Protective effects of exogenous bilirubin on ischemia-reperfusion injury in the isolated, perfused rat kidney

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HEME OXYGENASE (HO) is an enzyme that serves as the sole pathway for degradation of the heme molecule into iron, carbon monoxide (CO), and biliverdin (32, 51, 52). Biliverdin is then converted into bilirubin by biliverdin reductase. Three isoforms of HO have been described. HO-1 is an inducible form that has cytoprotective functions and is contained in the endoplasmic reticulum throughout all tissues (32). HO-2 and HO-3 are constitutive and are believed to serve as physiological regulators of normal function (24, 32). Research has focused on the protective activity of the HO-1 enzyme, which is induced by various injurious stimuli (reviewed in Refs. 2 and 49). One important protective action of HO-1 is in degradation of the heme molecule itself, which has prooxidant effects through the generation of free radicals and lipid peroxidation (7, 8, 23, 54). However, recent attention has focused on the beneficial actions of the eventual products of heme degradation, CO and bilirubin, in clinically relevant models of ischemia-reperfusion injury (IRI).

Experimental models have demonstrated that HO-1 activity is induced in response to IRI in the heart, brain, liver, and intestine and that the antioxidant and cytoprotective effects in these organs are mediated via bilirubin (12, 13, 16, 27). Exogenous administration of bilirubin in isolated, perfused rat heart and liver models had a protective effect against IRI at doses that ranged from 0.05 to 10 μM and the efficacy of this protective effect was improved by administration of bilirubin before or during the ischemic event (13, 27). Although other studies demonstrated the protective effects of HO-1 activity during ischemia- or toxin-induced injury in the kidney, it is not clear whether these effects are due to increased antioxidant activity through production of bilirubin or by CO-mediated vasodilation (1, 24, 33, 34, 37, 57). Interestingly, one recent study demonstrated that bile duct ligation-induced cholestasis is protective against glycerol-induced renal injury in a rat model, suggesting that bilirubin may play a significant role in renal IRI (28). Thus, although its protective effects have been demonstrated in other organs, no studies have definitively evaluated the effects of exogenous bilirubin on IRI in the rat kidney. The objectives of our study were to investigate the activity of exogenous bilirubin in the prevention of IRI using an isolated, perfused rat kidney (IPRk) system. Our hypotheses were that exogenous bilirubin would have a protective effect when administered before IRI and that this effect would be dose dependent.

METHODS

Animals. This study was approved by the University of Florida Institutional Animal Care and Use Committee and was performed in accordance with the Institute for Lab Animal Research Guide for the Care and Use of Laboratory Animals. Eighteen male Sprague-Dawley rats weighing between 350 and 400 g were used. Animals were euthanized by an overdose of pentobarbital sodium at the conclusion of the experiment.

Reagents. Bilirubin, bovine serum albumin, creatinine, 50× amino acid concentrate, sodium chloride (NaCl), potassium chloride (KCI), sodium phosphate (Na2HPO4), sodium bicarbonate (NaHCO3), calcium chloride (CaCl2), magnesium sulfate heptahydrate (MgSO4·7H2O), sodium hydroxide (NaOH), and dextrose were all obtained from Sigma (St. Louis, MO).

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**Perfusate.** Before kidney harvest, rats were randomly assigned to one of three groups. *Group 1* (*n* = 6 rats) would receive control perfusate, a modified Krebs-Henseleit buffer containing (in mM) 0.44 NaCl, 0.47 KCl, 0.17 NaHCO₃, 2.10 NaHCO₃, 0.33 CaCl₂, 0.29 MgSO₄·7H₂O, 2.90 dextrose, 6 bovine serum albumin, 0.4 creatinine, and a mixture of 20 amino acids as previously described (30). *Group 2* (*n* = 6 rats) would receive control perfusate + 1 µM bilirubin. *Group 3* (*n* = 6 rats) would receive control perfusate + 10 µM bilirubin. Immediately before the experiment, bilirubin was dissolved in 10 mM NaOH and added to the perfusate for *groups* 2 and 3. An equal volume of 10 mM NaOH was then added to the control perfusate. Perfusate was bubbled with a mixture of 95% oxygen-5% CO₂ until the pH was 7.4. All beakers and tubing were covered in aluminum foil during preparation of solutions and during the subsequent perfusion experiment to avoid degradation of bilirubin by exposure to light.

**Renal ischemia.** Rats were anesthetized using 5% isoflurane inhalant in 100% oxygen and maintained by mask administration of 2–3% isoflurane in 100% oxygen. Kidney harvest and perfusion were initiated using a modification of the methods described by Bowman (9). Briefly, a ventral midline celiotomy was performed and the viscera were exteriorized to expose the right kidney. Four-hundred units of heparin sulfate were administered into the caudal vena cava using a 25-gauge needle. The right ureter was transected 1 cm caudal to the kidney, dilated, and catheterized with a wire stylet and 22-gauge Intracath flexible catheter (Becton Dickinson, Sandy, UT) to allow urine collection. Before the stylet was removed, the catheter was secured by placing a single suture around the ureter and catheter. The pre- and postrenal vena cava and aorta were isolated using blunt dissection and a single suture was preplaced loosely around the right renal artery. The vena cava and aorta were ligated caudal to the renal artery and vein to prevent hemorrhage during cannulation. The cranial mesenteric artery (CMA) was ligated distally and a vascular clamp was placed on the prerenal section of the abdominal aorta. A 2-mm incision was made in the CMA and a 19-gauge steel cannula was inserted into the CMA and directed across the aorta, into the right renal artery. The right kidney was immediately flushed with 20 ml of control or experimental perfusate, producing a clear venous effluent. The preplaced suture was tied around the right renal artery to secure the arterial cannula and the kidney was removed from the retroperitoneal space. Renal injury was induced by maintaining the kidney at 37°C for 20 min of warm ischemia before initiating oxygenated machine perfusion.

**Isolated, perfused kidney model.** Following a 20-min period of warm (37°C) ischemia, normothermic (37°C) kidney perfusion was performed at a constant pressure of 100 mmHg for 2 h using a LifeSustainer 1000 automated organ perfusion workstation (LifeSystems, Redmond, WA) (46). Perfusate oxygen saturation was maintained at 300% using a hollow-fiber oxygenator and a mixture of 5% CO₂-95% oxygen. A total volume of 500 ml of recirculating perfusate was used for each experiment. During machine perfusion, urine was collected and quantified every 15 min. Two milliliters of perfusate were simultaneously collected at the venous outflow every 15 min. Urine and perfusate samples were stored at −20°C until the completion of the experiment and then transferred to −80°C until analyses were performed. At the conclusion of the experiment, a portion of kidney tissue was frozen to −80°C for subsequent analyses; the remainder of the kidney was preserved in 10% formalin for histological analysis.

**Assays.** Perfusate and urine creatinine assays were performed on a Hitachi 911 analyzer (Boehringer Mannheim) using a kinetic, modified Jaffe method. Perfusate and urine sodium, potassium, and chloride concentrations were measured on the same analyzer using ion-specific electrodes and internal reference solutions. Free radical activity was assessed in kidney tissue samples acquired at the completion of the 2-h perfusion period using the thiobarbituric acid reaction (TBAR), a method that is used to quantify lipid peroxidation (5). Frozen tissue samples weighing between 0.0125 and 0.0228 g were added to 100 µl of HPLC grade water and homogenized using a variable speed homogenizer (Tissue Tearor, Dremel, Racine, WI) for 60 s. Fifty microliters of this homogenate were combined with 50 µl of HPLC water to produce the 100-µl sample volume required for the assay. Each tissue sample was run twice and measurements were repeated until coefficient of variance between the two measurements was <10%. Tissue TBAR levels were expressed in nanomoles per gram of tissue protein. Tetraethoxypropane was used as the external standard.

**Organ viability.** Perfusate flow rate was recorded continuously as an assessment of vascular resistance in the isolated organ during constant pressure perfusion. Glomerular filtration rate (GFR) of the isolated kidney was estimated using exogenous creatinine clearance, and tubular function was evaluated by measuring fractional excretion of sodium. Transverse sections of the kidney taken at the level of the hylus were processed using hematoxylin and eosin staining and periodic acid-Schiff staining. Histological examination was performed by a nephropathologist blinded to the treatment group, and tubular injury was graded using the numerical scale reported by Aragno et al. (4) for IRI in the rat kidney, as follows: 0 = normal histology; 1 = slight alteration (loss of brush border, mild hydropic degeneration); 2 = mild (intensive hydropic degeneration, mild vacuolization); 3 = moderate (shrunken nuclei, intensive vacuolization); 4 = severe (necrotic/apoptotic cells, denudation/rupture of basement membranes); and 5 = necrosis (total necrosis of the tubule). Additional electron microscopy was performed in control (0 µM bilirubin) and bilirubin (10 µM)-treated kidneys to evaluate mitochondrial integrity in the medullary thick ascending limb (MTAL) following IRI.

**Statistical analyses.** Statistical calculations were performed using a computer software program (Statview, SAS Institute, Cary, NC). Comparisons of perfusate flow rate, urine output, creatinine clearance, and fractional excretion of sodium were made over time and between treatment groups using a two-way repeated-measures ANOVA. Individual ANOVAs were then performed to determine relationship between treatment groups at each time point and pairwise comparisons were performed using Fischer’s protected least significant difference. Tissue TBARs and histological scores were compared between groups using ANOVAs. A probability value of <0.05 was considered significant.

**RESULTS**

**Perfusate flow rate.** After cannulation, kidneys were flushed with control perfusate, 1 or 10 µM bilirubin, and were subjected to 20 min of warm ischemia. After induction of ischemic injury, a 2-h period of isothermic, constant pressure machine perfusion was instituted and perfusate flow rate was recorded as a measure of vascular resistance (Fig. 1, *time 0* indicates initiation of machine perfusion). A significant interaction was found between perfusate bilirubin concentration and perfusate flow over time (*P < 0.001*), suggesting that bilirubin caused a decrease in vascular resistance during reperfusion of the organ. Although bilirubin-treated kidneys had improved flow rates throughout the majority of the 2-h perfusion period, significant differences from control occurred only during the first 20 min of reperfusion. During this period, flow rate was significantly higher for kidneys treated with both 1 µM (*P < 0.001*) and 10 µM bilirubin (*P < 0.001*) compared with control perfusate, but there was no significant difference between groups treated with 1 and 10 µM bilirubin (*P = 0.09*).

**Urine output.** In three rats (1 from each group), obstruction or inadvertent dislodgement of the ureteral catheters occurred during the perfusion experiment. For the remaining 15 rats, urine output was minimal during the first 30 min after reper...
fusions and during the last 30 min of the 2-h isothermic perfusion period. As a result, data presented for urine output, and for subsequent analyses that required urine analysis (creatinine clearance and fractional excretion of sodium), represent samples obtained from 15 rats (n = 5 rats in each group) during the 45- to 90-min time period.

Significant changes in urine output were noted over time, with significantly increased urine production for kidneys perfused with 10 \( \mu \)M bilirubin compared with kidneys treated with 1 \( \mu \)M bilirubin (\( P = 0.035 \)) and to control (\( P = 0.0009 \); Fig. 2). Data suggest a dose-related response with increased urine output associated with increases in the bilirubin dose from 1 to 10 \( \mu \)M (Fig. 2).

**Creatinine clearance.** There was a significant effect of treatment on creatinine clearance over time (\( P = 0.035 \); Fig. 3). Similar to the data presented for urine output, a trend toward dose-dependent increase in creatinine clearance is evident in kidneys treated with 1 or 10 \( \mu \)M, although when analyzed at specific time points, these differences were statistically significant only at 75 min (\( P = 0.01, 0 \) vs. 10 \( \mu \)M bilirubin).

**Fractional excretion of sodium.** Fractional excretion of sodium was significantly decreased for kidneys treated with 10 \( \mu \)M bilirubin compared with control (\( P = 0.01 \); Fig. 4), suggesting improved tubular function after ischemic injury.

Treatment with 1 \( \mu \)M bilirubin also appeared to decrease fractional excretion of sodium compared with control, although this failed to reach statistical significance (\( P = 0.057 \)). No significant differences in fractional excretion of sodium were noted between kidneys treated with 1 and 10 \( \mu \)M bilirubin.

**Histological scoring.** After 20 min of ischemia and 2 h of reperfusion, histological scores of tubular injury were moderate overall, with mean scores of 2.83 \( \pm \) 0.41 for control kidneys, 2.40 \( \pm \) 0.55 for kidneys treated with 1 \( \mu \)M bilirubin, and 2.92 \( \pm \) 0.492 for kidneys treated with 10 \( \mu \)M bilirubin (Fig. 5). Although no significant effect of treatment was detected on overall histological scores of IRI using this classification system, electron microscopy of the outer medullary stripe suggested that bilirubin treatment provided a specific protective effect at the level of the MTAL. Electron microscopy revealed variable degrees of injury to the MTAL and proximal convoluted tubule, including cellular and mitochondrial swelling, luminal membrane blebbing, cytoplasmic vacuolation, and loss of brush border in all rats that were examined. However, the degree of tubular injury appeared less severe in rats treated with 10 \( \mu \)M bilirubin. Specific examination of the MTAL suggested that mitochondrial integrity (swelling and architecture of cristae) was better preserved in the rats treated with 10 \( \mu \)M bilirubin compared with control kidneys (Fig. 6).
Treatment with bilirubin had a significant effect on lipid peroxide levels after 2 h of reperfusion (P = 0.022 by repeated-measures ANOVA; Fig. 7). Pairwise comparisons between groups demonstrated that lipid peroxide levels were significantly lower for kidneys treated with 10 μM bilirubin vs. kidneys treated with 1 μM bilirubin (P = 0.0068). Although lipid peroxide levels were lowest in kidneys treated with 10 μM bilirubin, this effect was not statistically significant compared with control (P = 0.071).

DISCUSSION

The results of our study suggest that administration of bilirubin before and during IRI in the IPRK model caused significant improvements in vascular resistance, urine output, GFR, and fractional excretion of sodium. The simplicity of the IPRK model has specific advantages in studying the mechanisms of the protective effects of the products of HO-1 in the kidney compared with previous models. First, isolation of the kidney and administration of exogenous, near-physiological doses of bilirubin in an acellular perfusate were performed to minimize the potential for interactions with other chemical messengers involved in tissue injury. Previous experiments in the rat kidney using chemical inducers (e.g., hemin or hemoglobin) or inhibitors (e.g., tin or zinc protoporphyrin) of HO-1 (1, 37, 48) had been limited by the potential for interactions with other signaling systems such as nitric oxide synthase and guanylate cyclase, diminishing their ability to discern the mechanisms of action of HO-1 (19, 25). A second benefit of the current IPRK model is that it allows evaluation of the physiological effects of the products of HO-1 activity at the organ level without the potential for interactions with other organ systems or cellular messengers that are introduced by an in vivo model. Although GFR and tubular function in the IPRK do not directly approximate values measured in vivo (9, 31), the application of normothermic, oxygenated machine perfusion does maintain physiological function of the organ and thus allows for comparison of organ viability and physiological parameters between treatment groups. Vascular resistance, GFR, and other physiological parameters measured during machine perfusion in the current study have been shown to be predictors of kidney function in vivo after kidney harvest and preservation (14, 53).

The most effective dose of bilirubin for prevention of IRI is not known. One method to establish a proposed dose of exogenous bilirubin would be to approximate the physiological levels of bilirubin produced after induction of HO-1. Bilirubin production in kidney microsomes has been measured after induction of HO-1 by glycerol-induced injury (37) and IRI (48). Unfortunately, it is difficult to compare these tissue concentrations over time to the micromolar perfusate concentration of bilirubin used in our IPRK model. The HO-1 reaction results in the degradation of heme and the liberation of equimolar amounts of biliverdin, which is subsequently converted to bilirubin. Previous studies reported that in the glycerol model of acute renal injury, heme content increases in the kidney to ~10 μM within 3 h (38), a time point at which HO-1 is induced. Thus one could speculate that the activation of HO-1...
could lead to the liberation of equimolar concentrations of bilirubin. As with many previous studies (13, 18, 27, and 28), the exogenous bilirubin concentrations used in our study (0, 1, and 10 μM bilirubin) do fall within the reference range for serum bilirubin concentrations in the rat (0.0 to 0.64 mg/dl) (47). However, the most effective dose of exogenous bilirubin appears to vary widely between different organs and experimental models. For example, one study evaluated concentrations between 0.1 and 5 μM in a cell culture model with rat cardiac myocytes (18). This study indicated that 0.5 μM was the only concentration that offered a protective effect during hypoxia reoxygenation. A later study by the same group used 0.05 or 0.1 μM bilirubin in an isolated, perfused rat heart model and found that, of these two concentrations, 0.1 μM offered significant protection against IRI (13). Subsequent models of IRI in the liver (27) and kidney (28) suggested that much higher doses of bilirubin may be required to produce maximal protective effects in these organs. A clinically relevant model of rat liver transplantation similar to our study used a bilirubin rinse at doses ranging from 1 to 25 μM after 12 h of cold ischemia (27). In that study, the 5- and 10-μM doses of bilirubin produced maximal liver function following hypothermic liver preservation and transplantation. Effective concentrations of bilirubin have not been investigated in the intact kidney, but increasing the concentration of bilirubin from 2.5 to 10 μM caused a dose-dependent protective effect on renal tubular epithelial cells in vitro (28). Based on these latter two studies and consideration of the physiological range of serum bilirubin concentrations, we elected to use concentrations of 1 and 10 μM of exogenous bilirubin in the IPRK model. The data obtained in our study suggest that 10 μM bilirubin produced the most consistent improvements in vascular resistance, urine output, GFR, and tubular function after IRI in the rat kidney.

It is presumed that the protective effects of bilirubin in IRI are due to its antioxidant properties (16, 35, 50). Bilirubin has been described as the most powerful endogenous antioxidant substance in vitro (35), acting alone and complexed with serum albumin to serve as a superoxide scavenger and peroxyl radical trapping antioxidant (50). A study performed in neonatal Gunn rats documented that blood TBARs were inversely proportional to serum bilirubin levels and that it is bilirubin, not other serum antioxidants, that is responsible for the increased resistance to free radical injury in neonates (15). Although there is some difficulty in comparing clinical acute tubular necrosis in human beings to that created in various experimental models (45), it is believed that reactive oxygen species are also major contributors to the tubular injury associated with IRI and, similarly, in the IPRK (39). While the majority of studies performed in the kidney documented the beneficial effects of allopurinol, superoxide dismutase, dimethylthiourea, l-carnitine, l-arginine, and other free radical scavengers in preservation of renal function and morphology (6, 21, 22, 36, 40, 41, 43), others (20) produced contradictory results. Our results confirm the protective effect of bilirubin on renal function in the rat, although free radical activity in the tissues measured by TBARs and overall histological scoring of IRI did not correlate.
well with improved physiological function demonstrated in kidneys treated with 1 and 10 μM bilirubin. The lack of correlation between measures of tissue IRI and physiological function may be due to the fact that tissue samples were obtained at the end of the experiment, at a time when renal function had diminished precipitously and was not significantly different between control and treatment groups. Sampling of tissue during the period of maximal renal function (~60 min after initiation of machine perfusion) may be required to confirm a decrease in lipid peroxidation accompanying the improved organ function. Alternatively, lack of differences in overall histological scores noted in our study may fail to reflect the protective effect of bilirubin in the MTAL, the region of the nephron that is considered most susceptible to ischemic tubular injury in the IPRK due to the high metabolic requirements associated with ion transport (10, 11). In fact, ultrastructural examination was supportive of this hypothesis, suggesting that treatment with 10 μM bilirubin led to improved mitochondrial integrity in the MTAL after IRI in the rat kidney, which was similar to the protective effect noted by previous investigators in myocardial tissues subjected to IRI (13).

The vasoactive effects of increased HO-1 activity during IRI have been attributed to increased production of CO, an endothelium-derived vasodilator (42, 55). The beneficial vasoelastic effects of CO after IRI are accomplished through both cyclic guanosine monophosphate (cGMP)-dependent and cGMP-independent pathways (3, 44). However, little investigation has been focused on the potential vasoactive effects of bilirubin, the other reaction product of HO-1 activity. Based on the data obtained in our study, bilirubin treatment caused significant improvements in perfusate flow during the early stages of reperfusion, indicating a decrease in vascular resistance. The fact that these vasoactive effects were noted in our IPRK model in the absence of CO supplementation would suggest that micromolar concentrations of bilirubin may also contribute to decreased vascular resistance after IRI in the kidney. Further studies will be required to elucidate the mechanism of the bilirubin-mediated decrease in renal vascular resistance noted in the IPRK.

After 20 min of warm ischemia, GFR in the current model was decreased 8- to 10-fold compared with previously reported values for normothermic IPRKs (9, 31) and organ viability diminished rapidly over the 2-h perfusion period. Thus, although treatment with bilirubin significantly improved GFR, it failed to return renal function to values reported in isolated, perfused kidney circuits using similar perfusate and perfusion pressures. Two previous studies determined that rat kidneys subjected to 45 min (56) and 60 min (26) of warm ischemia experienced severe, but reversible, injury that was considered ideal for studies of IRI (56). Results of these in vivo studies of blood-perfused kidneys may not be directly comparable to the acellular perfusate and ex vivo IPRK model used in our study. In fact, it appeared that the 20-min period of warm ischemia used in our study was enough to cause severe compromise of renal function and no evidence of recovery during the 2-h reperfusion period. Renal injury may have been exacerbated in our model because we elected to avoid the use of preservation solutions or vasodilators that may have contributed to the antioxidant or vasoactive effects of the perfusate, making it difficult to evaluate the effects of bilirubin in the IPRK.

Complete recovery from renal injury would not be anticipated during the 2-h perfusion period described in our model; the reversible lesions described in previous studies performed in vivo required 2–15 days before return of renal function.

In conclusion, this study demonstrated that bilirubin administration caused significant improvements in objective measures of renal function after IRI. These findings may have future clinical applications, particularly in minimizing allograft injury suffered during organ harvest and ischemia. Compared with gene transfer or other methods currently being considered for molecular manipulation of HO-1 activity (49), bilirubin pretreatment could provide an inexpensive and simple method to minimize IRI in the kidney, particularly in the setting of organ transplantation.

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