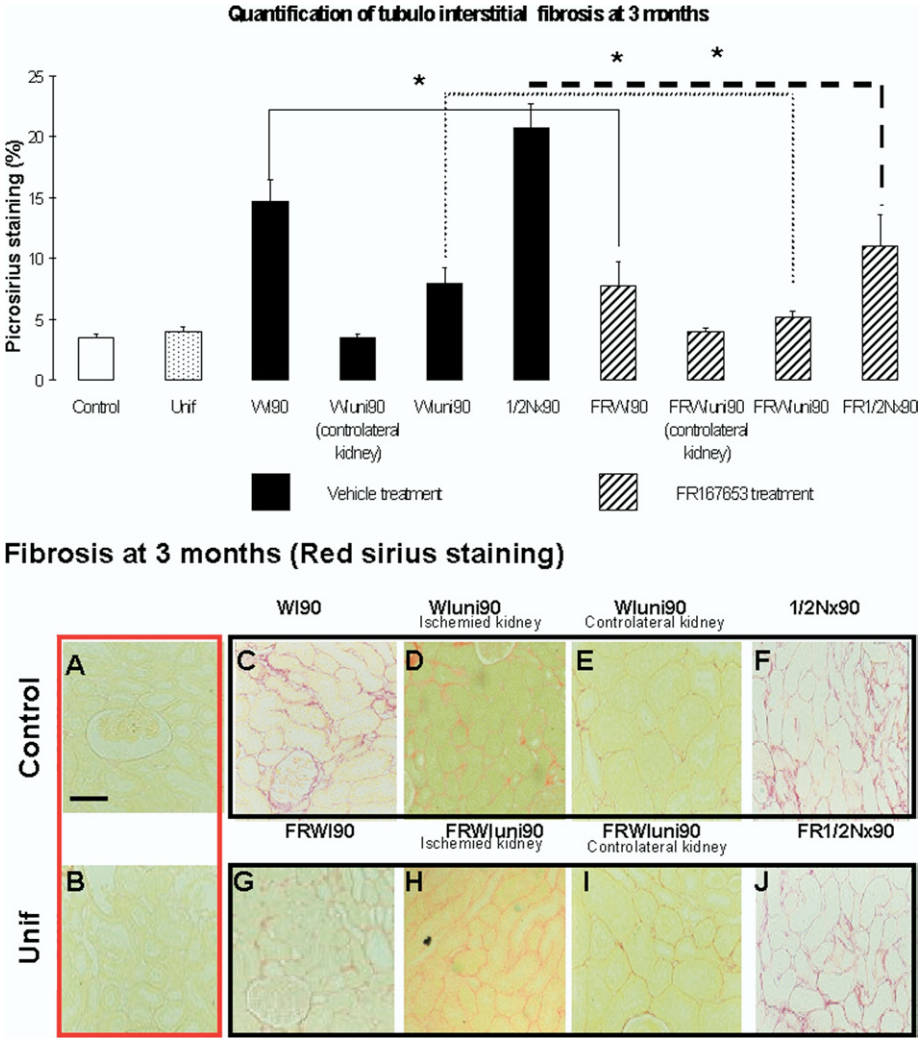


Fig 6. Effect of FR167653 *(FR)* treatment on the onset of tubulointerstitial fibrosis. **Upper Panel,** The percentage of kidney areas displaying interstitial fibrosis stained with picrosirius was measured during several weeks after warm ischemia within kidneys. The values reported are the mean ± standard error of the mean. *$P < .05$, FR-treated groups vs no-FR-treated groups. **Lower Panel,** Immunostaining for picrosirius in the different experimental groups. *A and B*, Uninephrectomy *(Unif)* group and control group, respectively. *C to F*, no-FR167653-treated groups: warm ischemia *(WI)* for 90 minutes *(WI90)*, 90 minutes of unilateral *(uni)* renal clamping after contralateral nephrectomy *(1/2N×90)*, WIuni90, and WIuni90 contralateral kidneys. *G to J,* FR-treated groups: FRWI90, FR1/2N×90, FRWIuni90, and FRWIuni90 contralateral kidneys (original magnification ×200; scale bar, 200 μm).

Real-time polymerase chain reaction using SYBR green for TNF-α. At 3 months after reperfusion, TNF-α messenger RNA (mRNA) in the cortex and outer medulla was measured to assess the influence of FR treatment, as previously described.[19] The primer sequences for PCR were as follows: TNF-α; upstream 5′-TCCTCACTCACACCATCAGC-3′, downstream, 5′-CCAAAATAGACCTGCCCAGA-3′. The relative amount of mRNA, normalized to an internal control 18S rRNA (primer sequences: forward, 5′-CGTCCCCGCCCCTTGCCTCT-3′, reverse 5′-GCTTTCGCTCTGGTCCGTCTT-3′) and relative to a calibrator (normal), was calculated by $2^{-\Delta\Delta CT}$. Real-time quantitative PCR results were quantified and expressed as the percentage change in copy numbers relative to the normal group.

Measurement of TNF-α and IL-1. Blood samples were collected from a jugular catheter before WI induction and at 1, 3, and 24 hours after reperfusion for TNF-α and IL-1 level determination. At 3 months, TNF-α was determined from blood a sample collected directly from a renal vein. IL-1 was assayed using a commercial enzyme-linked immunoabsorbent assay kit (Biosource Int, Camarillo, Calif) following manufacturer's instruction. TNF-α was determined with a Quantikine Porcine TNF-α/TNFSF2

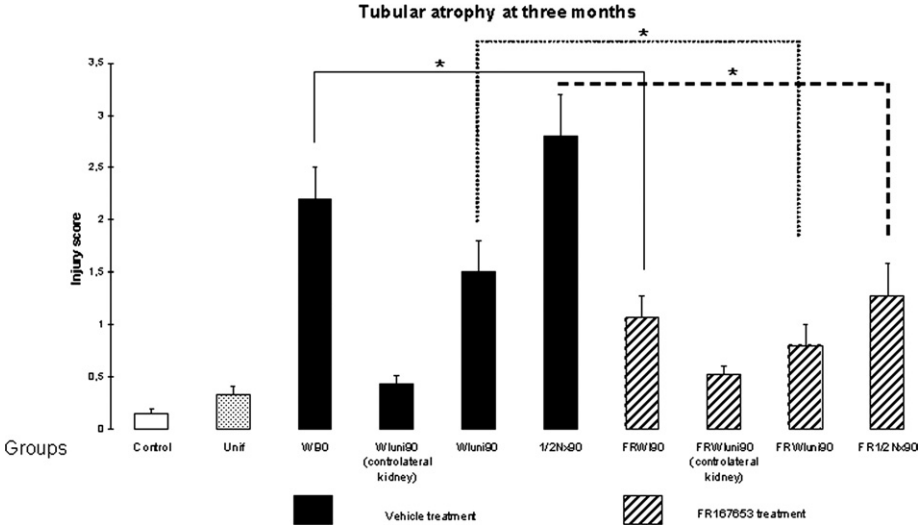


Fig 7. Effect of FR167653 *(FR)* treatment on tubular atrophy at 3 months (See Methods for details). The values reported are the mean ± standard error of the mean. *$P < .05$, FR-treated groups vs no-FR-treated groups. *1/2N×90,* 90 minutes of unilateral *(uni)* renal clamping after contralateral nephrectomy; *WI,* warm ischemia; *Unif,* Uninephrectomy.

immunoassay kit (R&D Systems, Minneapolis, Minn) as described in manufacturer's instructions.

Statistical methods. Results are shown as mean ± SEM. Comparisons within groups were performed by use of a paired *t* test. For the statistical analysis among groups, we used analysis of variance with Bonferroni correction for multiple comparisons, followed by an unpaired *t* test. Statistical significance was accepted for $P < .05$.

RESULTS

Effects of FR167653 and warm ischemic injury on early and long-term renal function and survival. The outcomes after WI and reperfusion differ markedly between groups. Three animals (43%) in the WI90 group and five (70%) in the 1/2N×90 group were euthanized at day 7 because of no urine production or poor condition. During this procedure, renal pedicle patency was controlled and no thrombosis was observed. Survival was 100% in control, Unif, WIuni90, and FR groups ($P < .05$ vs WI90 and 1/2N×90). Renal functional data are shown in Fig 2. During the first week, the creatinine concentration was significantly increased in no-FR-treated groups (day 1 to 7 after reperfusion; $P < .05$) compared with FR-treated groups (Fig 2). No difference was observed in the WIuni90 groups (with or without FR treatment) because of the contralateral functional kidney. After 7 days after ischemia, the creatinine concentration still remained increased in the reduced nephron mass (1/2N×90) group compared with FR-treated group (14 days and 1 and 3 months after reperfusion).

At 3 months a significantly high level of proteinuria was detected in the 1/2N×90 and in bilateral renal ischemia groups (WI90), suggesting the role of residual nephron mass and the level of injury compared with FR-treated groups (Fig 3). No difference was observed between groups with unilateral renal ischemia without contralateral nephrectomy (with or without FR treatment) compared with the control and Unif groups. In turn, a significantly high level of proteinuria was detected in the WI90 and 1/2N×90 groups compared with the control and Unif groups. These data suggest that FR is effective to protect kidneys from IRI consequences even in reduced renal mass, which represents the patient population particularly exposed to IRI.

Effect of FR167653 and WI on the cellular integrity of post-reperfused kidneys. Histologic analysis of biopsy samples from WI kidneys revealed differences in the cellular integrity at day 14 (Fig 4). Biopsy specimens from no-FR-treated groups (particularly 1/2N×90 groups) had marked injury showing tubular necrosis and tubular casts compared with FR-treated groups (Fig 4). In turn, FR-treated groups exhibited limitation of renal injury and a reduction of renal injury at day 14, suggesting that the injury level was reduced and the regeneration process was more efficient. No injury was detected in the control group and contralateral kidney (not ischemic) in the Wiuni90 groups. Kidneys from the different groups are presented in Fig 5. In the FR-treated groups, renal damage was reduced and brush border integrity was well preserved.

Effect of FR167653 on the onset of interstitial fibrosis and inflammation. Determination of interstitial fibrosis at 3 months after reperfusion is presented in Fig 6 (*upper panel*). The more intense percentage of interstitial fibrosis was observed in no-FR-treated groups, particularly within the 1/2N×90 group ($P < .05$). The lesions in these groups exhibited a focal-radial character and are character-

ized by patchy destruction of renal structures such as tubuli, glomeruli, and vessels (Fig 6, *lower panel*). In turn, FR reduced fibrosis development, particularly in all treated groups, including kidneys exposed to ischemia in the FR-WIuni90 group. Fibrosis development was accompanied by marked tubular atrophy in no-FR-treated groups compared with FR-treated groups (Fig 7). The protective effect of FR was marked in the 1/2N×90 group, which was the more severe experimental condition.

At 3 months, the major inflammatory cell population detected by immunohistochemical studies was CD3-positive ($CD3^+$) T lymphocytes. No infiltrating cells were detected in the control and Unif groups (data not shown). The number of cells staining $CD3^+$ increased in the no-FR-treated groups, particularly in the 1/2N×90 group (Fig 8, *upper panel*). Increased $CD3^+$ cell staining was also detected in ischemic kidneys from the WIuni90 pigs and some cells were also observed in the contralateral kidneys. After 4 weeks, $CD3^+$ slightly increased in the no-FR-treated groups. The FR-treated groups exhibited reduced inflammation, particularly in the FR1/2N×90 group at 3 months (Fig 8, *lower panel*).

Effect of FR167653 on the expression of TNF-α and p38-MAPK phosphorylation in warm ischemic tissue. At 3 months after reperfusion, FR treatment influenced inflammation in the warm ischemic tissue. This reduction of inflammation was accompanied by the modulation of TNF-α by FR treatment (Fig 9, *A* and *B: upper panel*). Production of TNF-α was predominantly detected in the WI90 and particularly in the 1/2N×90 groups. Within contralateral kidneys from WIuni90, TNF-α was not detected. FR treatment reduced TNF-α expression in all experimental groups, even in the WIuni90 group within exposed kidneys. At the end of the 3-month follow-up, TNF-α was also modulated by FR treatment (Fig 9, *C, middle panel*). No FR-treated groups showed an increase in the ratio of TNF-α mRNA, particularly in the 1/2N×90 group. In WI90 (bilateral renal ischemia model) pigs, TNF-α mRNA remained increase compared with the FR-treated pigs. In the Wiuni90 group, TNF-α messenger was increased in the ischemic kidneys compared with the FR-treated group. In the contralateral kidneys, no difference was noted and the values were at the same level observed in the control and Unif groups. FR reduced phospho-p38-MAPK expression in kidney tissue at 3 months (Fig 9, *C* and *D*).

Effect of FR167653 on cytokine release. As expected, the circulating level of TNF-α and IL-1 was attenuated in the FR-treated groups (Fig 10). At 3 months, the circulating level of TNF-α was attenuated in the FR-treated groups and paralleled the renal inflammation (Fig 11).

DISCUSSION

The WI-reflow model results in extensive necrosis that destroys the proximal tubules of the outer stripe of the outer medulla and is variable in extent, both with short and longer periods of obstruction.[5,8,28,30-32] The kidney has the ability to restore the structural and functional integrity of the proximal tubule, which undergoes extensive epithelial cell death after prolonged exposure to ischemia, but only to a limited extent. This ability is modulated by the resulting damage levels and the residual functional nephron mass.[33,34] A recent study demonstrated that nephron repair by surviving tubular epithelial cells is the predominant mechanism of repair after ischemic tubular injury in the adult mammalian kidney.[35] As a consequence, improving the ability of the kidney to tolerate ischemic injury would have important implications in different clinical situations such as organ transplantation or major aortic surgery for patients with pre-existing conditions of reduced nephron mass.

The current study showed FR is effective in improving renal recovery function, particularly after severe ischemia (WI for 90 minutes) and contralateral nephrectomy, which represents severe conditions in the current model. IRI in the uninephrectomized condition causes further reduction of nephrons, enough to accentuate the glomerular injury by glomerular hyperfiltration and hypertension, which finally promote the development of glomerulosclerosis. Injured tissues release cytokines that promote inflammation, proliferation of fibroblasts, and interstitial fibrosis [36] Interestingly, FR was effective to protect renal tissue from WI and its consequences when the contralateral kidney was in place.[34,35]

The long-term effect of FR is marked by a reduction of proteinuria excretion at 3 months, accompanied by a marked reduction of tubulointerstitial fibrosis and a reduction of tubular atrophy. Several investigators have reported the development of chronic and progressive renal dysfunction after severe acute kidney injury, and a review is presented by Basile.[8] Interstitial fibrosis, which is characterized by accumulation of extracellular matrix protein, is a common feature of progressive renal diseases and is correlated with structural damage. These data suggest that FR treatment has the ability to affect the events that lead to fibrosis, and likely at the initiation phase, by reducing IRI and protecting the remaining renal mass. The underlying mechanism could be related to the limitation of phosphorylated p38-MAPK expression, which is involved in the production of proinflammatory cytokines.

IRI is marked not only by necrosis or apoptosis, or both, but also by an inflammatory response that may occur by either antigen-dependent or antigen-independent insults.[37,38] Some controversy exists about the role of these inflammatory cells in the early phase after renal injury. Inflammation can result in a reduction in local blood flow to the outer medulla, with adverse consequences on tubule function and viability.[39,40] However, renal and extrarenal immune responses were recently demonstrated to occur after a single episode of severe IRI.[41] These changes could in turn have long-term consequences on the progression of renal disease in transplanted and native kidneys.

In the current study, WI and renal mass influenced detection of inflammatory cells. FR treatment significantly lowered cell infiltration in the early and long-term follow-up, suggesting that this drug attenuates inflammatory pro-

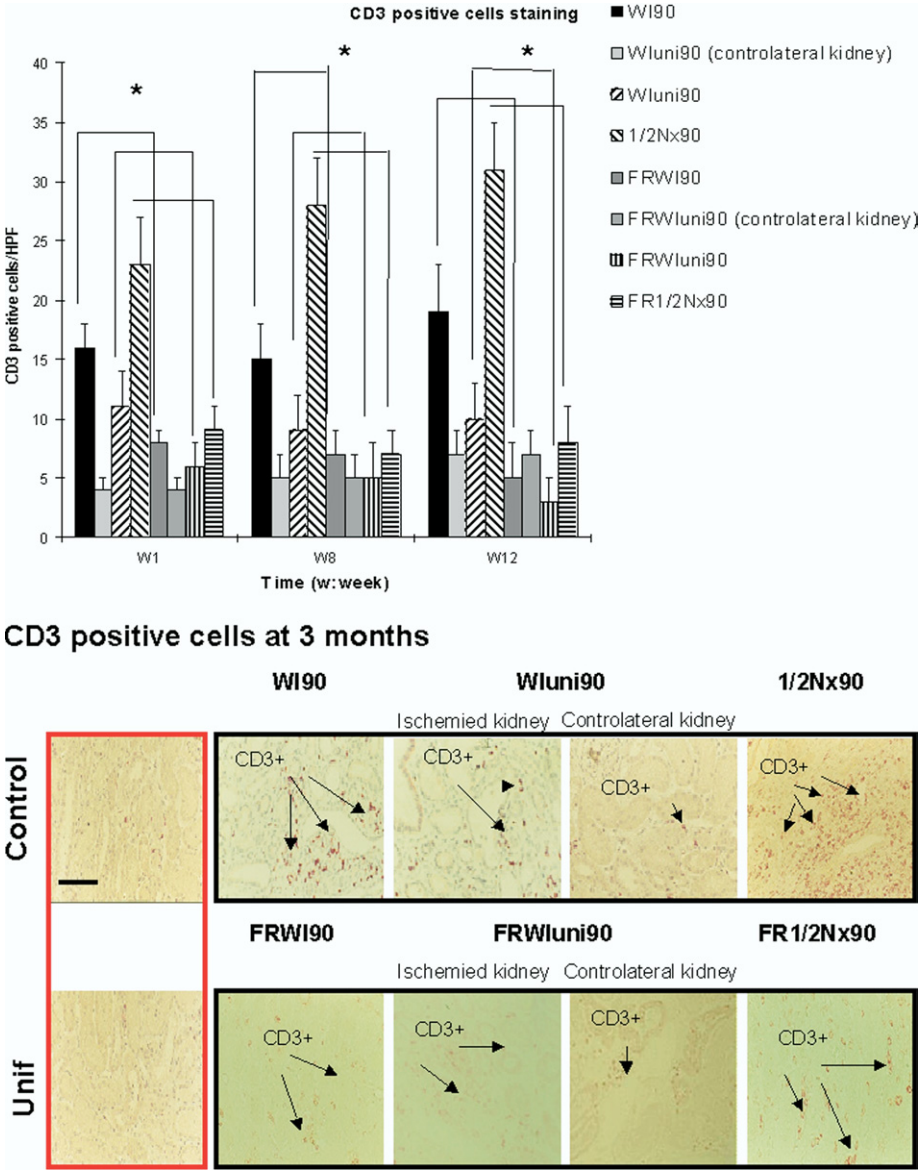
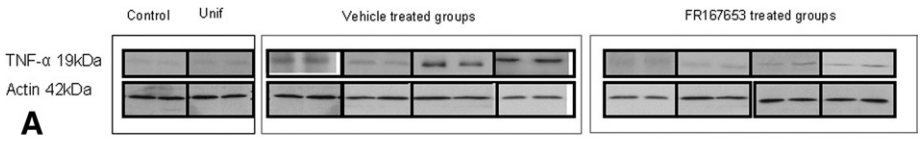


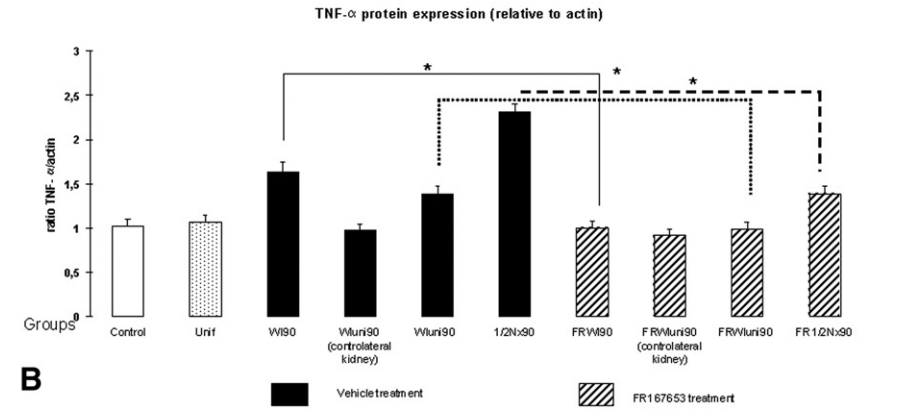
Fig 8. Effect of FR167653 *(FR)* treatment on inflammation in post-reperfused kidney. **Upper Panel,** The number of positively stained cells per surface area was counted on biopsy samples from experimental groups at 1 and 6 weeks and at 3 months. FR treatment reduced CD3-positive cells staining compared with no FR treatment. **Lower Panel,** Immunostaining for CD3-positive cells at 3 months *(black arrow)* in the different experimental groups, uninephrectomy (Unif) group, and control group, respectively. No-FR167653-treated groups: *WI90,* 90 minutes of unilateral *(uni)* renal clamping after contralateral nephrectomy *(1/2N×90)*, 90 minutes of unilateral warm ischemia *(WIuni90)*, and WIuni90 contralateral kidneys. FR-treated groups: FRWI90, FR1/2N×90, FRWIuni90, and FRWIuni90 contralateral kidneys (original magnification ×200; scale bar, 200 μm).

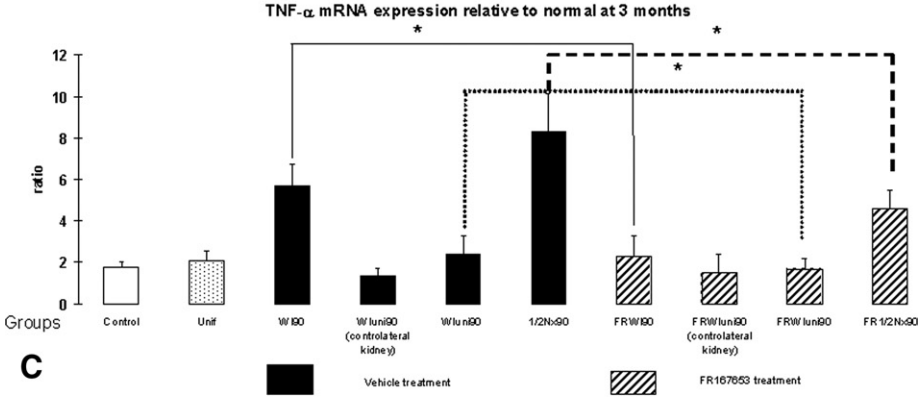
cess associated with IRI. This effect is particularly interesting because IRI is an inflammatory condition that profoundly affects both early and long-term function of the kidney, and the acute phase of IRI has been increasingly viewed as part of the innate immune response.[6,15]

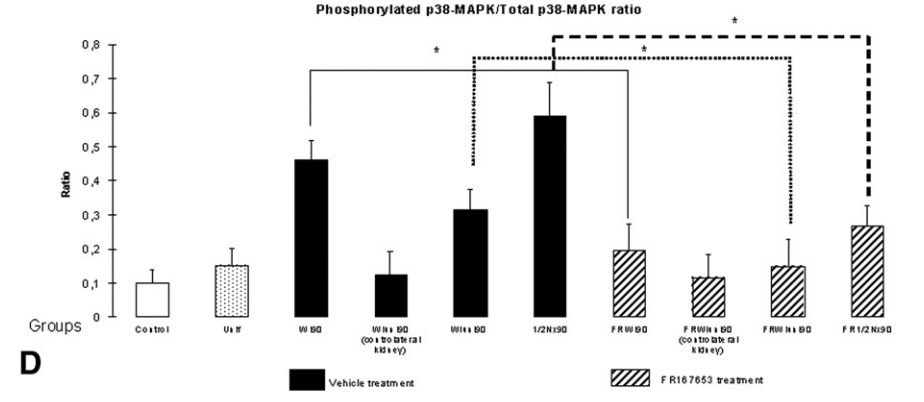
A number of proinflammatory cytokines and chemokines have been consistently implicated in the pathophysiology of acute renal failure, among the most prominent of which is TNF.[14] TNF-α is implicated in the pathogenesis of different renal diseases and can promote renal dysfunction by direct cytotoxicity, vasoconstriction, and inflammatory cells recruitment.[13,35,42,43] Up-regulation of mRNA and protein levels of TNF occurs at a whole-organ level within minutes to hours of the onset of IRI.[13]

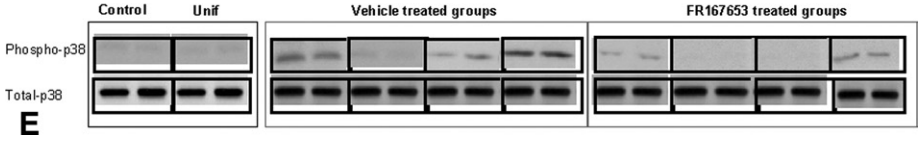
FR was discovered as a cytokine production inhibitor of IL-1 and TNF-α. Its target molecule was demonstrated to


Control
Unif
Vehicle treated groups
FR167653 treated groups
TNF-α 19kDa
Actin 42kDa
A


TNF-α protein expression (relative to actin)
ratio TNF-α/actin
Groups
Control
Unif
Wl90
Wluni90 (controlateral kidney)
Wluni90
1/2Nx90
FRWl90
FRWluni90 (controlateral kidney)
FRWluni90
FR1/2Nx90
Vehicle treatment
FR167653 treatment
B


TNF-α mRNA expression relative to normal at 3 months
ratio
Groups
Control
Unif
Wl90
Wluni90 (controlateral kidney)
Wluni90
1/2Nx90
FRWl90
FRWluni90 (controlateral kidney)
FRWluni90
FR1/2Nx90
Vehicle treatment
FR167653 treatment
C


Phosphorylated p38-MAPK/Total p38-MAPK ratio
Ratio
Groups
Control
Unif
Wl90
Wluni90 (controlateral kidney)
Wluni90
1/2Nx90
FRWl90
FRWluni90 (controlateral kidney)
FRWluni90
FR1/2Nx90
Vehicle treatment
FR167653 treatment
D


Control
Unif
Vehicle treated groups
FR167653 treated groups
Phospho-p38
Total-p38
E

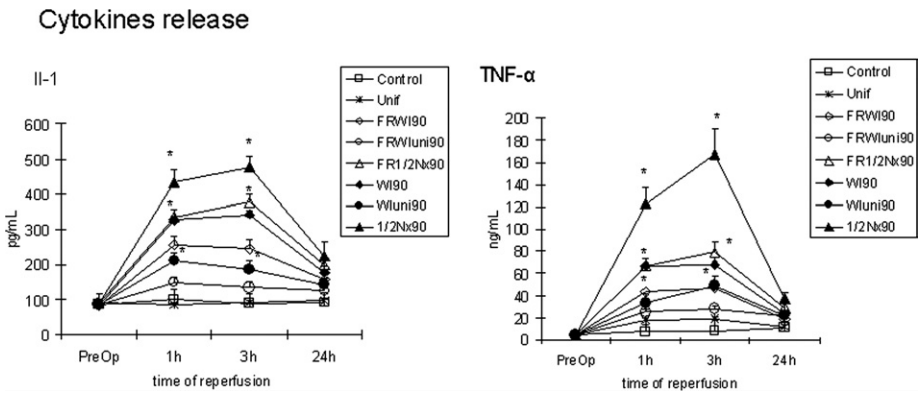


Fig 10. Effect of FR167653 *(FR)* treatment on **(Left)** interleukin-1 *(IL-1) and* **(Right)** tumor necrosis factor-α *(TNF-α)* release. FR167653 treatment reduced TNF-α and IL-1 release. The values reported are the mean ± standard error of the mean. *1/2N×90*, 90 minutes of unilateral renal clamping after contralateral nephrectomy; *Unif,* uninephrectomized group; *WIuni90,* unilateral warm ischemia for 90 minutes. **P* < .05 FR-treated groups vs no-FR-treated groups.

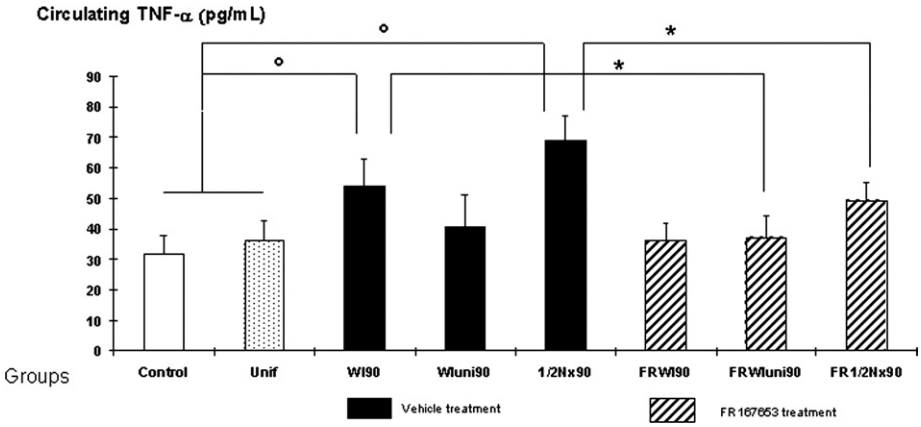


Fig 11. Quantification of circulating tumor necrosis factor-α (*TNF-α*) is presented. The values reported are the mean ± standard error of the mean. *1/2N×90*, 90 minutes of unilateral renal clamping after contralateral nephrectomy; *Unif,* uninephrectomized group; *WIuni90,* unilateral warm ischemia for 90 minutes. **P* < .05, groups treated with FR167653 (FR) vs untreated groups, °*P* < .05 WI and 1/2N×90 groups vs control and Unif groups.

be p38α-MAPK (α isoform of p38-MAPK), a key proinflammatory mediator that contributes to TNF-α production at a post-transcriptional step under various conditions.[16,22,41] Our study showed FR reduced p38-MAPK protein expression and TNF-α at the messenger and protein level, accompanied by a limitation of inflammatory process.

Current data suggest also a potential influence of nephron mass, which is involved in the IRI process. According with data reported by Cruzado et al,[34] our results

Fig 9. Effect of FR167653 *(FR)* treatment on tumor necrosis factor-α (*TNF-α*) protein, messenger RNA expression, and on phosphorylated p38-mitogen activated protein kinase (MAPK) within renal tissue from different experimental conditions. **A,** Representative immunoblots of TNF-α from the different experimental groups. **B,** Quantification of renal protein. FR treatment reduced TNF-α expression. **C,** The ratio of TNF-α. **D,** Quantification of phosphorylated p38-MAPK (phospho-p38)/total p38-MAPK (total-p38) is presented. **E,** Representative immunoblot of phospho-p38 and total p-38 respectively. The values reported are the mean ± standard error of the mean. *1/2N×90*, 90 minutes of unilateral renal clamping after contralateral nephrectomy; *Unif,* uninephrectomized group; *WIuni90,* unilateral warm ischemia for 90 minutes. **P* < .05, FR-treated groups vs no-FR-treated groups, °*P* < .05 WI and 1/2N×90 groups vs control and Unif groups.

demonstrate that nephron mass is directly correlated with the outcome of the ischemic kidneys. However, we have observed that bilateral ischemia induces less severe chronic renal lesions compared with WI associated with contralateral nephrectomy. Exposed nephron mass paralleled with the TNF-α expression within tissue collected from different experimental conditions. We have also observed that nephrons were incompletely repaired in the 1/2N×90 group. This suggests that the different compensatory mechanisms are not fully adapted in this condition. The reduced nephron mass could also be related with a limitation of the surviving tubular epithelial cells involved in the mechanism of repair after ischemic tubular injury.[30,35] Nevertheless, the current study demonstrates that FR is a renoprotective treatment for the remaining kidney in a large animal model of IRI.

The results collectively demonstrate that a single administration of FR, before WI induction and during the early stage of reperfusion, is efficient to reach a protective level against inflammation and fibrogenesis, which are the two determinants of the progression of renal fibrosis. Such pharmacologic treatments have a potential indication for renal protection to safely improve WI tolerance during open or laparoscopic surgeries, particularly for exposed patients with reduced nephron mass.

We would like to thank the following individuals: Professor Lilach O. Lerman and Dr Christophe Jayle for their contribution to this work, Professors Gérard Mauco, Michel Eugene, and Michel Carretier for their valuable comments, and Sandrine Joffrion, Dominique Lochon, Sylvette Devant, Valérie Lépine, Florent Ribardière, William Hébrard, Catherine Henry, and Alexandre Bati for their excellent technical assistance.

AUTHOR CONTRIBUTIONS

Conception and design: JC, KZ, JG, TH
Analysis and interpretation: JC, FF, KZ, JR, JG, TH
Data collection: FF, KZ, GR, GF, JG, TH
Writing the article: JC, FF, KZ, GR, GF, JG, TH
Critical revision of the article: JC, FF, KZ, GR, GF, JR, JG, TH
Final approval of the article: JC, FF, KZ, GR, GF, JR, JG, TH
Statistical analysis: FF, JBR
Obtained funding: TH
Overall responsibility: TH

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Submitted Mar 12, 2008; accepted Sep 27, 2008.

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