Peroxisome Proliferator-Activated Receptor β/δ Exerts a Strong Protection from Ischemic Acute Renal Failure

Emmanuel Letavernier,* Joëlle Perez,* Elisabeth Joye,† Agnès Bellocq,* Jean-Philippe Haymann,* Didier Heudes,‡ Walter Wahli,† Béatrice Desvergne,† and Laurent Baud*  
*INSERM U489, Tenon Hospital, Paris, France; † Center for Integrative Genomics, NCCR Frontiers in Genetics, University of Lausanne, Lausanne-Dorigny, Switzerland; and ‡INSERM U430, Cordeliers Institute, Paris, France

Ischemic acute renal failure is characterized by damages to the proximal straight tubule in the outer medulla. Lesions include loss of polarity, shedding into the tubule lumen, and eventually necrotic or apoptotic death of epithelial cells. It was recently shown that peroxisome proliferator-activated receptor β/δ (PPARβ/δ) increases keratinocyte survival after an inflammatory reaction. Therefore, whether PPARβ/δ could contribute also to the control of tubular epithelium death after renal ischemia/reperfusion was tested. It was found that PPARβ/δ−/− and PPARβ/δ−/−/− mutant mice exhibited much greater kidney dysfunction and injury than wild-type counterparts after a 30-min renal ischemia followed by a 36-h reperfusion. Conversely, wild-type mice that were given the specific PPARβ/δ ligand L-165041 before renal ischemia were completely protected against renal dysfunction, as indicated by the lack of rise in serum creatinine and fractional excretion of Na+. This protective effect was accompanied by a significant reduction in medullary necrosis, apoptosis, and inflammation. On the basis of in vitro studies, PPARβ/δ ligands seem to exert their role by activating the antiapoptotic Akt signaling pathway and, unexpectedly, by increasing the spreading of tubular epithelial cells, thus limiting potentially their shedding and anoikis. These results point to PPARβ/δ as a remarkable new target for preconditioning strategies.


the proximal straight tubule in the outer medulla of the kidney is particularly susceptible to ischemia/reperfusion injury, which remains the leading cause of acute renal failure (1,2). Damages to this segment are characterized initially by the disruption of tight junctions that control both paracellular permeability and cell polarity (3,4). The loss of cell polarity is responsible for the redistribution of integrin subunits from the basolateral to the apical membrane, contributing to the shedding of cells into the tubule lumen (4). Both increase in paracellular permeability and desquamation lead to back-leakage of glomerular filtrate (5). With more sustained ischemia/reperfusion, epithelial cells of the proximal tubule undergo necrotic or apoptotic cell death (6). Epithelial cells that do not die participate in the regeneration of tubular epithelium and the restoration of renal function (2,7). They use integrins to flatten, spread, and migrate into areas denuded by exfoliation, where they dedifferentiate, proliferate, and differentiate again (8).

Peroxisome proliferator-activated receptor β/δ (PPARβ/δ; called PPARβ hereafter) is a ligand-activated transcription factor that belongs to the nuclear hormone receptor family (9). It plays a key role in cell survival (10–14). For example, PPARβ activation leads to the expression of genes that increase the resistance of keratinocytes to apoptotic death (11,13). In these cells, PPARβ expression is also involved in the control of cell adhesion to the extracellular matrix and migration (10). All of these properties may account for the finding that PPARβ expression and activation participate in skin wound repair (10).

By contrast with PPARα and PPARγ, PPARβ is ubiquitously expressed in all nephron segments within the kidney. In particular, it is the predominant PPAR isotype in the proximal straight tubule (15). Because of this high expression, we assessed whether PPARβ would contribute to the survival of proximal tubular cells in a model of ischemic acute renal failure. We found that PPARβ-deficient mice were remarkably susceptible to renal ischemia/reperfusion injury. Conversely, mice that were given PPARβ ligand pretreatment were completely protected. To identify a possible mechanism involved in this protection, we performed in vitro experiments that emphasized the critical role of both antiapoptotic Akt signaling pathway and cell spreading. These results provide proof that PPARβ is a promising pharmacologic target for preconditioning strategy in ischemic acute renal failure.

Materials and Methods

Cell Cultures

Human proximal tubular epithelial cells (HK-2 cell line; American Type Culture Collection, Manassas, VA) were cultured at 37°C in a serum-free keratinocyte-SFM medium supplemented with human recombinant EGF and pituitary bovine extract (Life Technologies, Cergy Pontoise, France), under a 5% CO2 and 95% air atmosphere. These cells were exposed to specific PPARβ agonists, including carboxprostacyclin.

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Address correspondence to: Dr. Laurent Baud, INSERM U489, Hôpital Tenon, 4 rue de la Chine, Paris, France 75020. Phone: 33-1-5601-7951; Fax: 33-1-5601-7003; E-mail: laurent.baud@mn.ap-hop-paris.fr

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25 g were used for genetic approaches. To maintain fluid balance and volume status, mice were given 0.3 ml of warm saline intraperitoneally. Finally, to maintain fluid balance and volume status, mice were given 0.3 ml of warm saline intraperitoneally. After 36 h of reperfusion, they were re-anesthetized, blood and urine samples were collected, and kidneys were removed for morphologic analyses. For genetic approaches, wild-type and mutant C57BL/6 × 129/SV had identical surgical procedures except that they were anesthetized with xylazine (10 mg/kg) and ketamine (75 mg/kg).

Assessment of Renal Function
Samples of serum and urine were collected from all mice, and creatinine and Na⁺ levels were measured using an autoanalyzer. The fractional excretion of Na⁺ was calculated as the ratio of Na⁺ clearance to creatinine clearance.

Evaluation of Renal Histology
Kidneys were fixed in 4% paraformaldehyde and processed for paraffin embedding. Sections of 3-μm thickness were made and stained with the periodic acid-Schiff (PAS) reagent. Tubular injury was scored by estimating the percentage of tubules in the cortex or the outer medulla that showed epithelial necrosis or had luminal necrotic debris and tubular dilation, as follows: 0, none; 1, <5%; 2, 5 to 25%; 3, 25 to 75%; and 4, >75% (20). All evaluations were made on 10 fields per section and 10 sections per kidney, by two different blinded observers. In addition, tubular dilation, a hallmark of renal ischemia/reperfusion injury (1), was assessed by quantitative morphometric analysis, with the use of an automated image analyzing system. Measurements were performed on randomly selected sections that were stained with hematoxylin and eosin (five per kidney). The lumen area of cortical tubules was measured at ×40 magnification from digital images (Nikon Eclipse 800 light microscope and Dxm 1200 digital camera; Nikon, Champigny sur Marne, France) and image analysis software (Image J 1.33; http://rsb.info.nih.gov/iij/download/). Results were expressed as the surface occupied by tubular lumens relative to the total cortical area.

Induction of Ischemic Acute Renal Failure
The studies were conducted by following established guidelines for animal care, and all protocols were approved by the INSERM and by the Service Vétérinaire Cantonal de Lausanne. For pharmacologic approaches, C57BL/6 mice were anesthetized by intraperitoneal administration of Avertin (Sigma-Aldrich, St. Louis, MO) to detect externalized phosphatidylserine or the caspase 3 activity detection kit (Oncogene Research Products, Merck Biosciences, Nottingham, UK). The antiapoptotic role of PPARβ via the Akt1 signaling cascade was assessed by measuring Akt1 phosphorylation with the Fast Activated Cell–based ELISA Kit (Active motif, Rixensart, Belgium) according to the manufacturer’s instructions and by analyzing the effect of Akt1 inhibitor (Calbiochem, San Diego, CA) on L-165041 antiapoptotic efficiency.

Animals
Male C57BL/6 mice that weighed 20 to 25 g were used for pharmacologic approaches. They were given L-165041 (30 mg/kg per d) or vehicle alone (0.5% carboxymethylcellulose) by gavage for 2 d before the induction of acute renal failure and during the reperfusion period. These doses of L-165041 are sufficient to produce the activation of PPARβ but not PPARα and PPARγ (17,18). Male PPARβ-deficient mice (11) and C57BL/6 × 129/SV wild-type control mice that weighed 20 to 25 g were used for genetic approaches.

Detection of Neutrophils and Macrophages Using Myeloperoxidase Assay
Snap-frozen kidney samples were thawed, added to ice-cold 50 mM PBS (pH 6.0) supplemented with 0.5% hexadecyltrimethylammonium bromide up to concentrations of 0.1 g renal tissue/ml, and homogenized with 20 strokes in a glass homogenizer. The lysates then were freeze-thawed three times and centrifuged at 20,000 × g for 1 h at 4°C. Supernatants were assayed for myeloperoxidase (MPO), as described previously (21). Results were expressed as OD/min per mg wet tissue.

Statistical Analyses
Results are expressed as mean ± SEM. Comparisons between groups of values were made with the t test for unrelated groups. A difference between groups of P < 0.05 was considered significant.

Results
PPARβ⁺/⁻ and PPARβ⁻/⁻ Mice Developed More Severe Ischemic Acute Renal Failure Than Wild-Type Mice
We first performed an in vivo ischemia/reperfusion experiment in wild-type (PPARβ⁺/⁺), heterozygous (PPARβ⁺/⁻), and null (PPARβ⁻/⁻) mice. Serum creatinine levels, measured before surgery, did not differ among the three groups of ani-
mals (26.5 ± 3.5, 22.3 ± 1.8, and 28.8 ± 2.7 μM, respectively; n = 3 in each group). Then mice of each genotype underwent bilateral renal artery clamping for 30 min under deep anesthesia, after which they showed normal activity in the initial first hours. However, the reperfusion period had to be limited to 36 h because of rapid worsening of clinical symptoms (e.g., shivering and drowsiness) in mutant mice. At that time point, PPARβ+/− and PPARβ−/− mice exhibited a much worse kidney dysfunction than PPARβ+/+ mice, as indicated by significantly higher serum creatinine levels (Figure 1). This renal dysfunction was corroborated by the occurrence of increased histologic damage in the cortex and the outer medulla in mutant mice as compared with wild-type counterparts (Figure 2). A semiquantitative tubular necrosis scoring method indicated an increased damage, albeit not reaching statistical significance. In addition, a quantitative morphometric analysis of tubular lumen areas in the cortex showed a statistically significant increase in tubular dilation in PPARβ−/− mice as compared with wild-type mice and PPARβ+/− mice, respectively (wild-type mice 0.202 ± 0.020; PPARβ−/− mice 0.172 ± 0.028; PPARβ+/− mice 0.264 ± 0.024; P < 0.05). Together, these observations indicate that PPARβ is required to fight renal injury in ischemic acute renal failure.

**PPARβ Ligands Modulated the Phenotype of Proximal Tubular Cells**

Defects in epithelial cells of the proximal straight tubule are the histologic hallmark of renal ischemia/reperfusion injury. Main alterations are the disruption of the epithelial cell cytoskeleton, loss of cell polarity, and ultimately cell death. Thus, we examined these cytopathologic events using the human proximal tubular cell line HK-2 as an experimental tool. These cells showed abundant expression of PPARβ, the level of which was not modified by PPARβ ligands, including carprofenstacyclin (data not shown) and L-165041 (Figure 3A). In the absence of treatment, HK-2 cells displayed a rounded shape with few cellular extensions (Figure 3B). Actin fibers were sparse, distributed mainly at the cell periphery, and focal adhesion plaques with vinculin were condensed. Exposure of these cells to the PPARβ ligand carprofenstacyclin (data not shown) or L-165041 (Figure 3B) for 24 h resulted in a marked alteration of this morphology. Cells became flattened and spread, concomitant with the coalescence of actin fibers into stress fibers. In addition, the focal adhesion component vinculin was now scattered throughout the basal cell membrane, in close contact with actin fibers. These results dramatically emphasized the role of PPARβ in the structural organization of the epithelial cytoskeleton, which, in turn, is responsible for cell spreading and adhesion to extracellular matrix.

**PPARβ Ligands Modulated the Viability of Proximal Tubular Cells**

We then analyzed the impact of the PPARβ ligand on HK-2 cell survival. Twenty-four-hour exposure to effective concentrations of L-165041 did not affect growth and viability of these cells (Figure 4A). Because oxygen-derived free radicals are known to dramatically reduce epithelial survival in ischemic acute renal failure (22), we analyzed the impact of the PPARβ ligand on cell survival during oxidative stress. Apoptosis, which was minimal in control cells, reached 29.9 ± 5.6% in cells that were exposed for 24 h to 0.5 mM H2O2 (Figure 4B).
death response was dramatically decreased in a dose-dependent manner in cells that were pretreated with L-165041. Prominent among the signaling pathways involved in the resistance to apoptosis is the ubiquitous phosphatidylinositol-3-kinase/Akt1 signaling pathway (23). L-165041 dose-dependently increased active phosphorylated Akt1 levels without modifying total Akt1 expression (Figure 5A). Remarkably, a specific Akt1 inhibitor prevented the antiapoptotic effect of L-165041 completely (Figure 5B). These results demonstrate that activated PPAR\beta exhibits a major antiapoptotic effect on HK-2 cells exposed to oxidative stress, via activation of the phosphatidylinositol-3-kinase/Akt1 signaling pathway.

Wild-Type Mice Given L-165041 Pretreatment Developed Less Severe Ischemic Acute Renal Failure

Acute renal failure occurs mainly in the context of various functional alterations of several organs. Although it severely aggravates the vital prognosis, the damage caused is reversible in case of survival, the epithelium being healed via mechanisms that include induction of heat-shock proteins and production of growth factors that induce tubular regeneration (1,2). These processes have been proposed as targets for preconditioning treatments that would protect the kidney in situations of imminent ischemic renal failure. The renal prejudice observed in PPARβ mutant mice, together with the demonstration of the important action of PPARβ on cell morphology and protection against apoptosis, identifies PPARβ as a putative target for preconditioning treatments. To test this hypothesis, we pretreated a group of C57BL/6 mice with L-165041 (30 mg/kg per d for 2 d before and during the reperfusion period). This treatment completely prevented the ischemia/reperfusion-de-
ependent rise in serum creatinine and fractional excretion of Na\textsuperscript{+}, which are markers of glomerular and tubular dysfunction, respectively (Figure 6). Remarkably, this dysfunction abrogation was associated with a significant reduction in tubular injury (including necrosis and sloughing of epithelial cells) in the cortex and the outer medulla as assessed by semiquantitative analysis (Figure 7) and with a significant decrease in dilation of the cortical tubules as assessed by quantitative morphometric analysis (wild-type mice 0.123 ± 0.025; wild-type mice given L-165041 0.064 ± 0.010; \(P < 0.05\)).

Furthermore, there was a reduction in monocytes/macrophages and neutrophils influx, as reflected by the decrease in MPO activity (Figure 8). The PPAR\(\beta\) ligand also had a major effect on the occurrence of apoptosis. Kidneys from mice that were subjected to ischemic acute renal failure showed extensive TUNEL-positive staining, predominantly in the tubules of the outer medulla, in sharp contrast with mice that were pretreated with L-165041 before the induction of the failure (Figure 9). These results demonstrate a very strong protective role of PPAR\(\beta\), which designates it as a very promising pharmacologic target for preconditioning strategies in ischemic acute renal failure.

\section*{Discussion}

This study identifies PPAR\(\beta\) as a newly recognized participant in the control of renal ischemia reperfusion injury. We found that PPAR\(\beta^{++}\) and PPAR\(\beta^{+-}\) mice had a much worse kidney dysfunction than PPAR\(\beta^{++}\) mice. Conversely, wild-type mice that were pretreated with L-165041 were functionally and histologically protected against ischemic acute renal failure. Although the mechanisms underlying this protection are conceivably numerous, our results suggest that PPAR\(\beta\) expression and activation are required mainly to limit epithelial cell death in the proximal straight tubule.

Various reports have shown previously that PPAR\(\beta\) activation protects cells against apoptosis. Colon carcinoma cells that are exposed to PPAR\(\beta\) ligand become resistant to growth factor withdrawal–induced apoptosis (14). Keratinocytes that are exposed to proinflammatory cytokines TNF-\(\alpha\) and IFN-\(\gamma\) express high levels of PPAR\(\beta\) and produce endogenous PPAR\(\beta\) ligands, which increase the resistance of these cells to TNF-\(\alpha\)-induced apoptosis (11). Within the kidney, medullary interstitial cells that are exposed to hypertonic stress produce PGI\textsubscript{2}, which, in turn, increases their survival through a PPAR\(\beta\)-mediated process (12). In the present study, we extended the list of PPAR\(\beta\) target cells by demonstrating the antiapoptotic role of PPAR\(\beta\) ligand in HK-2 cells that were exposed to oxidative stress. The apoptotic process in these human proximal tubular cells, which are known to be much more sensitive to oxidative stress than...


Figure 7. Wild-type mice that were given L-165041 pretreatment developed less severe ischemic renal lesions. Mice were pretreated without or with L-165041 (30 mg/kg per d for 2 d before and during the reperfusion period) and then subjected to 30 min of renal ischemia followed by 36 h of reperfusion. (A) Representative PAS-stained sections of kidneys from untreated (left) and L-165041–pretreated (right) mice are shown. Compared with kidneys from untreated mice, kidneys from L-165041–pretreated mice exhibited much less extensive tubular damage (arrows). The inset shows a magnification of tubular change in the kidney section from an L-165041–pretreated mouse: Cells spread out, covering areas of denuded epithelium. (B) Semiquantitative analysis of tubular necrosis (*P < 0.05 versus untreated mice). Magnification, ×200 in A.

Figure 8. Wild-type mice that were given L-165041 pretreatment developed less severe ischemia-induced inflammation. Mice were pretreated without or with L-165041 (30 mg/kg per d for 2 d) and then subjected to 30 min of renal ischemia followed by 36 h of reperfusion. Influx of monocytes/macrophages and neutrophils was reflected by the increase in myeloperoxidase (MPO) activity. *P < 0.05 versus untreated mice.

Figure 9. Wild-type mice that were given L-165041 pretreatment developed less severe ischemia-induced tubular apoptosis. Mice were pretreated without or with L-165041 (30 mg/kg per d) and then subjected to 30 min of renal ischemia followed by 36 h of reperfusion. Tubular apoptosis was reflected by the increased number of transferase-mediated dUTP nick-end labeling (TUNEL)-positive nuclei per field (*P < 0.05 versus untreated mice). A representative picture of the distribution of TUNEL-positive nuclei (top) versus propidium iodide–positive nuclei (bottom) in kidney of sham (left), untreated (center), and L-165041–pretreated (right) mice is shown. (Inset) High magnification of TUNEL-positive nuclei in the epithelium of a tubule.

The apoptotic process in HK-2 cells may also result from their limited interaction with the extracellular matrix (anoikis) (7). In this study, we demonstrated for the first time that PPARβ ligands increase cell spreading, raising the possibility that increased cell interaction with the extracellular matrix may play a part in PPARβ-induced resistance to apoptosis. Most important, PPARβ-induced cell flattening and spreading are potentially important in vivo during ischemic acute renal failure for preventing back-leakage of the glomerular filtrate (5). Although the molecular mechanisms involved in this process remain to be characterized, the role of Akt is suggested by the recent observation that Akt signaling pathway promotes integrin recycling and cell spreading (25).

Distal tubular cells (22), results from an increase in the activity of extracellular signal-regulated kinase-1 and-2 and a decrease in phosphorylated Akt (24). We have demonstrated the importance of Akt phosphorylation in the antiapoptotic role of PPARβ. These results are consistent with our previous demonstration of Akt participation in the molecular mechanism by which PPARβ protects keratinocytes against apoptosis (13).

The apoptotic process in HK-2 cells may also result from their limited interaction with the extracellular matrix (anoikis) (7). In this study, we demonstrated for the first time that PPARβ activation increases cell spreading, raising the possibility that increased cell interaction with the extracellular matrix may play a part in PPARβ-induced resistance to apoptosis. Most important, PPARβ-induced cell flattening and spreading are potentially important in vivo during ischemic acute renal failure for preventing back-leakage of the glomerular filtrate (5). Although the molecular mechanisms involved in this process remain to be characterized, the role of Akt is suggested by the recent observation that Akt signaling pathway promotes integrin recycling and cell spreading (25).

Apoptotic cell death contributes in part to the inflammatory response observed after renal ischemia/reperfusion (26). Thus, the anti-inflammatory effect of PPARβ ligands in our model, as demonstrated by a decrease in MPO activity, could be related to the PPARβ-dependent abrogation of epithelial cell apoptosis. Alternatively, PPARβ ligands could trigger directly inflammatory and/or endothelial cells. Along with this hypothesis, spe-
pecific PPARβ ligands have been shown to inhibit the expression of proinflammatory cytokines (e.g., monocyte chemoattractant protein-1) in both macrophages and endothelial cells (27,28). Because vascular endothelium activation and injury contribute to reduce renal perfusion and, hence, to decrease GFR (29,30), PPARβ ligands would protect renal function by preventing these mechanisms as well.

Apart from PPARβ, the other PPAR isotypes may affect tubular cell apoptosis and, thereby, renal ischemia/reperfusion lesions. PPARγ is expressed mainly in the inner medullary collecting duct and to a lesser extent in the proximal tubule (15,31). Administration of rosiglitazone and ciglitazone, two specific PPARγ agonists, have been shown to reduce renal dysfunction and injury associated with ischemia/reperfusion of the rat kidney (32). However, in sharp contrast to these in vivo experiments, PPARγ ligands have been shown to stimulate rather than prevent proximal tubular cell apoptosis in vitro, as did overexpression of PPARγ (31,33). PPARα is also expressed in the convoluted part of the proximal tubule (15). Because ischemic lesions affect predominantly the straight part of proximal tubule (S3), the mechanism by which PPARα agonists exert beneficial effects in experimental models of ischemic acute renal failure (34,35) remains to be elucidated.

In summary, our data demonstrate that PPARβ activation has highly beneficial effects in ischemic acute renal failure, as a result of its combined action on cell survival and cytoskeleton reorganization. Importantly, a short preconditioning strategy with PPARβ agonists was highly protective. Thus, PPARβ agonists should be considered seriously as a novel means of conferring renal protection in clinical situations that carry a high risk for ischemic renal failure occurrence. New strategies of preconditioning or prevention are indeed badly needed for this syndrome, which still has a mortality of >50%.

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