Cytochrome P-450 as a source of catalytic iron in minimal change nephrotic syndrome in rats

HUA LIU,1 SUDHIR V. SHAH,2 AND RADHAKRISHNA BALIGA1
1Department of Pediatrics, University of Mississippi Medical Center, Jackson, Mississippi 39216; and 2Department of Medicine, University of Arkansas for Medical Sciences, Little Rock, Arkansas 72205

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Liu, Hua, Sudhir V. Shah, and Radhakrishna Baliga. Cytochrome P-450 as a source of catalytic iron in minimal change nephrotic syndrome in rats. Am J Physiol Renal Physiol 280: F88–F94, 2001.—We have recently demonstrated an important pathogenic role for glomerular catalytic iron in the puromycin aminonucleoside (PAN) induced minimal change nephrotic syndrome (MCNS). The source of this catalytic iron capable of catalyzing free radical reactions is not known. We examined the role of cytochrome P-450 (CYP) as a source of catalytic iron in a model MCNS induced by single injection of PAN to rats. Treatment of PAN resulted in a marked increase in the catalytic iron associated with significant loss of glomerular CYP content. Administration of CYP inhibitors significantly prevented the injury-induced loss of CYP content and the increase in the catalytic iron in the glomeruli accompanied by a marked decrease in proteinuria. In an in vitro study utilizing glomerular epithelial cells (GEC), CYP inhibitors also markedly prevented the PAN-induced increase in the catalytic iron and hydroxyl radical formation accompanied by significant protection against PAN-induced cytotoxicity. Taken together our data indicate that the CYP, a group of heme protein, may serve as a significant source of this catalytic iron.

reactive oxygen metabolites; puromycin aminonucleoside; glomerular epithelial cells; cytochrome P-450 inhibitors

MINIMAL CHANGE NEPHROTIC SYNDROME (MCNS) IS THE MOST COMMON NEPHROTIC SYNDROME that affects children between 2 to 6 years of age. Reactive oxygen metabolites (ROM) are important mediators of this renal injury (5, 31). The molecular mechanism for the generation of ROM is not clear. Hence, the treatment of the MCNS is empirical, requiring mostly corticosteroids and at times immunosuppressive agents. A better understanding of the mechanism of this glomerular injury may lead to therapy with fewer complications.

Iron, a transition metal, plays an important role in vivo and in vitro models of reactive oxygen metabolites-mediated tissue injury (4–7, 17, 31). The case with which iron is reversibly oxidized and reduced, while essential for its metabolic functions, also makes iron potentially hazardous because of its ability to participate in the generation of powerful oxidant species, hydroxyl radical (the metal catalyzed Haber-Weiss reaction) and/or in the generation of the highly reactive iron oxygen complexes such as ferryl or perferryl ion (17). In vivo most of the iron is bound to heme and non-heme proteins and does not freely catalyze the generation of hydroxyl radicals or similar oxidants (17). Gutteridge et al. (14–17) have described an assay based on the use of the antibiotic bleomycin to detect iron complexes capable of catalyzing free radicals in biological fluids. By using this assay we have reported a marked increase in the bleomycin-detectable iron in several models of acute renal failure (4, 6, 7) including puromycin aminonucleoside (PAN)-induced MCNS (33).

The source of this iron capable of catalyzing free radical reactions is currently not known. Iron storage protein ferritin, transferrin, iron-rich mitochondria, and under certain circumstances, extracellular heme protein such as hemoglobin and myoglobin have all been suggested as possible sources for this iron (25, 27). Recent studies, including that of ours, indicate that the iron rich enzyme, cytochrome P-450 (CYP), may serve as a potential source of iron in models of tissue injury (8, 10, 26). However, the role of CYP in any glomerular disease model including PAN-induced MCNS has not been previously examined. Hence, the current study was designed to examine the role of CYP, especially as a source of catalytic iron, in an in vivo model of MCNS induced by a single injection of PAN to rats and in an in vitro model of PAN-induced cytotoxicity to glomerular epithelial cells (GEC).

METHODS

In vivo studies

PAN-induced nephrotic syndrome. Male Sprague-Dawley rats weighing 200–250 g were injected with saline or a single intravenous injection of PAN (Sigma, St. Louis) in a dose of 7.5 mg/100 g body wt. (BW; day 0) as in our previous study (33). Animals were housed in separate metabolic cages and allowed free access to rat chow (Purina). Daily urine protein

Address for reprint requests and other correspondence: R. Baliga, Dept. of Pediatrics, Division of Nephrology, Univ. of Mississippi Medical Center, 2500 North State St., Jackson, MS 39216–4505 (E-mail: rbaliga@ped.umsmed.edu).
Excretion was determined and animals were killed on day 7. Blood was obtained for the measurement of serum albumin and the evaluation of renal function as measured by blood urea nitrogen (BUN) and plasma creatinine. Cell fraction of the glomeruli was prepared for bleomycin-detectable iron assay. The microsome fraction was utilized for the measurement of CYP content.

**Inhibition of cytochrome P-450.** Two different inhibitors of CYP were used. Cimetidine (CM) has imidazole and cyano groups that inhibit CYP by interacting with the heme moiety (29). This effect of CM is specific for CYP, as it does not interact with other heme enzymes (2). To determine the effect of the CYP inhibitors in PAN-induced nephrotic syndrome, CM (120 mg/kg BW) was administered intraperitoneal 1h prior to PAN injection and then twice a day. Another CYP inhibitor, piperonyl butoxide (PB), which yields a metabolite that binds to the heme moiety of CYP (1, 12), was given intraperitoneal (400 mg/kg BW) 4 h before PAN injection and then every other day. All experimental procedures were conducted in accordance with our institutional guidelines.

**Isolation of glomeruli.** Glomeruli were isolated by a combination of sieving and differential centrifugation as in our previous studies (3, 33). Glomeruli isolated from two rats were pooled together (as n = 1) for the isolation of microsomes.

**Bleomycin-detectable iron assay.** Iron capable of catalyzing free radical reactions was measured by bleomycin-detectable iron assay as described by Gutteridge et al. (15, 16) and as detailed in previous studies from our laboratory (6–8, 33).

**Cytochrome P-450 content in microsome.** Glomeruli isolated from rat kidneys were suspended in an extraction buffer containing 20 mM Tris·HCl, pH 7.4, 0.25 M sucrose, 1 mM EDTA and protease inhibitor cocktail (5 μl/100 mg wet weight, Sigma, St. Louis, MO) and frozen at −80°C. Subsequently, the glomeruli were thawed and sonicated (22). The homogenate was centrifuged at 15,000 g for 20 min at 4°C and the precipitate was discarded. The microsomes were sedimented by centrifugation of the supernatant at 105,000 g for 60 min at 4°C. The firmly packed pellet of microsomes was resuspended in above extracting buffer at a concentration of ~10 mg protein/ml (32). CYP content was measured by the method of Omura and Sato (24). In brief, suspension of microsome from the glomeruli was diluted to about 1 mg of protein/ml with the assay buffer (0.1 M potassium phosphate buffer, pH 7.25, 20% glycerol, and 0.2% tergitol). After the baseline was recorded, the sample was reduced with a few crystals of dithionite, and followed by CO bubbling for ~1 min. The CO difference spectrum of reduced microsomes was recorded on a Shimadzu UV-2101PC spectrophotometer. The peak absorbance at 450 nm was measured, and the amount of CYP was determined by using the extinction coefficient of 91 mM/cm.

**In vitro studies**

**Cell culture.** Rat GEC (kindly provided by Dr. Saulo Klahr, Washington University School of Medicine, St. Louis, MO) were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum, 15 mM HEPES, insulin, penicillin, streptomycin, and L-glutamine in a humidified atmosphere of 95% air-5% CO2 at 37°C and fed at intervals of 3 days (20). The cells were maintained in 75-cm² tissue culture flasks and the monolayers were subcultured by using 0.05% trypsin-0.53 mM EDTA in Hank’s balanced salt solution (HBSS). For
the experimental study, the cells were grown in 12-well tissue culture plate until confluence.

**PAN-induced cytotoxicity.** On the day of experiment, the medium was discarded and the confluent GEC monolayer was washed twice with HBSS. The cells were then incubated with various concentrations of PAN (0, 0.1, 0.5, 1.0, 1.5, and 2.0 mM in HBSS) for different periods of time (0, 6 h, 1, 2, 3, and 4 days) at 37°C. At the end of the incubation, the incubation medium was discarded and the GEC monolayer was harvested by trypsinization with 0.05% trypsin-0.53 mM EDTA for 5 min at 37°C. Isolated cells were suspended in HBSS to give 10^6 cells/ml. Cell viability was determined by use of trypan blue exclusion assay as in our previous study (9).

**Effect of CYP inhibitors on the PAN-induced cytotoxicity.** Confluent GEC monolayers were washed twice with HBSS and then incubated with various concentrations of CM, ranitidine ([RN], as a control for CM) for 30 min, and PB for 60 min at 37°C. After the incubation, the cell monolayers were washed twice with HBSS and then incubated with cytotoxic dose of PAN in HBSS for a period of time necessary to induce consistent cytotoxicity (1.5 mM/ml, 48 h, based on the concentration and time course studies) at 37°C. PAN-induced cytotoxicity on GEC was measured by trypan blue exclusion assay.

**Effect of CYP inhibitors on PAN-induced catalytic iron release.** Confluent cell monolayer was washed three times with Chelex-treated HBSS to remove as much contaminating iron as possible. The GEC monolayer was then incubated in Chelex-treated HBSS with a cytotoxic dose of PAN for a period of time at 37°C before substantial cell killing and after significant iron release occurs (1.5 mM, 60 min, on the basis of a time course study on iron release induced by PAN, data not shown). The incubation medium was then collected for the measurement of catalytic iron by using bleomycin-detectable iron assay as mentioned above. To determine the effect of CYP inhibitors on the iron release, GEC monolayer was preincubated with CM (2 mM), RN (1 mM, as a control for CM) or PB (25 μM) for 60 min in Chelex-treated HBSS at 37°C. After the incubation, the medium with CYP inhibitor was discarded and then the cell monolayers were washed twice with chelate-treated HBSS prior to the incubation of PAN.

**Effect of CYP inhibitors on PAN-induced hydroxyl radical formation.** Confluent GEC monolayer was washed twice with HBSS and then incubated with 1.5 mM PAN in HBSS for a period of time before substantial cell killing occurs (1 h) at 37°C. 2-Deoxy-D-ribose in a final concentration of 3 mM was added to the medium just prior to the incubation. At the end of the incubation, the incubation medium was collected for the measurement of hydroxyl radical formation by deoxyribose degradation method as in our previous study (9). To determine the effect of CYP inhibitors on the hydroxyl radi-

![Fig. 3. Effect of CYP inhibitors on the bleomycin-detectable iron content in the glomeruli of PAN-treated rats. Values are means ± SE. *P < 0.05 compared with control rats. **P < 0.05 compared with PAN treatment alone.](image3)

![Fig. 4. Effect of CYP inhibitors on CYP content in the glomeruli of PAN-treated rats. Values are means ± SE. N/D, not detectable.](image4)

![Fig. 5. Effect of CYP inhibitors on the proteinuria in rats treated with PAN. Values are means ± SE. **P < 0.05 compared with the rats treated with PAN alone. +PB, piperonyl butoxide; +CM, citimetidine.](image5)

![Table 1. Weight loss/gain of rats before and after treatments](table1)
cal formation, GEC monolayers were preincubated with CM, RN, or PB as mentioned above.

**Statistical analysis.** Values are expressed as means ± SE. Statistical analysis was performed by using unpaired t-test (for only two groups) and analysis of variance (for more than two groups). Statistical significance was considered at \( P < 0.05 \).

**RESULTS**

Intravenous administration of PAN resulted in nephrotic range proteinuria by day 7 (Fig. 1A). The catalytic iron content as measured by the bleomycin-detectable iron assay was significantly elevated from a control value of 39 to 160 nmol/mg protein (\( n = 3, P < 0.01 \)) in the glomeruli obtained from rats injected with PAN (Fig. 1B). If CYP serves as a source of this catalytic iron, there would be a marked reduction in the CYP content in the glomeruli in the PAN-treated rats. Indeed the CYP content in the PAN-treated rats was not detectable in the glomeruli (Fig. 2). This loss of CYP content in the glomeruli was injury specific because there was no difference in the level of CYP content in the liver between the untreated and the PAN-treated animals (Fig. 2).

We postulated that the heme moiety of CYP may serve as a significant source of iron in this model of injury. Hence, we examined the effect of CYP inhibitors CM and PB (which interact with the heme moiety of CYP) on the bleomycin-detectable iron content in the glomeruli in PAN-treated rats. Both CM and PB significantly prevented the increase in this iron in the glomeruli in rats subjected to PAN injection (Fig. 3). If the heme moiety of CYP serves as a significant source of this catalytic iron then one would anticipate that the CYP inhibitors that prevent the increase in bleomycin-detectable iron will also preserve the loss of CYP in the glomeruli. We therefore measured the effect of these inhibitors on CYP content in PAN-treated animals. Both CM and PB significantly preserved the loss of CYP content in the glomeruli in the rats subjected to PAN injection (Fig. 4). We next examined the effect of

![Fig. 6. Effect of CYP inhibitors on the serum albumin in rats treated with PAN. Values are means ± SE. *P < 0.05 compared with the control rats.](image)

![Fig. 7. Renal function as measured by serum creatinine (A) and blood urea nitrogen (BUN) (B) in rats injected with PAN with or without CYP inhibitors. Values are means ± SE. *P < 0.05 compared with the control rats.](image)

![Fig. 8. Effect of CYP inhibitors on the catalytic iron release from glomerular epithelial cells (GEC) exposed to PAN. Confluent GEC was incubated with cytotoxic dose of PAN (1.5 mM) for a period of time before substantial cell killing occurs (1 h). CYP inhibitors PB (25 \( \mu \)M), CM (2 mM) and ranitidine (RN) (1 mM, as a control for CM) were preincubated with GEC for 30–60 min and then washed with chelexed Hank's balanced salt solution (HBSS) followed by addition of PAN. Values are means ± SE. *P < 0.05 compared with the control; †P < 0.05 compared with GEC exposed to PAN alone.](image)

![Fig. 9. Effect of CYP inhibitors on the hydroxyl radical generation in GEC exposed to PAN. Confluent GEC were incubated with cytotoxic dose of PAN (1.5 mM) for a period of time before substantial cell killing occurs (1 h). CYP inhibitors PB (25 \( \mu \)M), CM (2 mM), and RN (1 mM, as a control for CM) were preincubated with GEC for 30–60 min and then washed with HBSS followed by addition of PAN. Values are means ± SE. *P < 0.05 compared with the control; †P < 0.05 compared with PAN alone.](image)
these inhibitors on PAN-induced proteinuria. Administration of PAN resulted in significant proteinuria on the fourth day with marked increase thereafter throughout the course of the study (Fig. 5). Both CM and PB provided substantial protection against PAN-induced proteinuria (Fig. 5).

PAN-treated rats had significant weight gain at the time of death compared with the control animals and those treated with CYP inhibitors CM and PB (Table 1). The serum albumin in the PAN-treated animals was markedly decreased and this decrease was prevented by the CYP inhibitors CM and PB (Fig. 6). Renal function as measured by serum creatinine was similar in all the groups while the BUN was slightly but significantly elevated in the rats treated with PAN and CYP inhibitors compared with the control animals (Fig. 7).

We also examined the role of CYP in an in vitro model of PAN-induced cytotoxicity to GEC. Exposure of GEC to PAN (1.5 mM) resulted in a significant increase in the bleomycin-detectable iron content (Fig. 8). Both the CYP inhibitors, CM (2 mM) and PB (25 μM), markedly prevented the increase in the bleomycin-detectable iron content (Fig. 8). RN (1 mM), which has a similar structure as CM but is a weak inhibitor of CYP, did not prevent the marked increase in the bleomycin-detectable iron content (Fig. 8).

Iron has been shown to participate in the generation of powerful oxidant species, such as the hydroxyl radical via the metal catalyzed Haber-Weiss reaction (5). We hence examined the potential role of hydroxyl radical in PAN-induced cytotoxicity to GEC. As shown in Fig. 9, exposure of GEC to PAN led to a significant increase in the hydroxyl radical formation. CYP inhibitors CM and PB, but not RN, markedly reduced the PAN-induced hydroxyl radical formation in the GEC.

PAN was cytotoxic to the GEC in a time and a dose-dependent manner as measured by the trypan blue exclusion assay (Fig. 10). Both CM and PB but not RN significantly reduced PAN-induced cytotoxicity to the GEC (Fig. 11).

DISCUSSION

The role of CYP in any model of glomerular disease has never been examined. Our study indicates an important role of CYP in PAN-induced MCNS. A single intravenous injection of PAN to rats results in marked proteinuria and renal morphological changes similar to MCNS in humans (23). We have demonstrated, in our previous study, a significant increase in catalytic iron (as measured by bleomycin-detectable iron assay) in the glomeruli in PAN-treated rats, and the iron chelator deferoxamine markedly reduced the increase in the catalytic iron associated with significant protection against proteinuria (33). Our present data suggest that the major role of CYP in this model of MCNS is to serve as a significant source of catalytic iron.

Administration of PAN resulted in a significant increase in the catalytic iron accompanied by loss of CYP content in the glomeruli. This loss of CYP content was specific to the glomeruli as there was no difference noted in the levels of CYP content in the liver between PAN and control group of animals. If the heme moiety...
of CYP does serve as a significant source of this iron in PAN-induced MCNS, then the CYP inhibitors that interact with the heme moiety of CYP should prevent the increase in the bleomycin-detectable iron and preserve the loss of CYP in this model. We used two different inhibitors of CYP to increase the specificity of our observation. Both CM and PB prevented the increase of the bleomycin-detectable iron in the glomeruli and the PAN-induced loss of CYP content. They also provided significant protection against PAN-induced proteinuria and the marked decrease in the serum albumin. We also conducted an in vitro study by using GEC, which is the specific site of injury in MCNS. Exposure of GEC to PAN resulted in a significant increase in the bleomycin-detectable iron content that was prevented by both CYP inhibitors CM and PB. Because we have shown the relationship between the loss of CYP content and the increase in catalytic iron after administration of PAN in rats, we did not repeat this study in vitro. Preincubation of the cells with CM and PB also provided significant reduction of PAN-induced cytotoxicity. RN, which has three times the H$_2$O$_2$ receptor blocking activity as CM but is a weak inhibitor of CYP, did not exhibit any protection.

One of the important mechanisms by which iron mediates tissue injury is the generation of hydroxyl radical via the iron catalyzed Haber-Weiss reaction (5, 17). The protective effects of iron chelators and hydroxyl radical scavengers have been generally taken as evidence for the participation of hydroxyl radical in PAN-induced MCNS (5). We demonstrated that exposure of GEC to PAN resulted in a significant increase in the hydroxyl radical formation. CYP inhibitors, both CM and PB but not RN, significantly reduced the PAN-induced hydroxyl radical formation. We have observed in our previous studies that CYP inhibitors CM and PB do not chelate the catalytic iron and scavenge hydroxyl radical in a cell-free system (9). Hence, we speculate that the protective effect of CYP inhibitors is due to their binding with CYP hemeprotein that protects the heme moiety from oxidative injury and consequently prevents release of catalytic iron. Taken together, these data indicate an important role of CYP and support the notion that CYP serves as a critical endogenous source of iron, capable of catalyzing free radical reactions in PAN-induced MCNS. Our study however, does not exclude the other roles of CYP in this model of renal injury. CYP participates in the bioactivation and detoxification of a wide variety of substances. The administration of CYP inhibitors may also influence such functions of CYP that are relevant to the expression of renal injury by mechanism(s) independent of iron.

The mechanism(s) responsible for the loss of CYP is not well defined. In our previous studies, we have shown that the incubation of the microsomes isolated from LLC-PK$_1$ (renal proximal tubular cells) with hydrogen peroxide resulted in a marked increase in iron mobilization. In addition, CYP inhibitor CM (but not RN) significantly reduced the iron mobilization from the microsome fraction exposed to hydrogen peroxide (9). Recent studies have demonstrated enhanced generation of hydrogen peroxide from kidney slice cultures and GEC exposed to PAN (21, 30). It is likely that such an increase in the generation of hydrogen peroxide could result in direct oxidative attack on the heme moiety of CYP, promoting the heme destruction and the release of iron (19).

Our data does not exclude other intracellular sources of iron from participating in ROM-mediated glomerular injury. For instance, mitochondrial cytochromes, iron-sulfur protein, and other iron containing proteins may be an alternative source of iron. Shah et al. found that isolated renal cortical mitochondria released iron when exposed to the nephrotoxic gentamicin (34). Zager and coworkers suggested that the formation of iron/H$_2$O$_2$-based reactive intermediates in mitochondria may be responsible for the cell damage in an in vitro model of myoglobin cytotoxicity (35). However, our study indicates a close relationship between the catalytic iron formation and the content of microsomal CYP, which is mainly involved in the drug metabolism (18). In addition, CYP inhibitors were markedly effective in reducing PAN-induced proteinuria in rats as well as PAN-induced cytotoxicity to GEC. Thus we suggest that microsomal CYP is the major source of the catalytic iron.

MCNS accounts for >75% of cases of nephrotic syndrome in children and 30% of nephrotic syndrome in adults (11, 13, 28). Treatment of minimal change disease is empirical because the underlying mechanism(s) that cause glomerular injury are not well known. Corticosteroids and/or immunosuppressive agents are often used to treat minimal change disease (11, 13, 28). Our results indicate that CYP inhibitors have a beneficial effect on proteinuria and may have future clinical application not only in patients with MCNS but also in other models of nephrotic syndrome as well.

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REFERENCES


