Transplant International ISSN 0934-0874

ORIGINAL ARTICLE

PPARα agonists improve renal preservation in kidneys subjected to chronic *in vitro* perfusion: interaction with mannitol

Travis C. Jackson,[1] Zaichuan Mi,[1] Sheldon I. Bastacky,[2] Teresa McHale,[2] Mona F. Melhem,[2] Prajakta A. Sonalker,[3] Stevan P. Tofovic[1] and Edwin K. Jackson[3]

1 Department of Medicine, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA
2 Department of Pathology, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA
3 Department of Pharmacology, Center for Clinical Pharmacology, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA

Keywords
kidney, mannitol, organ preservation, PPARα, renal preservation, transplantation.

Correspondence
Edwin K. Jackson, PhD, Department of Pharmacology, Center for Clinical Pharmacology, University of Pittsburgh School of Medicine, 100 Technology Drive, Suite 450, Pittsburgh, PA 15219-3130, USA.
Tel.: +412 648 1505; fax: +412 648 1837;
e-mail: edj@pitt.edu

Received: 26 July 2006
Revision requested: 5 September 2006
Accepted: 30 October 2006

doi:10.1111/j.1432-2277.2006.00431.x

Summary

We developed methods for prolonged (12 h), sterile, normothermic perfusion of rat kidneys and screened compounds for renal preservation including: mitochondrial transition pore inhibitor (decylubiquinone); caspase inhibitor (Z-VAD); peroxisome proliferator-activated receptor-alpha (PPARα) agonists (gemfibrozil, WY-14643); antioxidants (trolox, luteolin, quercetin); growth factors (HGF, PDGF, EGF, IGF-1, VEGF, transferrin); calpain inhibitor (Z-Val-Phe-CHO); calmodulin inhibitor (W7); K_{ATP} opener (minoxidil, minoxidil sulfate); PARP inhibitor (3-aminobenzamide); calcium channel blocker (verapamil); V_2 agonist (DDAVP); diuretics (acetazolamide, hydrochlorothiazide, furosemide, mannitol); peroxisome proliferator-activated receptor-beta agonist (L-165041); dopamine agonist (dopamine); essential fatty acid (linolenic acid); β-NAD; urea; uric acid; and aldosterone. In pilot studies, only PPARα agonists and mannitol provided promising results. Accordingly, these agents were investigated further. Fifteen rat kidneys were perfused for 12 h with L-15 media at 37 °C in the absence or presence of mannitol, gemfibrozil, gemfibrozil + mannitol or WY-14643. Chronic perfusion in untreated kidneys caused destruction of glomerular and tubular architecture (light and electron microscopy), disappearance of Na^+-K^+-ATPase-α_1 (Western blotting), and apoptosis (Apoptag staining). Gemfibrozil and WY-14643 marginally improved some biomarkers of renal preservation. However, the combination of gemfibrozil with mannitol markedly improved all parameters of renal preservation. We conclude that PPARα agonists, particularly when combined with mannitol, protect organs from normothermic, perfusion-induced damage.

Introduction

Identification of drugs that preserve organs would have important implications. Effective drugs added to organ preservation solutions, such as University of Wisconsin solution, Celsior, St. Thomas Hospital 2 solution, Ringer-lactate, Euro-Collins solution or Bretschneider HTK solution, could extend the time for extracorporeal survival of grafts prior to transplantation, decrease the incidence of primary graft dysfunction and augment the pool of available donors [1]. Moreover, such drugs may find utility for the prevention and treatment of acute organ failure, for example acute renal failure for which no effective pharmacological agent is currently known [2]. Finally, preservation drugs, if sufficiently efficacious, could allow for the prolonged *in situ* perfusion of organ systems with solutions to restore organ function.

The purpose of the present investigation was to explore several available drug classes for possible organ preservation. In this regard, we developed a method for perfusing rat kidneys in a rigorously sterile environment such that the kidney can be maintained at 37 °C for prolonged periods of time without infection. Using this model system, we can reproducibly induce by 12-h perfusion at 37 °C severe acute tubular necrosis as well as deterioration of the glomerular and renal interstitial elements. Using this relatively inexpensive and convenient model system, we began systematically examining a number of drug classes for possible efficacy. Here we describe our model system and provide a summary of our screening efforts.

In our screening, the osmotic diuretic mannitol and the peroxisome proliferator-activated receptor-alpha (PPARα) agonists gemfibrozil and WY-14643 demonstrated promise. Previous studies have recognized mannitol as an effective drug for improving organ preservation [3–11]. However, to our knowledge the identification of PPARα agonists as organ-preserving agents is a novel finding. Accordingly, we engaged a more detailed examination of PPARα agonists. Also, because mannitol is already employed in some organ preservation solutions [1], we investigated the interaction between mannitol and PPARα agonists on kidney preservation.

Methods

Perfusion system

A glass perfusion system (Hugo Sachs Elektronik-Harvard Apparatus GmbH, March-Hugstetten, Germany) was disassembled, autoclaved (35 min at 135 °C), reassembled in a laminar flow hood (Baker Company, model SG-400, Sanford, ME, USA), and irradiated with ultraviolet light. The system, shown schematically in top panel of Fig. 1, included the following components: a jacketed glass perfusate reservoir for warming and initial oxygenation of perfusate; a jacketed glass-oxygenator for additional oxygenation; a Windkessel chamber for dampening of pulsations; a jacketed heat exchanger for warming the perfusate and trapping bubbles; and a jacketed organ chamber to keep the kidney's surrounding environment at 37 °C. The Hugo Sachs system was modified in five ways. First, the plexiglass framework that came with the system was eliminated and replaced with a custom-built aluminum framework that could withstand ultraviolet irradiation. Second, the plastic inline filter holder that came with the system was eliminated and replaced with two high-pressure stainless steel filter holders (filter diameter 47 mm, filtration area, 11.2 cm^2, catalog number c-06644–50, Cole-Palmer, Vernon Hills, IL, USA; glass fiber filters, size 0.7 μm, catalog number GF7547MM, Advantec MFS, Inc., Pleasanton, CA, USA) that could be autoclaved and that provided a greater surface area for filtration (to prevent back-pressure) and that were configured in parallel (also to reduce back-pressure) between the oxygenator and Windkessel. Third, the tubing that came with the system that connected the glass components was replaced with Tygon® Norprene® tubing (catalog number 72–0948; Harvard Apparatus, Holliston, MA, USA) that could withstand repeated autoclaving. Fourth, a plastic platform was inserted into the organ chamber upon which the kidney rested to prevent kinking of the renal artery and ureter. Fifth the various sections of tubing were either connected with plastic male Luer adapters (Harvard Apparatus) or metal stopcocks (Harvard Apparatus) or disposable plastic stopcocks (Cole-Palmer). The perfusate was pumped with a roller pump (model ISM834A, Ismatec, Glattbrug-Zurich, Switerland), and the jacketed devices were maintained at 37°C with a thermostatic circulator (model 1136, VWR Scientific, Niles, IL, USA). Tygon roller pump tubing (Harvard Apparatus) was used to pump perfusate from the reservoir toward the oxygenator (catalog number 731839), from the overflow of the oxygenator back toward the reservoir (catalog number 730155), from the main outflow of the oxygenator toward the Windkessel (catalog number 731836), and from the organ chamber back toward the reservoir (catalog number 730155).

Drug preparation

Leibovitz L-15 cell culture medium (Sigma, St. Louis, MO, USA) was mixed according to manufactures specifications to make 2 l, and 120 mg of penicillin was added. We used L-15 as the perfusate for four reasons. First, L-15 maintains vascular integrity and function in small skeletal arteries for 48 h [12]. Second, preglomerular microvessels are better maintained in L-15 compared with Tyrode's solution [13]. Third, L-15 maintains human renal tissue in tissue culture for up to 9 days [14]. Fourth, the pH of L-15 can be maintained at 7.4 without gassing with carbon dioxide; therefore, 100% oxygen, rather than 95% oxygen, can be used to gas the perfusate. The pH of 1 l of L-15 was adjusted to 7.4, and this solution was used during the kidney isolation procedure. The other liter was further prepared with the addition of various pharmacological agents, and then the pH adjusted to 7.4. Both solutions were filtered (0.22 μm) into 1 l containers. The solution with the experimental drug was perfused through the kidney perfusion system at a rate of 2.6 ml/min. The other solution was placed underneath a second laminar flow hood (catalog number 3740002; Labconco, Kansas City, MO, USA) where the surgery was performed.

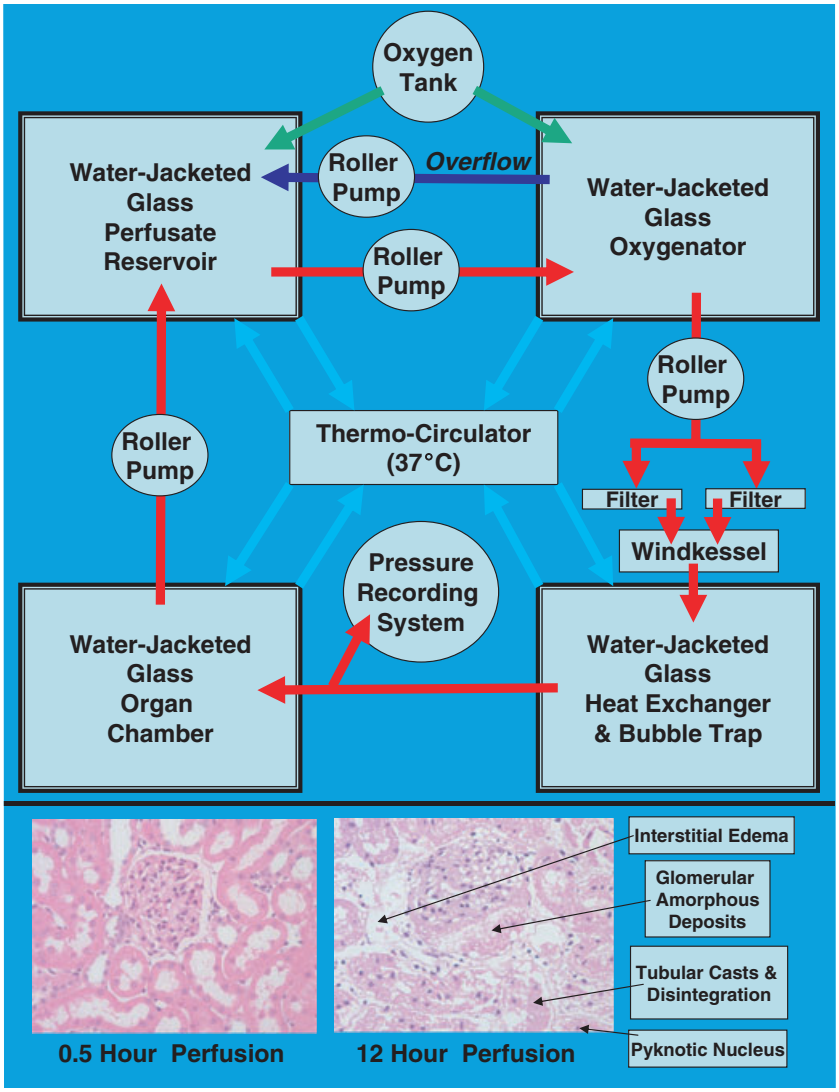

Figure 1 The top panel is a schematic diagram of the kidney perfusion system. Perfusate was pumped from a water-jacketed glass reservoir to a water-jacketed glass oxygenator. Oxygen (100%) was delivered to both the reservoir and oxygenator via an oxygen tank. Some perfusate was pumped through parallel filters, whereas excess perfusate was diverted back to the reservoir with a pump. After inline filtration, the perfusate entered a Windkessel and then a heat exchanger/bubble trap. From the heat exchanger, the perfusate entered the kidney which resided in a water-jacketed glass organ chamber. Venous effluent from the kidney was collected in a funnel and pumped back to the reservoir. Urine was collected separately and was not returned to the system. For clarity, the diagram shows four separate pumps; however, the system utilized only one pump with four channels for pumping four different lines simultaneously. All the aforementioned components resided in a sterile laminar flow hood and were sterilized by autoclaving before each experiment. A single thermo-circulator (placed under the laminar flow hood) was used to circulate warm (37 °C) water to the jackets of all jacketed glass components. Also, a pressure monitoring system was connected to the kidney inflow line to measure continuously renal perfusion pressure. The bottom panel shows hematoxylin and eosin stain of kidney sections obtained from rat kidneys perfused with the system described above. The left section shows the histology of a renal section from a kidney that was not chronically perfused, but only flushed for 30 min with perfusate. This section provides an image of 'baseline' histology of a kidney not damaged by 12 h of perfusion at 37 °C. Histology is normal except for tubular dilatation due to the short-term flush. The right section shows the histology of a renal section from kidney that was chronically perfused (12 h at 37 °C), but in the absence of mannitol or gemfibrozil. The section shows nearly complete destruction of glomerular architecture, tubular disintegration, tubular casts, pyknotic nuclei, diminished number of nuclei and interstitial edema (see notational arrows on right side of section).

Kidney isolation

Kidneys were isolated from adult male Sprague-Dawley rats under a laminar flow hood (Labconco). Animals were anesthetized with Inactin (90 mg/kg, ip), the abdomen was shaved, washed with 75% alcohol, and covered with autoclaved towels. A sterile catheter was assembled, placed in a roller pump and filled with L-15. The catheter

consisted of three sequentially smaller sections, the middle of which was the pump tubing (Harvard Apparatus). The smallest of the sections (consisting of PE-50 tubing) was for insertion into the renal artery. The largest size tubing was placed into the 1 l of room temperature L-15. The animal's left kidney was exposed, and the ureter was cleaned and cannulated with a short section of sterile PE-10 tubing connected to a longer section of sterile PE-50 tubing. All side branches of the renal artery and veins were ligated and cut. The aorta, vena cava, renal artery and renal vein were dissected free, and ligatures were loosely placed around these vessels. The infra-renal aorta was ligated, a small clamp was placed on the aorta just above the ligation but below the renal artery, a cut was made between the clamp and ligation, and the PE-50 tubing was inserted, secured with a ligature and advanced into the left renal artery. The roller pump was activated to perfuse the kidney with room temperature L-15 at 5 ml/min. The supra-renal aorta and renal vein were immediately ligated and severed. The kidney, now completely isolated from the cardiovascular system of the animal, was freed from surrounding tissue, placed in sterile saline solution in a sterilized Petri dish, and transferred to the perfusion apparatus within seconds of interrupting flow. The ureter catheter was directed to a urine collection system. The kidney was then perfused for 12 h at 37 °C with L-15 gassed with 100% oxygen. Throughout the 12-h experiment, perfusion pressure was time-averaged every 5 min using a Digi-Med (Louisville, KY, USA) model BPA digital pressure measuring system, and pressure data were captured with a computer running the Digi-Med software package.

Treatments

To identify possible pharmacological agents for organ preservation, we screened ($n = 1$–2) a number of pharmacological agents including: decylubiquinone (16 mg/l; mitochondrial transition pore inhibitor); Z-VAD (2.5 mg/l; caspase inhibitor); gemfibrozil and WY-14643 (50 and 16 mg/l, respectively; PPARα agonists); trolox, luteolin and quercetin (2.5 g/l, 3 mg/l and 3 mg/l, respectively; antioxidants); recombinant hepatocyte growth factor, recombinant platelet-derived growth factor-BB, recombinant epidermal growth factor, recombinant fibroblast growth factor-basic, recombinant vascular endothelial growth factor, recombinant insulin-like growth factor-1 and transferrin (5 μg/l, 5 μg/l, 10 μg/l, 25 μg/l, 10 μg/l, 10 μg/l and 5 mg/l, respectively; growth factors); Z-Val-Phe-CHO (4 mg/l; calpain inhibitor); W7 (50 mg/l; calmodulin inhibitor); minoxidil and its active metabolite, minoxidil sulfate (20 mg/l and 25 mg/l, respectively; K_{ATP} openers); 3-aminobenzamide [680 mg/l; inhibitor of poly (ADP-ribose) polymerases]; verapamil (50 mg/l; calcium channel blocker); DDAVP (1 μg/l; selective V_2 agonist); acetazolamide, hydrochlorothiazide, furosemide and mannitol (22 mg/l, 30 mg/l, 33 mg/l and 20 g/l, respectively; diuretics); L-165041 (241 μg/l; peroxisome proliferator-activated receptor-beta agonist); dopamine (10 mg/l; dopamine receptor agonist); linolenic acid (14 mg/l; essential fatty acid); β-NAD (340 mg/l; involved in energy metabolism); urea and uric acid (300 mg/l and 40 mg/l, respectively; normally exist in high concentrations in renal interstitium); and aldosterone (1 μg/l, respectively; hormone). Both PPARα agonists and mannitol appeared to provide moderate histological preservation as assessed by light microscopy, and aldosterone, urea, uric acid, 3-aminobenzamide and minoxidil sulfate demonstrated mild protection. The remaining agents either had no effect or worsened the histological outcome.

Because PPARα agonist showed the most promising results and because mannitol is known to enhance organ preservation, we investigated in a formal protocol the protecting effects of PPARα agonists in comparison to mannitol as the 'gold-standard' for preservation. Also, because mannitol is used in some renal preservation solutions to which could be added a PPARα agonist, we also investigated the efficacy of mannitol combined with a PPARα agonist. Accordingly, we randomized 12 kidneys for treatment with L-15 alone (damaged control; Group A) or L-15 containing mannitol (20 g/l; Sigma; Group B), gemfibrozil (50 mg/l; Sigma; Group C) or gemfibrozil + mannitol (50 mg/l and 20 g/l, respectively; Group D). These kidneys were perfused as described above and processed for light microscopy, electron microscopy, Apoptag staining, and Western blotting. An additional three kidneys were treated with WY-14643 (16 mg/l; Biomol, Plymouth Meeting, PA, USA) and examined by light microscopy only.

Collection of kidneys

A timed urine collection was performed at the beginning and end of each perfusion experiment, and urine was also collected throughout the 12 h of perfusion. After 12 h, the kidney was removed and rapidly sectioned into three pieces. One piece was placed in 10% buffered formalin for light microscopy and Apoptag staining, another slice was placed in Karnovsky's fixative for electron microscopy, and the third section was rapidly frozen and stored at −80 °C for Western blot analysis.

Light microscopy

The portion of kidney stored in 10% buffered formalin was processed and embedded in paraffin. Two-μm thick

sections were cut and stained with hematoxylin–eosin. Specimens were examined on a Nikon 50i compound microscope, and images were captured using a color digital camera (RT-KE Slider F Mount Camera) that was controlled by a computerized imaging system (SPOT RT-RE Camera Controller and Board) (all microscope and accessories from Fryer Company, Huntley, IL, USA).

Electron microscopy

Separate 1 mm^3 pieces of fresh rat kidney tissue were placed in Karnovsky's fixative, rinsed in buffer, post-fixed in osmium tetroxide, processed and embedded in Epon. Thin sections were cut on copper grids using an ultramicrotome, stained with uranyl acetate and lead citrate and examined on a FEI/Philips CM12 electron microscope (Eindhoven, The Netherlands). Digital images were obtained using an AMT digital camera (Danvers, MA, USA).

Apoptag staining

Five-micron sections were cut from the paraffin-embedded tissue blocks, and these sections were heated in an oven (55–58 °C for 45 min), deparaffinized and hydrated. A commercial ApopTag Peroxidase *In situ* Apoptosis Detection Kit (Chemicon International, Temecula, CA, USA) was used to detect apoptotic cells. Detection of apoptosis is based on the TUNEL method and the use of an anti-digoxigenin antibody that is conjugated to a peroxidase reporter molecule. Apoptotic cells showed brown nuclear staining on microscopic examination.

Western blotting

Tissues were homogenized in lysis buffer (Tris–HCl, 2% SDS, glycerol, PMSF and protease inhibitors). Protein concentrations were measured using the BCA protein assay. The proteins in whole homogenates were solubilized at 60 °C for 15 min in Laemmli sample buffer. SDS-PAGE was performed on gradient polyacrylamide gels (4–12%) loaded with 20 μg protein per lane. For immunoblotting, proteins were transferred electrophoretically to PVDF membranes. Membranes were blocked in 5% milk for 2 h, probed overnight at 4 °C with primary antibody (anti-Na^+-K^+-ATPase-α_1, 1:5000, Chemicon) in PBS containing 1% milk. Membranes were probed with anti-β-actin (1:10 000, Sigma) for 1 h to determine loading efficiency. Subsequently, membranes were exposed to a secondary HRP-conjugated donkey anti-rabbit polyclonal antibody (1:5000, Pierce Biotechnology Inc., Rockford, IL, USA) in PBS containing 1% milk for 1 h at room temperature. Bound antibodies were visualized using a luminol-based enhanced chemiluminescence substrate (SupersignalWest Dura Extended Duration Substrate, Pierce) before exposure to X-ray film (Kodak 165–1579; Eastman Kodak Co., Rochester, NY, USA). Densitometric analysis was performed using ImageQuant TL (Amersham Biosciences, Piscataway, NJ, USA) and band densities were normalized to β-actin.

Statistics

Data were normally distributed and were analyzed with a 1-factor or 2-factor analysis of variance using NCSS 2004 statistical package (Kaysville, UT, USA). $P < 0.05$ was the criterion of significance.

Results

Some kidneys were not perfused chronically but were only briefly perfused (30 min) with L-15 and then processed for light microscopy. Figure 1 shows the typical histology of such a kidney and provides a standard for the very best achievable histological picture for a 12-h perfused kidney. The histological image of a merely 'flushed' kidney is normal with the exception of tubular dilatation caused by the flushing.

Figure 1 also shows the typical histology of kidneys perfused for 12 h at 37 °C with L-15 gassed with 100% oxygen. Twelve-hour perfusion resulted in nearly complete destruction of the renal architecture. Bowman's space and the glomerular capillary loops were collapsed and/or filled with an amorphous substance, the tubules were fragmented and occluded with cellular debri/casts, tubular epithelial cells were detached from basement membrane, nuclei were pyknotic and the interstitial space was edematous.

Figure 2 shows representative light microscopy results from all 12 kidneys randomized to L-15 alone (Group A), mannitol (Group B), gemfibrozil (Group C) and gemfibrozil + mannitol (Group D). As is clearly evident from the images from Group A, 12 h of perfusion obliterated the glomerular, tubular and interstitial renal architecture in all three kidneys perfused with L-15 alone. At the light microscopic level, histology was modestly improved by mannitol (Group B) or gemfibrozil (Group C) alone, but was markedly improved by the combination of gemfibrozil + mannitol (Group D). In this regard, in the combination group Bowman's space and capillary lumens were observable, tubules were mostly intact, few epithelial cells were detached from the underlying basement membrane, cells appeared less pyknotic and the interstitium contained much less edema. However, the histological picture did not achieve that in the 30-min flushed kidney (Fig. 1) and damage was still evident.

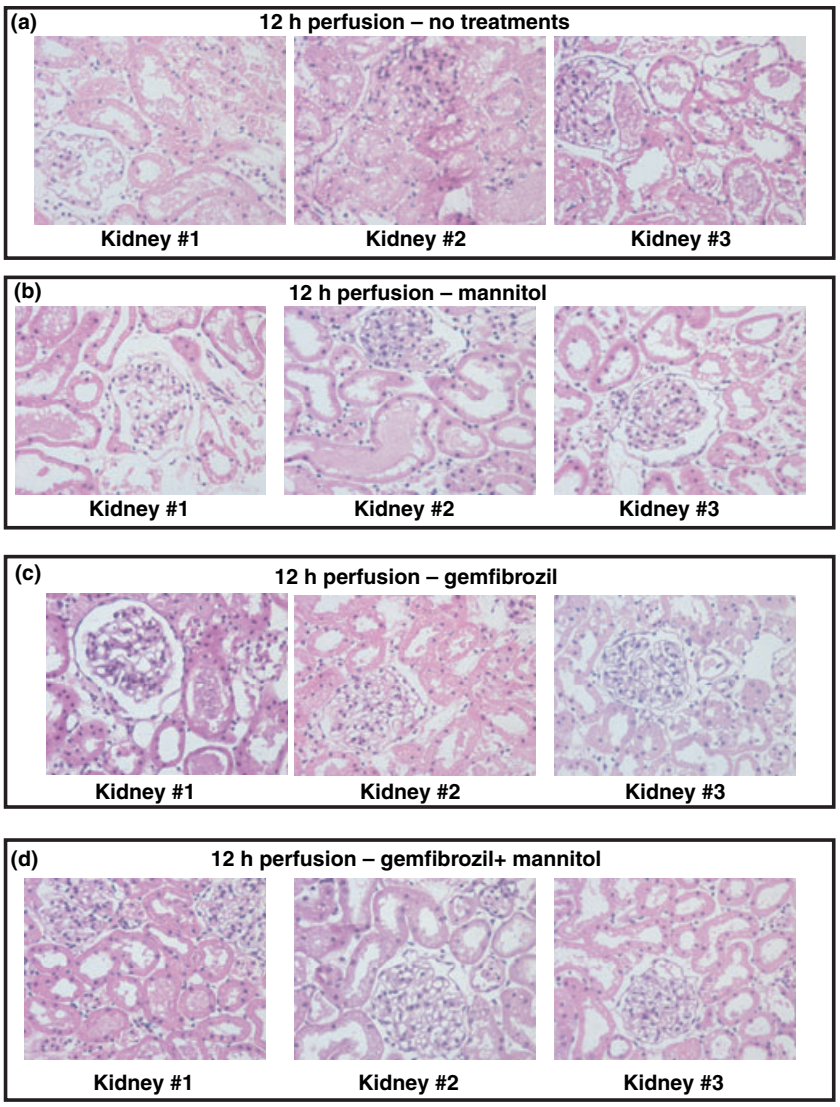

Figure 2 Hematoxylin and eosin stain of kidney sections obtained from isolated, perfused rat kidneys chronically perfused (12 h at 37 °C) in the absence (a) and presence of mannitol (b), gemfibrozil (c) or both (d). In all three untreated kidneys, there was nearly complete destruction of glomerular architecture, tubular disintegration, tubular casts, pyknotic nuclei, diminished number of nuclei and interstitial edema. Histology was slightly better preserved in the mannitol-treated and gemfibrozil-treated kidneys, and was much better preserved in the kidneys treated with both mannitol and gemfibrozil.

In an attempt to semi-quantify the effects of mannitol, gemfibrozil and gemfibrozil + mannitol, a histology scoring system was devised in which 0 represented histological damage that was so severe that the micro-structure was unrecognizable and 10 represented the histology evident in a 30-min flushed kidney. A score was then assigned by a blinded observer that considered the extent and severity of all of the aforementioned histopathology. As shown in Fig. 3, mannitol's effect *per se* did not achieve statistical significance. However, gemfibrozil statistically significantly improved the histological score in both non-mannitol-treated and mannitol-treated kidneys. However, the effects of gemfibrozil were statistically significantly greater in mannitol-treated kidneys than in non-mannitol-treated kidneys.

Four kidneys, one from each group, were randomly selected for electron microscopic studies. Figure 4 shows glomerular ultrastructure. At the electron microscopic level, in the L-15 alone kidney (Group A), Bowman's space was filled with cellular detritus, capillary loops were obliterated, and visceral epithelial cells were absent. Glomerular ultrastructure appeared slightly improved in both the mannitol-treated (Group B) and gemfibrozil-treated (Group C) kidneys and was markedly improved in the mannitol + gemfibrozil-treated kidney (Group D). The combination treatment resulted in many glomeruli with open capillaries, a Bowman's space with little cellular detritus, a covering of visceral epithelial cells, unfragmented podocytes with foot processes extending to the glomerular basement membrane and endothelial cells with large non-condensed nuclei.

As shown in Fig. 5, the proximal tubular epithelial structures were destroyed by 12 h of perfusion with L-15 alone (Group A). In this regard, no brush border was

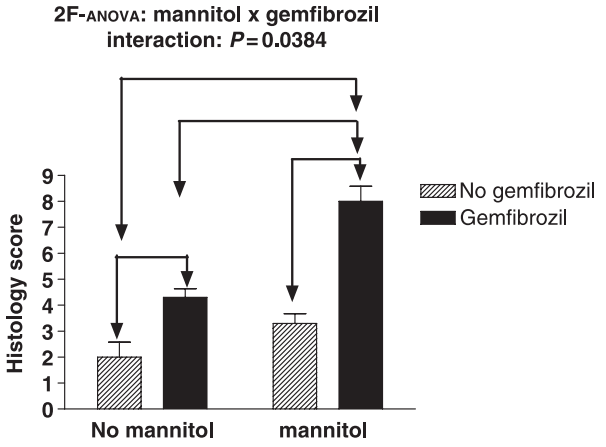

Figure 3 Histology scores in kidney sections obtained from isolated, perfused rat kidneys chronically perfused (12 h at 37 °C) in the absence and presence of mannitol, gemfibrozil or both. Scoring was calibrated with 0 being complete destruction of glomerular and tubular architecture and 10 being a histological pattern similar to kidneys flushed only for 30 min. Arrows connect statistically different groups ($P < 0.05$, Fishers' Least Significant Difference test).

evident, tubular epithelial cells were unrecognizable and the tubular lumen was filled with amorphous casts. This severe histopathology was evident throughout the observed section, with no areas showing preservation. Tubular ultrastructure was only marginally better in mannitol-treated (Group B) and gemfibrozil-treated (Group C) kidneys. Notably, in the gemfibrozil + mannitol-treated kidney (Group D), ultrastructure was highly preserved in many, but not all, tubules. As shown in Group D, proximal tubular epithelial cells with normal brush border and tight junctions, large and numerous mitochondria and a normal basement membrane were evident.

The light and electron microscopic images provided supporting evidence for preservation by gemfibrozil, particularly when combined with mannitol. To test the hypothesis of gemfibrozil-induced preservation using an objective criterion, we examined by Western blotting and densitometry the expression of Na^+-K^+-ATPase-α_1. Na^+-K^+-ATPase provides the driving force for tubular reabsorption in renal epithelial cells and therefore is richly expressed in the basolateral membrane of all renal epithelial cells. Damage of epithelial cells would be expected to be associated with a reduction in the expression of this protein. As shown in Fig. 6, Na^+-K^+-ATPase-α_1 was strongly expressed in normal (freshly isolated) kidneys. However, Na^+-K^+-ATPase-α_1 expression was nearly undetectable in kidneys perfused with L-15 alone and in mannitol-treated kidneys, but was slightly higher in gemfibrozil-treated kidneys. Importantly, Na^+-K^+-ATPase-α_1 was statistically, significantly greater in gemfibrozil + mannitol-treated kidneys compared with kidneys treated with L-15 alone, mannitol alone or gemfibrozil alone. Although gemfibrozil + mannitol markedly increased Na^+-K^+-ATPase-α_1 expression, the combination treatment did not normalize the expression of Na^+-K^+-ATPase-α_1.

In our model system, we used a renal perfusion flow rate that was low but within the normal limits for an adult rat. This lower flow rate allowed the addition of mannitol to the L-15 without unacceptable back-pressure behind the 0.7 μm inline filters. Because of the low flow rate and low viscosity of L-15 relative to blood, the initial perfusion pressure in L-15 alone kidneys was approximately 50 mmHg, increased rapidly and within 30 min to approximately 65 mmHg, and thereafter changed little (Fig. 7). In kidneys receiving mannitol, either alone or with gemfibrozil, initial renal perfusion pressure was lower (approximately 25 mmHg). The rapid increase in perfusion pressure observed in L-15 alone kidneys was delayed by gemfibrozil, more so by mannitol and even more by the combination of gemfibrozil with mannitol (Fig. 7).

Figure 8 illustrates the effects of mannitol, gemfibrozil and gemfibrozil + mannitol on initial urine flow rate, terminal urine flow rate and total urine volume. Both gemfibrozil and mannitol increased total urine volume; however, the diuretic effects of mannitol were greater than those observed with gemfibrozil, and the diuretic actions of gemfibrozil + mannitol were less than those observed for mannitol *per se*. Thus there was a statistically significant interaction between gemfibrozil and mannitol on total urine volume. Similar trends were observed with respect to the initial and terminal urine flow rates; however the interaction did not achieve statistical significance with the terminal urine flow rates and none of the treatments significantly altered terminal flow rates.

As shown in Fig. 9, staining for apoptosis with Apoptag revealed diffuse areas of apoptosis in L-15 alone kidneys (Group A). Mannitol (Group B) and gemfibrozil (Group C) reduced the degree of apoptosis, and the combination (Group D) appeared to be even more effective in this regard.

An additional three kidneys were perfused with the potent PPARα agonist WY-14643 and compared with the L-15 alone group (Fig. 10). At the light microscopic level, histological preservation was improved by WY-14643 compared with L-15 alone. These results extended the findings with gemfibrozil to a structurally different PPARα agonist, thus supporting the conclusion that the preserving effects of gemfibrozil are shared by the pharmacological class of PPARα agonists.

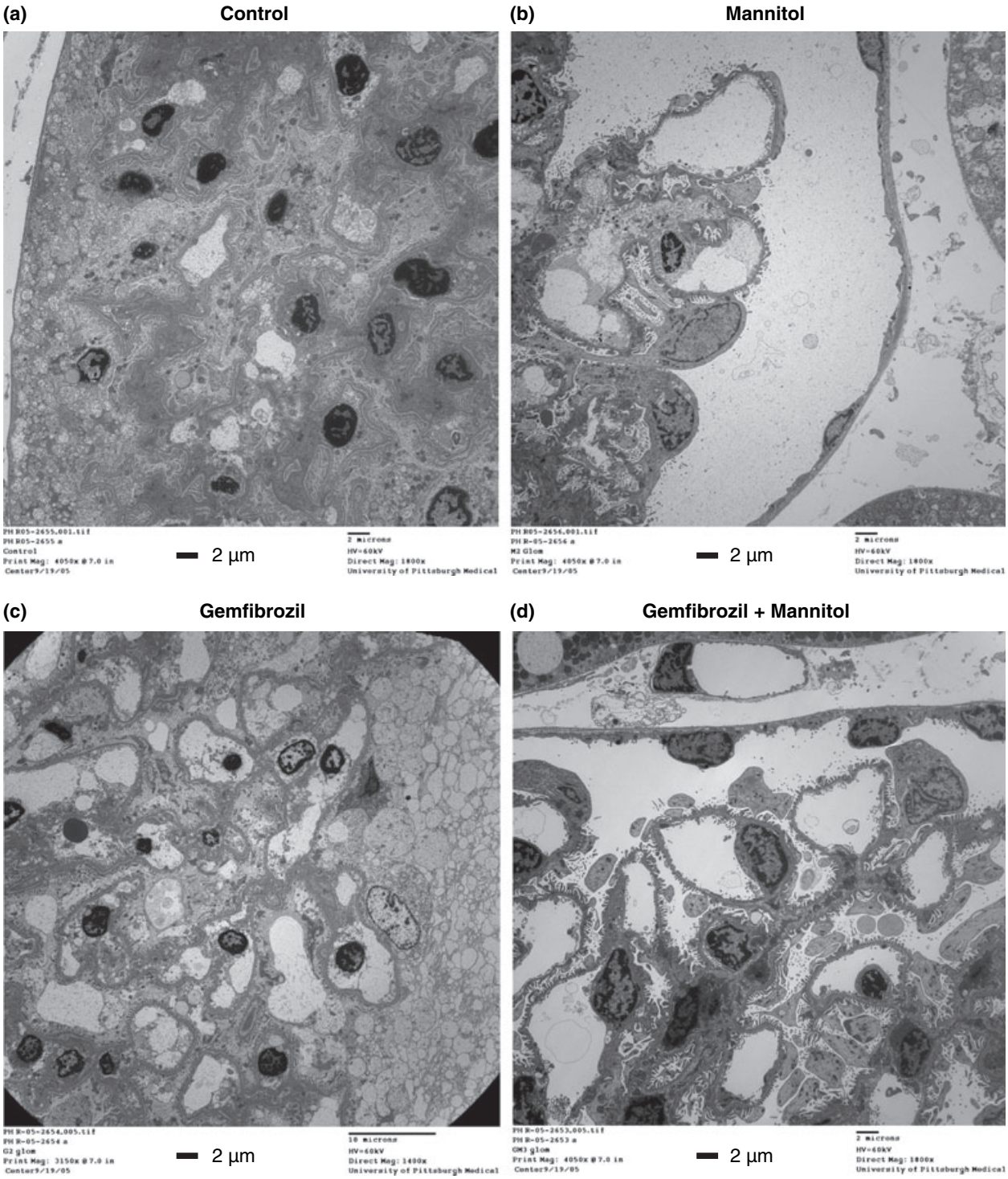

Figure 4 Electron micrographs of glomeruli obtained from isolated, perfused rat kidneys chronically perfused (12 h at 37 °C) in the absence (a) and presence of mannitol (b), gemfibrozil (c) or both (d). Note the nearly complete destruction of glomerular architecture in the untreated glomerulus (a) and the near normal glomerular structure in the mannitol + gemfibrozil-treated glomerulus (d) (open Bowman's space with parietal and visceral epithelial cells intact, open capillary loops with intact endothelial cells and well-preserved foot processes). Ultrastructure in the mannitol-treated (b) and gemfibrozil-treated (c) glomeruli was improved, but not as much as the combination treatment.

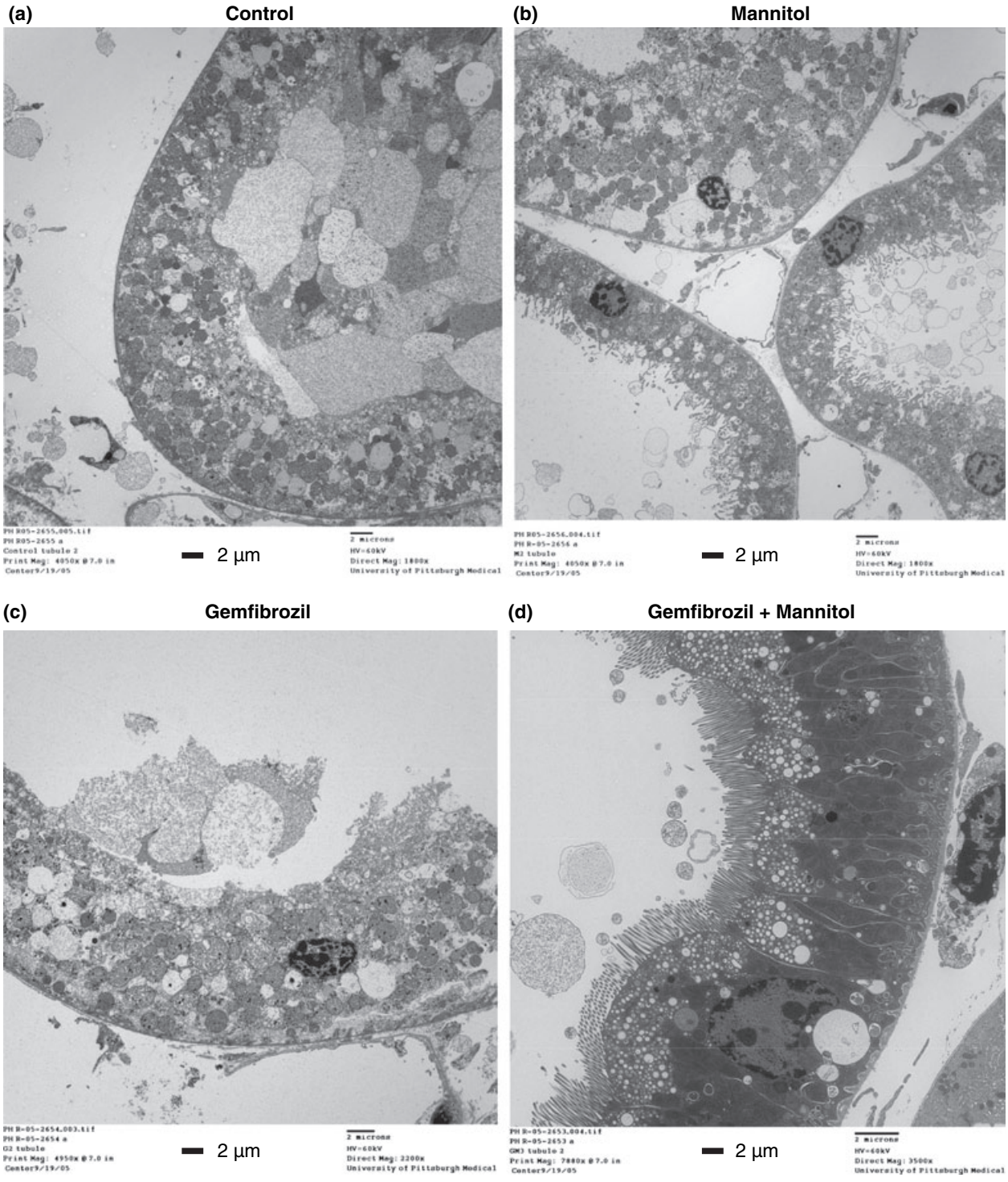

Figure 5 Electron micrographs of proximal tubular epithelial cells obtained from isolated, perfused rat kidneys chronically perfused (12 h at 37 °C) in the absence (a) and presence of mannitol (b), gemfibrozil (c) or both (d). Note the nearly complete destruction of tubular epithelial ultrastructure in the untreated (a), mannitol-treated (b) and gemfibrozil-treated (c) tubular epithelial cells, and the near normal epithelial ultrastructure in the mannitol + gemfibrozil-treated epithelial cells (d) (open lumen and intact microvilli (brush border), tight junctions, mitochondria, nuclei, vacuoles and basement membrane).

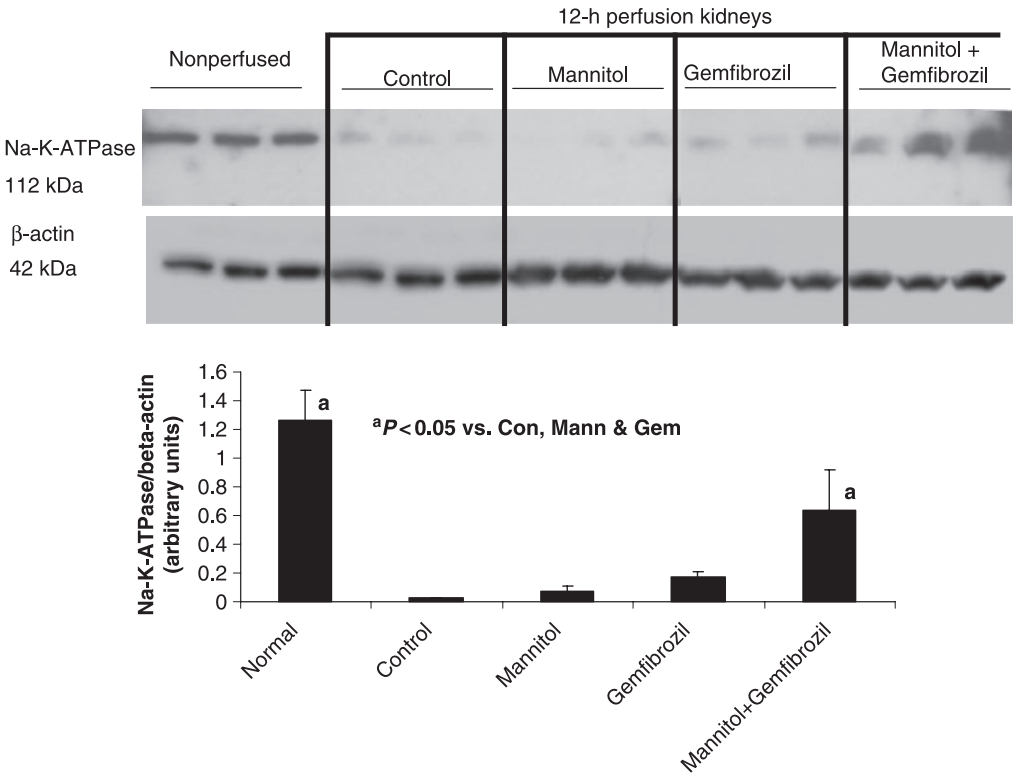

Figure 6 Western blots of Na^+-K^+-ATPase-α_1 in protein samples obtained from isolated, perfused rat kidneys chronically perfused (12 h at 37 °C) in the absence and presence of mannitol, gemfibrozil or both. For comparison, non-perfused kidneys were included. Top panel shows blots and bottom panel illustrates densitometry results. *P*-values are from Fisher Least Significant Difference text. Na^+-K^+-ATPase-α_1 was highly expressed in non-perfused kidneys, but expression was barely detectable in 12-h control kidneys. Mannitol and gemfibrozil *per se* appeared to slightly enhance expression; however, in kidneys perfused for 12 h in the presence of mannitol + gemfibrozil expression was markedly increased relative to either 12-h control kidneys or relative to kidneys treated with either mannitol or gemfibrozil alone.

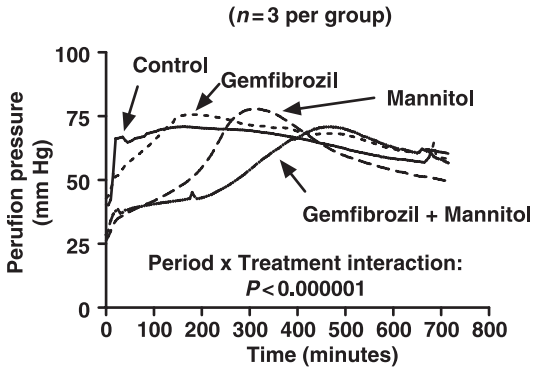

Figure 7 Perfusion pressure in isolated, perfused rat kidneys chronically perfused (12 h at 37° C) in the absence and presence of mannitol, gemfibrozil or both.

Discussion

The goal of this study was to identify new pharmacological strategies to preserve organ systems. Our approach was to develop a method for chronically perfusing rat kidneys under normothermic conditions *in vitro* without the complication of infection, and then use this model system to screen existing pharmacological agents for efficacy using histology as the outcome measure. We selected normothermic, rather than hypothermic, perfusion in order to accelerate the biochemical processes leading to organ damage so that agents could be screened more rapidly. We screened a number of drug classes, and nearly all of the results were disappointing, with little or no improvement or a worsening of histology with the majority of agents. However, screening studies with mannitol and PPARα agonists provided encouraging results, so we conducted a detailed examination of these two agents. The results of this more detailed study were consistent with our preliminary screening study and indicate that PPARα agonists preserve organs and that the efficacy of PPARα agonists in this regard is improved by combining PPARα agonists with mannitol.

Our observation that mannitol *per se* provides some, albeit marginal, benefit in the 12-h perfused rat kidney

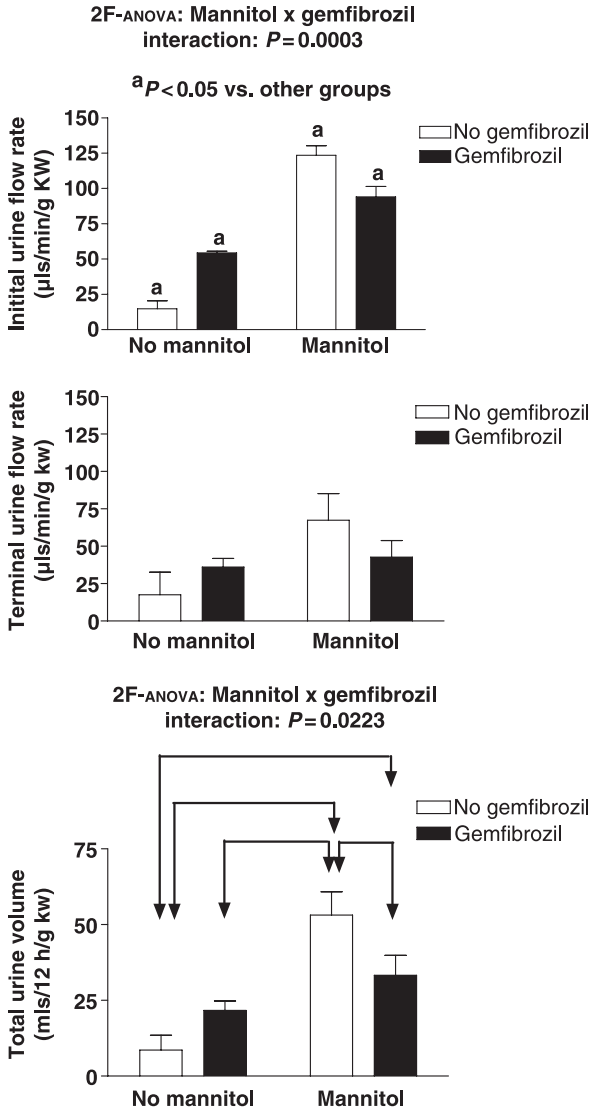

Figure 8 Urine flow rates at the beginning and end of perfusion and over the entire 12-h period in isolated, perfused rat kidneys chronically perfused (12 h at 37 °C) in the absence and presence of mannitol, gemfibrozil or both. Arrows connect statistically different groups ($P < 0.05$, Fishers' Least Significant Difference test).

model is not novel, and this finding is entirely consistent with an extensive literature regarding mannitol as a cell-impermeable solute in organ preservation [3–9]. For example, administration of mannitol to human donors prior to interruption of flow for harvesting the organ reduces postoperative acute tubular necrosis [5]. Similarly, flushing and storing rat kidneys in a mannitol-containing solution increases functional outcomes in transplantation studies [7], and perfusing human donor kidneys with a mannitol solution decreases the incidence of acute renal failure [8]. Flushing with a mannitol solution improves histology in rat kidneys stored under cold ischemia [9], and cold storage in a mannitol-containing solution improves cortical hemoglobin oxygenation in a rabbit renal transplant model [10]. Indeed, a flushing-storage solution containing mannitol as the cell-impermeable solute is patented [11], and mannitol-containing organ preservation solutions are widely employed [1]. Although our findings with mannitol are not novel, the consistency between the present findings and previous reports of efficacy with mannitol (and cell-impermeable solutes in general) supports the predictability of our model system and the validity of extrapolating findings in our model system to organ preservation in other model systems and humans.

The present study strongly suggests that PPARα agonists provide organ preservation and that there is a striking synergy between PPARα agonists and mannitol in this regard. To our knowledge, both these observations are novel findings. An exhaustive Medline search and patent database search did not uncover any prior publications regarding the organ preserving efficacy of PPARα agonists, either alone or in combination with mannitol. Therefore, the present findings represent a potentially important advance in the development of solutions for organ preservation.

When given chronically to patients with dyslipidemias, PPARα agonists, such as gemfibrozil, lower plasma triglycerides and increase high-density lipoprotein cholesterol levels. Consequently, PPARα agonists are widely prescribed for post-transplant hyperlipidemia and appear entirely safe in such a setting [15–18]. In fact, therapy with PPARα agonists may provide renoprotection from the damaging effects of chronic dyslipidemia [19]. Therefore, it is highly likely that the addition of gemfiborzil or other PPARα agonists to organ preservation solutions would carry little, if any, risk of adverse effects to the transplanted organ or its recipient.

Peroxisome proliferator-activated receptor-alpha is a ligand-activated transcriptional factor that is a member of the nuclear receptor family [20]. PPARα modulates the expression of multiple genes that participate in fatty acid metabolism including fatty acid transport protein, acyl-coA synthetase, fatty acid binding protein, medium chain acyl-coenzyme A dehydrogenase, acyl-coA oxidase, cytochrome P450 fatty acid ω-hydroxylase and carnitine palmitoyl transferase I [20]. It is conceivable that the organ-preserving effects of PPARα agonists are related to enhancing the utilization of fatty acids as an alternative energy source in organs with a reduced energy supply. Further studies are required to address this issue. Also, PPARα agonists increase the expression of nephrin by glomerular podocytes [21]. Importantly, very recent studies by Baker *et al.* [22] have identified in blood high

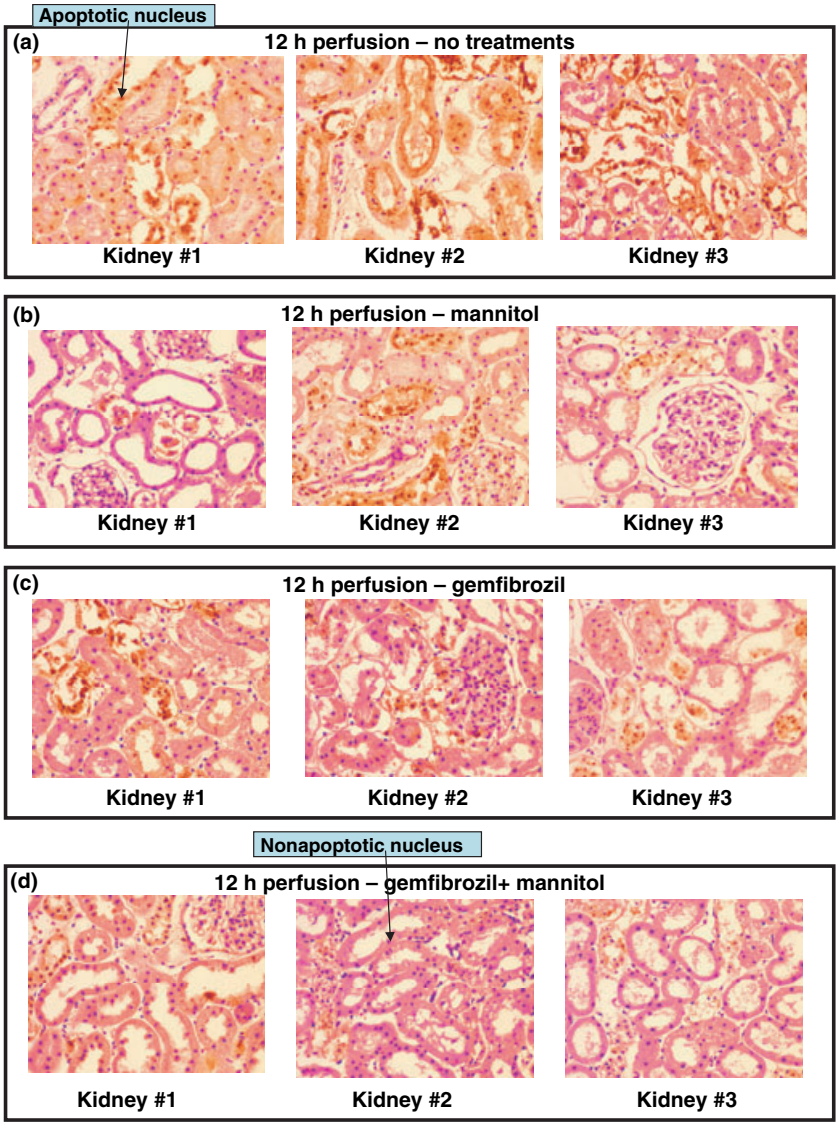

Figure 9 Apoptag stain of kidney sections obtained from isolated, perfused rat kidneys chronically perfused (12 h at 37 °C) in the absence (a) and presence of mannitol (b), gemfibrozil (c) or both (d). Reddish-brown and blue nuclei represent positive and negative staining for apoptosis, respectively. In control kidneys, many of the nuclei stained reddish-brown, whereas in the mannitol + gemfibrozil-treated kidneys, most nuclei stained blue.

concentrations of nitroalkene derivatives of unsaturated fatty acids that are potent endogenous activators of PPARs, including PPARα. Thus, it appears that *in vivo* organs are continually exposed to high concentrations of PPAR activators. It is conceivable that the absence of endogenous PPAR activators in the preservation solution is in part why isolated organs rapidly deteriorate.

The results of the present studies have several important implications. The addition of PPARα agonists to organ preservation solutions could extend the time for extracorporeal survival of grafts prior to transplantation, decrease the incidence of primary graft dysfunction or delayed function and augment the pool of available donors. Because PPARα agonists synergize with mannitol, the benefit derived from the addition of PPARα agonists may be particular efficacious with preservation solutions containing mannitol, such as Celsior and Bretschneider HTK solution [1]. Moreover, recent years have seen the development of innovative devices, such as the Life Port® Transporters developed by Organ Recovery Systems (Des Plaines, IL, USA), that provide constant, normothermic perfusion of organs during transport to the organ recipient. Addition of PPARα agonists to the solutions of such systems may increase the recovery of organs from marginally acceptable donors and thereby expand the available supply of organs for transplantation. In addition to improving organ preservation for transplant, it is conceivable that treatment of acute renal failure patients with a combination of mannitol and PPARα agonists could improve clinical outcomes in such patients. Although mannitol is used for the treatment of acute renal failure, mannitol *per se* does not improve outcome [2]. It will be

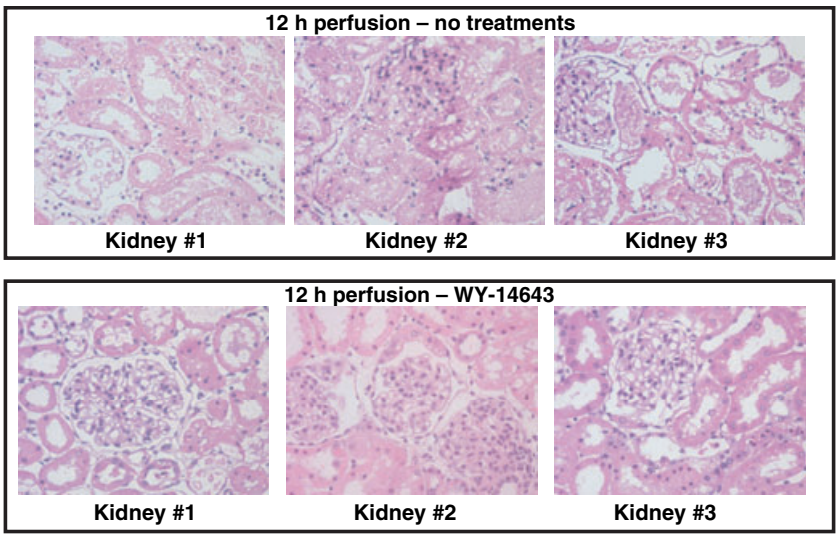

Figure 10 Hematoxylin and eosin stain of kidney sections obtained from isolated, perfused rat kidneys chronically perfused (12 h at 37 °C) in the absence and presence of WY- 14643 showing protective effects of WY-14643 (glomerular and tubular architectures were preserved).

important to test this concept *in vivo*. Finally, protecting drugs, if sufficiently efficacious, could allow for the prolonged *in situ* perfusion of organ systems with solutions to restore organ function or to deliver selectively other drugs or molecular biological constructs (plasmids, viral vectors, siRNAs, anti-sense oligonucleotides) directly to the target organ.

As noted above, we used normothermic perfusion to hasten organ injury and facilitate more rapid screening of potentially protective compounds. Nonetheless, it is possible that the results are applicable to hypothermic organ preservation as well because the biochemical mechanisms of injury in normothermic and hypothermic conditions likely overlap. Also, pretreatment of hypothermically maintained organs with PPARα agonists would provide high levels of PPARα activation during the critical rewarming period following transplantation. However, additional experiments are required to clarify whether PPARα agonists would protect organs preserved by cold storage and subsequently subjected to ischemic/reperfusion injury.

References

1. Nydegger UE, Carrel T, Maumonier T, Mohacsi LP. New concepts in organ preservation. *Transpl Immunol* 2002; **9**: 215.
2. Dishart MK, Kellum JA. An evaluation of pharmacological strategies for the prevention and treatment of acute renal failure. *Drugs* 2000; **59**: 79.
3. Cerilli J, Lauridsen P, Saurbrey J, Husfeldt E, Wandall HH. Effect of mannitol, saline, and urea on the function of a recently autotransplanted kidney. *Arch Surg* 1966; **92**: 178.
4. Weimar W, Geerlings W, Bijnen AB, *et al.* A controlled study on the effect of mannitol on immediate renal function after cadaver donor kidney transplantation. *Transplantation* 1983; **35**: 99.
5. Tiggeler RG, Berden JH, Hoitsma AJ, Koene RA. Prevention of acute tubular necrosis in cadaveric kidney transplantation by the combined use of mannitol and moderate hydration. *Ann Surg* 1985; **201**: 246.
6. Grino JM, Miravitlles R, Castelao AM, *et al.* Flush solution with mannitol in the prevention of post-transplant renal failure. *Transplant Proc* 1987; **19**: 4140.
7. Kline R, Churchill M, Churchill P, Bidani A, Schwartz M. High osmolality-low pH flush solutions improve renal transplant function in rats. *Urol Res* 1991; **19**: 81.
8. Lopez-Costea MA, Grino JM, Seron D, *et al.* Mannitol solution (M-400) versus mannitol solution combined with allopurinol in the prevention of post-transfusion acute renal failure. *Actas Urol Esp* 1992; **16**: 446.
9. Herrero I, Torras J, Carrera M, *et al.* Evaluation of a preservation solution containing fructose-1,6-diphosphate and mannitol using the isolated perfused rat kidney. Comparison with Euro-Collins and University of Wisconsin solutions. *Nephrol Dial Transplant* 1995; **10**: 519.
10. Lane NJ, Thorniley MS, Manek S, Fuller BJ, Green CJ. Effect of mannitol and polyethylene glycol on the action of frusemide during renal storage and transplantation. *Transplantation* 1996; **62**: 575.
11. Churchill PC, Churchill MC. *Flush-storage solution for donor organs*. United States Patent 5,834,178, 1998.
12. Bolz SS, Pieperhoff S, De Wit C, Pohl U. Intact endothelial and smooth muscle function in small resistance arteries after 48 h in vessel culture. *Am J Physiol* 2000; **279**: H1434.
13. Jackson TC, Mi Z, Jackson EK. Modulation of cyclic AMP production by signal transduction pathways in preglomer-

ular microvessels and microvascular smooth muscle cells. *J Pharmacol Exp Ther* 2004; **310**: 349.
14. Briere N. Human foetal kidney explants in serum-free organ culture. *Anat Embryol* 1987; **176**: 105.
15. Bastani B, Robinson S, Heisler T, *et al.* Post-transplant hyperlipidemia: risk factors and response to dietary modification and gemfibrozil therapy. *Clin Transplant* 1995; **9**: 340.
16. Vergoulas G, Miserlis G, Solonaki F, *et al.* Combined treatment of hypercholesterolemia of renal transplant allograft recipients with fluvastatin and gemfibrozil. *Transpl Int* 2000; **13**(Suppl 1): S64.
17. Kasiske BL, Heim-Duthoy KL, Singer GG, Watschinger B, Germain MJ, Bastani B. The effects of lipid-lowering agents on acute renal allograft rejection. *Transplantation* 2001; **72**: 223.
18. Chan TM, Cheng IK, Tam SC. Hyperlipidemia after renal transplantation: treatment with gemfibrozil. *Nephron* 1994; **67**: 317.
19. Izzedine H, Launay-Vacher V, Baumelou A, Deray G. Renal effects of PPARα-agonists. *Minerva Urol Nefrol* 2004; **56**: 339.
20. van Raalte DH, Li M, Pritchard PH, Wasan KM. Peroxisome proliferator-activated receptor (PPAR)-α: a pharmacological target with a promising future. *Pharm Res* 2004; **21**: 1531.
21. Ren S, Xin C, Beck KF, *et al.* PPARα activation upregulates nephrin expression in human embryonic kidney epithelial cells and podocytes by a dual mechanism. *Biochem Biophys Res Commun* 2005; **338**: 1818.
22. Baker PRS, Lin YM, Schopfer FJ, *et al.* Fatty acid transduction of nitric oxide signaling: Multiple nitrated unsaturated fatty acid derivatives exist in human blood and urine and serve as endogenous peroxisome proliferator-activated receptor ligands. *J Biol Chem* 2005; **280**: 42464.