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Cytochrome P-450 mediates tissue-damaging hydroxyl radical formation during reoxygenation of the kidney

(oxygen free radical/heme/iron)

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ABSTRACT **Renal reperfusion injury results from oxygen radical generation. During reoxygenation of hypoxic kidney cells, xanthine oxidase produces superoxide radical, which eventuates in hydroxyl radical formation by the Fenton reaction. This reaction, catalyzed by transition metals such as iron, is particularly important because hydroxyl radical is highly reactive with a wide variety of biomolecules. We tested the hypothesis that this catalytic function is fostered by iron released from the heme moiety of cytochrome P-450. Primary cultures of rat proximal tubule epithelial cells studied in a subconfluent stage were subjected to 60 min of hypoxia and 30 min of reoxygenation. When cells were pretreated with one of three cytochrome P-450 inhibitors (piperonyl butoxide, cimetidine, or ketoconazole), lethal cell injury was attenuated. There was the expected increase in $O_2^{-\cdot}$ production during hypoxia/reoxygenation that cytochrome P-450 inhibitors did not prevent; on the other hand, inhibitors did prevent reoxygenation-induced hydroxyl radical formation. Analogously, the increase in catalytic iron (bleomycin-detectable iron) that accompanies hypoxia/reoxygenation did not occur in the presence of cytochrome P-450 inhibitors. *In vivo* studies confirmed a protective effect of cytochrome P-450 inhibition because glomerular filtration rate was better preserved in rats pretreated with cimetidine and then subjected to renal artery occlusion. In summary, several chemically distinct cytochrome P-450 inhibitors reduced iron release, and thereby, hydroxyl radical formation and reoxygenation-induced lethal cell injury, without inhibiting superoxide radical formation. We conclude that highly labile P-450 may act as an Fe-donating catalyst for Fenton reaction production of HO·-mediated reperfusion injury.**

Oxidative injury is an important mechanism in reperfusion injury of a number of organs including the kidney (1–5). The molecular mechanisms for generation of reactive oxygen species have recently received intense scrutiny. In the kidney, during hypoxic conditions, the high-energy molecule ATP is sequentially dephosphorylated and then further degraded to nucleosides to eventually yield the base hypoxanthine. Cytoplasmic xanthine oxidase converts hypoxanthine to xanthine and then to uric acid. A parallel phenomenon that occurs during hypoxia is the conversion of xanthine dehydrogenase to xanthine oxidase through limited proteolysis by a calcium/calmodulin-dependent serine protease (6, 7). Xanthine oxidase uses molecular oxygen as an electron acceptor during these reactions to yield superoxide radical ($O_2^{-\cdot}$), which is subsequently dismutated to hydrogen peroxide and further reduced to the highly reactive hydroxyl radical ($HO^{\cdot}$) in the Fenton reaction (8). This final step of the pathway is catalyzed by transition metals such as iron or copper.

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There are several potential cellular sources of reactive iron. The iron storage protein ferritin is an obvious candidate (9). The kidney, particularly in the proximal tubule, is rich in ferritin (10, 11). However, recently, there has been considerable debate regarding the ease and number of iron molecules that can be released from ferritin during oxidative stress (12). Heme proteins are the second most abundant source of iron within the kidney and include mitochondrial cytochromes, cytochromes P-450, and catalase. Under certain circumstances extrarenal heme proteins, such as hemoglobin and myoglobin, might also serve as sources of iron (13, 14). Also, iron could be derived from the [4 Fe/4 S] clusters of dehydratases (15). One reason for paying particular attention to cytochrome P-450 is that it is labile, and in certain pathologic settings, it is degraded, releasing free heme. For example, endotoxin, a factor commonly associated with the development of acute renal failure, induces heme oxygenase resulting in degradation of and a lower steady-state concentration of cytochrome P-450 (16).

The purpose of this study was to determine whether renal cytochrome P-450 participates in reoxygenation injury of the kidney by serving as a source of catalytic iron. Indeed, our studies support this hypothesis. In addition to serving as a source of reactive iron, cytochrome P-450 might also cause injury by serving as a direct source of superoxide radical by the univalent reduction of molecular oxygen (17, 18). This possibility was also examined but not validated.

MATERIALS AND METHODS

Cell Culture. Rat (male Sprague–Dawley, Harlan Laboratories, Madison, WI) renal proximal tubular segments were isolated by Percoll density gradient centrifugation after collagenase digestion by the method of Gesek *et al.* (19) as employed in this laboratory (20). The tubule fragments were suspended in RPMI 1640 culture medium to which 10% (vol/vol) fetal calf serum, penicillin (100 microunits/ml), streptomycin (100 μg/ml), epidermal growth factor (10 ng/ml), transferrin (5 μg/ml), insulin (5 μg/ml), and 10 nM dexamethasone (final concentrations) were added. Suspended tubule fragments were plated onto collagen gel (type 1, Sigma)-coated plastic 12 (4.5 cm^2)-multiwell plates (Costar). Primary cultures were employed for all studies.

Hypoxia/Reoxygenation Injury. Cells were studied in a subconfluent stage three days after initial establishment of the culture. To reduce the likelihood of conversion to anaerobic metabolism and to enhance the susceptibility to hypoxic injury, the culture medium was changed to a glucose-free formulation of the usual medium after 24 h (20). Two hours before the study, the medium was changed to glucose-free medium also devoid of growth factors and serum (incubation medium). Cell plates were placed in an airtight chamber that was fitted with a one-way valve under a continuous flow of

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humidified gas and maintained at 37°C and subjected to hypoxia (95% N_2/5% CO_2) for 60 min followed by reoxygenation (95% O_2/5% CO_2) for 30 min. This protocol has been demonstrated to induce prompt reoxygenation without producing hyperoxia (20).

To ascertain the degree of renal proximal tubule cell injury after hypoxia/reoxygenation, lactate dehydrogenase (LDH) release into the incubation medium was measured and was expressed as the percent of total cellular LDH (supernatant LDH plus cell-fraction LDH) recovered in the incubation medium (21).

Inhibition of Cytochrome P-450. Three chemically distinct competitive inhibitors of cytochrome P-450 were employed. These chemicals bind reversibly to the substrate binding site of cytochrome P-450 or, for piperonyl butoxide, yield a metabolite that binds with high affinity and under a wide variety of conditions (22). Cimetidine has imidazole and cyano groups that bind to the heme iron (23). The cells were exposed to the cytochrome P-450 inhibitor for 60 min prior to the induction of hypoxia. The inhibitors used were piperonyl butoxide (10 μM), cimetidine (5 mM), and ketoconazole (10 μM) (22–26). Preliminary studies revealed that these concentrations of inhibitors had no effect on the assays for LDH, $O_2^{\cdot-}$, $HO^{\cdot}$, or bleomycin-detectable iron.

Measurement of Reactive Oxygen Species. Cells were examined for the release into the incubation medium of either superoxide radical or hydroxyl radical (20). Superoxide production was measured spectroscopically as the superoxide dismutase-inhibitable reduction of ferricytochrome *c* (27). Hydroxyl radical formation was detected by the degradation of 2-deoxyribose, which when attacked by hydroxyl radical yields malondialdehyde, which can be detected by the thiobarbituric acid assay (28). This is a sensitive assay for extracellularly derived hydroxyl radical but will miss radicals generated intracellularly because intracellular radicals will react with cellular biomolecules rather than the detector molecule. Therefore, it underestimates total hydroxyl radical formation.

Measurement of Catalytic Iron. Iron capable of catalyzing the Haber–Weiss reaction was detected by the bleomycin assay developed by Gutteridge *et al.* (29, 30). Although it would be desirable to apply the assay to cellular homogenates, in initial studies we found troubling variability in results when assaying cell homogenates or cytoplasmic fractions. We, therefore, turned to measuring catalytic iron released into the incubation medium during the experimental period. As was true for the determinations of reactive oxygen species concentrations, this measurement would tend to underestimate net mobilization of catalytic iron.

Cytochrome P-450 Concentration. The concentration of cytochrome P-450 was determined by the differential absorbance at 450 nm of the CO adduct of reduced hemoprotein in a microsomal fraction derived from either cells or kidney homogenate as described by Estabrook and Werringloer (31). In the presence of the cytochrome P-450 inhibitor piperonyl butoxide, cytochrome P-450 content could not be measured (preliminary data).

***In Vivo* Studies.** Rats were allowed unlimited access to food and water until the time of study. Rats were anesthetized with sodium pentobarbital (60 mg/kg, i.p.). Bilateral flank incisions were made and the right kidney was removed. The left kidney perirenal fat was removed and the left renal artery was exposed. A nontraumatic vascular clamp was then placed across the renal artery for 40 min. After removal of the clamp, the animal was sutured and allowed to recover until needed for further studies.

To inhibit cytochrome P-450, rats were given cimetidine in a dose of 120 mg/kg i.p. every 12 h for three doses (two doses on the day prior to ischemia and the final dose 1 h prior to ischemia) (32). Control animals received an injection of vehicle (0.5 ml of saline). The effect of ischemia on renal function was measured by plasma creatinine 24 and 48 h after ischemia or by inulin clearance 24 h after ischemia as described (2).

Statistical Methods. All data are presented as the mean ± SEM. Statistical comparisons were made by unpaired *t* test. Because most analyses involved multiple groups, modified critical values for the *t* distribution using Bonferroni's method were employed.

RESULTS

The Effect of Cytochrome P-450 Inhibitors on Lethal Cell Injury During Hypoxia/Reoxygenation. All three cytochrome P-450 inhibitors had a substantial ability to reduce the lethal cell injury produced by hypoxia/reoxygenation. As shown in Fig. 1, in this experiment basal LDH release was 8.6 ± 1.3%. Under normoxic conditions, the cytochrome P-450 inhibitor piperonyl butoxide had no significant effect on basal LDH release (12.4 ± 1.5%). A substantial number of cells were irreversibly injured by hypoxia/reoxygenation, as shown by release of 57.6 ± 2.8% of total cellular LDH. Previous studies using nigrosine exclusion or monitoring the ability of cells to maintain normal transcellular calcium gradients suggest that a similar percentage of cells developed irreversible lethal permeability defects, whereas the remaining cells remained potentially viable during the experiment (7, 20). Cytochrome P-450 inhibition dramatically limited lethal cell injury by ≈65%.

The other cytochrome P-450 inhibitors tested, cimetidine and ketoconazole, had similar beneficial effects on lethal cell injury (Table 1). They reduced hypoxic injury by 74–80%. Of note, ketoconazole had an apparently mild toxic effect on normoxic cells. Despite this, ketoconazole still protected against hypoxia/reoxygenation injury.

Previous studies of this model have demonstrated an increase in superoxide radical production during the early minutes of reoxygenation of hypoxic renal epithelial cells (20). In the current studies, this phenomenon was again observed, with an increase in $O_2^{\cdot-}$ production from 165.2 ± 22.7 in normoxic cells (n = 10) to 262.0 ± 28.0 nmol per mg of protein per 90 min in hypoxia/reoxygenation (n = 22; $P <$ 0.05). Cytochrome P-450 inhibition with either piperonyl butoxide or cimetidine had no effect on the reoxygenation-mediated superoxide radical production (piperonyl butoxide, 294.6 ± 34.0 nmol per mg of protein per 90 min, n = 12;

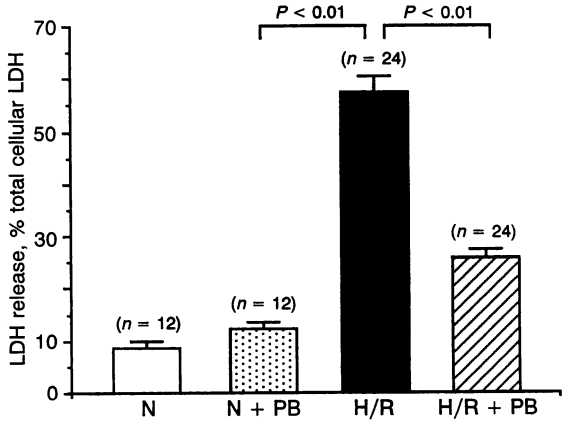

FIG. 1. Effect of the cytochrome P-450 inhibitor piperonyl butoxide on lethal injury of renal proximal tubule epithelial cells by hypoxia/reoxygenation. Cells were exposed to 10 μM piperonyl butoxide (PB) for 60 min prior to being subjected to hypoxia for 60 min and reoxygenation for 30 min (H/R). Irreversible injury was quantitated as percent total LDH released. N, normoxia.

Table 1. Effect of cytochrome P-450 inhibitors on renal cell injury during hypoxia/reoxygenation

Condition	n	LDH release, % total cellular LDH	Reduction of injury by P-450 inhibitor, %
Normoxia	12	24.8 ± 1.6	
Normoxia + cimetidine	6	21.6 ± 1.5	
H/R	18	58.6 ± 4.0*	
H/R + cimetidine	17	33.3 ± 2.8†	74.9
Normoxia	6	27.8 ± 1.5	
Normoxia + ketoconazole	8	36.8 ± 1.5*	
H/R	8	50.4 ± 2.6*	
H/R + ketoconazole	8	32.5 ± 2.3†	79.2

H/R, hypoxia/reoxygenation. Percent inhibition of injury by P-450 inhibitor = $[(H - N) - (H + I) - N]/(H - N) \times 100$, where H is LDH released by H/R, N is LDH released by normoxia, and I is LDH released by P-450 inhibitor. The term $(H - N)$ is the specific LDH release due to hypoxia/reoxygenation. *, $P < 0.05$ vs. normoxia; †, $P < 0.05$ vs. hypoxia/reoxygenation.

cimetidine, 232.6 ± 24.7 nmol per mg of protein per 90 min, $n = 24$). Therefore, cytochrome P-450 does not appear to be a major source of superoxide radical during reoxygenation of hypoxic renal epithelial cells.

On the other hand, cytochrome P-450 inhibitors substantially attenuated hydroxyl radical formation during reoxygenation. Fig. 2 demonstrates the expected increase of hydroxyl radical during reoxygenation of hypoxic renal epithelial cells (20). However, the cytochrome P-450 inhibitors piperonyl butoxide and cimetidine reduced this stimulated hydroxyl radical formation by 60%.

The observations that cytochrome P-450 inhibitors did not affect the generation of superoxide radical but markedly attenuated the reoxygenation-induced increase in hydroxyl radical formation suggest a role of cytochrome P-450 in

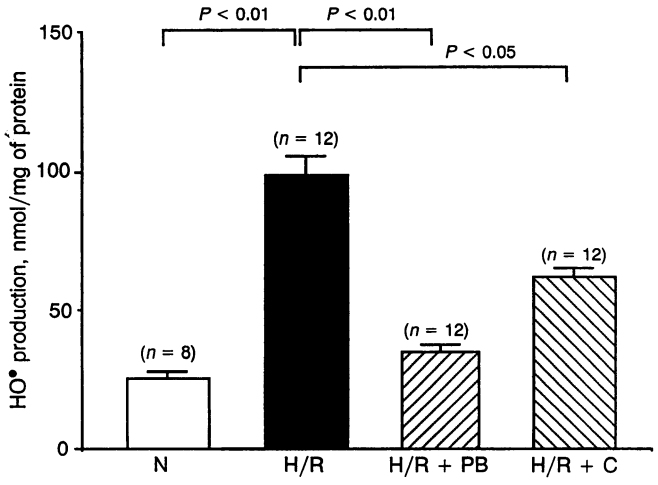

FIG. 2. Effect of the cytochrome P-450 inhibitors piperonyl butoxide and cimetidine on hydroxyl radical formation by renal proximal tubule epithelial cells during hypoxia/reoxygenation. Cells were exposed to 10 μM piperonyl butoxide (PB) or 5 mM cimetidine (C) for 60 min prior to being subjected to hypoxia for 60 min and reoxygenation for 30 min (H/R). Incubation medium was changed prior to hypoxia. Hydroxyl radical produced during H/R (total 90 min) and released into the incubation medium (or generated in the medium) was determined by the 2-deoxyribose assay. There was no effect of either cytochrome P-450 inhibitor on hydroxyl radical formation by normoxic cells. H/R plus cimetidine was significantly different from normoxia ($P < 0.05$), whereas H/R plus piperonyl butoxide was not significantly different from normoxia. N, normoxia.

catalyzing the Haber–Weiss reaction, perhaps by serving as a critical source of iron. Direct measurement of the release of catalytic iron by the bleomycin assay confirmed these suspicions (Fig. 3). During hypoxia/reoxygenation, release of catalytic iron from cells was clearly increased. Cimetidine prevented this excess release of iron during hypoxia/reoxygenation.

That catalytic iron is generated from cytochrome P-450 breakdown is supported by findings that cytochrome P-450 concentration of renal epithelial cells decreased by ≈90% during hypoxia/reoxygenation (Fig. 4). These observations also appear relevant to renal ischemia and reperfusion *in vivo* since the same pattern of ischemia-induced loss of cytochrome P-450 was observed in renal cortex. That is, nonischemic kidneys contained 0.128 ± 0.007 nmol of cytochrome P-450 per mg of protein, whereas kidneys subjected to ischemia/reperfusion contained only 0.08 ± 0.006 nmol per mg of protein, a 37% reduction of cytochrome P-450 content ($P = 0.002$).

Cytochrome P-450 Inhibition During Renal Ischemia. The importance of cytochrome P-450 in hypoxic renal cell injury was also investigated in the more clinically relevant model of renal ischemia *in vivo*. As shown in Table 2, rats subjected to 40 min of renal artery occlusion suffered a dramatic impairment in glomerular filtration rate whether measured indirectly as plasma creatinine or directly by inulin clearance. Compared with vehicle, the cytochrome P-450 inhibitor cimetidine substantially lessened the decrement in glomerular filtration rate in animals subjected to renal ischemia. Although there was a trend toward a higher urine/plasma creatinine ratio in cimetidine-treated rats compared with controls (11.4 ± 2.9 vs. 6.5 ± 1.0), there were no differences in mean arterial pressure (131 ± 2.8 vs. 126 ± 2.6 mmHg), urine volume (26 ± 3.5 vs. 21 ± 2.9 −μl/min), or fractional excretion of sodium (12.0 ± 2.8 vs. 11.7 ± 2.1%) between the two groups of animals.

DISCUSSION

These studies describe an important role of cytochrome P-450 in mediating hypoxia/reoxygenation injury that, to our

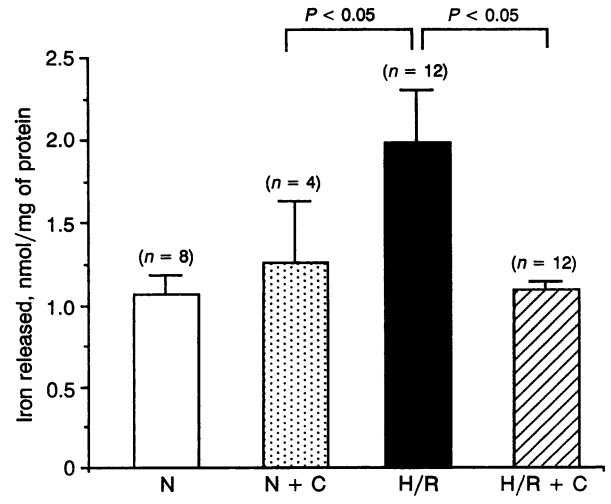

FIG. 3. Effect of the cytochrome P-450 inhibitor cimetidine on catalytic iron release by renal proximal tubule epithelial cells during hypoxia/reoxygenation. Cells were exposed to 5 mM cimetidine (C) for 60 min prior to being subjected to hypoxia for 60 min and reoxygenation for 30 min (H/R). Incubation medium was changed prior to hypoxia. Catalytic iron mobilized during H/R (total 90 min) and released into the incubation medium was determined by the bleomycin assay. Neither H/R + C nor normoxia (N) + C was significantly different from normoxia.

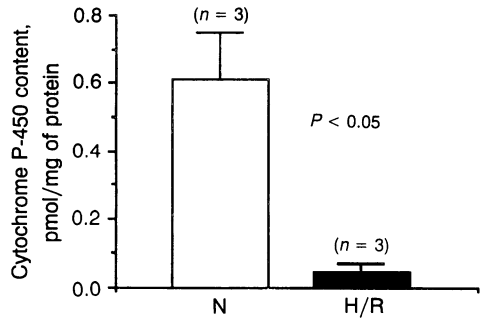

FIG. 4. Effect of hypoxia/reoxygenation on cytochrome P-450 content of renal proximal tubule epithelial cells. Cells were exposed to hypoxia for 60 min and reoxygenation for 30 min (H/R). Incubation medium was changed prior to hypoxia. Cytochrome P-450 content was determined by the differential absorbance at 450 nm of the CO adduct of the reduced cytochrome. Hypoxia/reoxygenation caused a 92% decrease in cytochrome P-450 content. N, normoxia.

knowledge, has not previously been described in the kidney. An important role of cytochrome P-450 to mediate reperfusion injury was first suggested by Bysani *et al.* (33) studying reperfusion injury in the isolated perfused lung. Whether this mechanism is important in other organs has not been studied and is important since the source of reactive oxygen species during reoxygenation injury appears to be different from one organ to another.

A role of iron in renal hypoxic or ischemic injury has been suggested (20, 34, 35). Infusion of the iron chelator deferoxamine during postischemic reperfusion of the kidney improved glomerular filtration rate and reduced histologic injury (34). Conversely, infusion of the iron complex ferric EDTA during postischemic reperfusion enhanced injury (34). By using the bleomycin assay, a 10- to 20-fold increase in catalytic iron was detected in the urine during reperfusion of ischemic kidneys (34). Similarly, in our *in vitro* model of hypoxia/reoxygenation of proximal tubule epithelial cells, hydroxyl radical formation and lethal cell injury were inhibited by inclusion of the iron chelator deferoxamine (20). This protection by iron chelators was correlated with an increase in ferrozine-detectable iron in homogenates of cells subjected to hypoxia/reoxygenation (35).

The cellular source of this iron has not been identified. Although exogenous sources of iron, such as the heme proteins hemoglobin and myoglobin, can clearly provide catalytic iron for Fenton reactions, *in vitro* work suggests that reoxygenation injury probably requires an endogenous source of iron (20, 35). The present studies demonstrate that cytochrome P-450 inhibitors limit lethal cell injury while concomitantly reducing bleomycin-detectable iron and hydroxyl radical formation (but not superoxide radical production) and support the notion that cytochrome P-450 serves as a critical endogenous iron donor.

Table 2. Effect of the cytochrome P-450 inhibitor cimetidine on renal function after renal ischemia

	Plasma creatinine, mg/dl			Inulin clearance, μl/min
	Baseline	24 h	48 h	
Vehicle	0.43 ± 0.04	3.7 ± 0.2	4.6 ± 1.1	134 ± 36
Cimetidine	0.42 ± 0.02	2.4 ± 0.5	3.5 ± 1.1	372 ± 104
P value	NS	0.03	NS	0.04

One group of rats was used for plasma creatinine studies ($n = 6 + 6$). Cimetidine at 120 mg/kg i.p. every 12 h for three doses (two doses on the day prior to ischemia and the final dose 1 h prior to ischemia) was used. Plasma creatinine was measured 24 and 48 h after ischemia. A second group of rats was used for inulin clearance determination 24 h after ischemia ($n = 10 + 10$). NS, not significantly different. The normal value (no ischemia) for inulin clearance for our laboratory is 1000 ± 80 μl/min.

These findings do not exclude other cellular sources of iron from also participating in reperfusion injury through the formation of reactive oxygen species. For instance, mitochondrial cytochromes are another similar source of iron and Shah and coworkers (36) found that isolated renal mitochondria released iron when exposed to the nephrotoxin gentamicin. Their finding suggests that mitochondria can release iron during injury, but whether mitochondria are a source of iron when intact cells are exposed to nephrotoxins, such as gentamicin, or hypoxia is unclear. In addition, ferritin also remains a potential source of catalytic iron during reperfusion injury, although recent studies suggest iron release from ferritin may not be very facile (12). Although the current study demonstrates that cytochrome P-450 is likely a critical source of iron to promote Fenton chemistry after renal ischemia, we acknowledge that cytochrome P-450 may not be the exclusive source of this highly toxic iron. The magnitude of release of bleomycin-detectable iron suggests additional sources of iron. However, because cytochrome P-450 inhibitors were so effective in limiting injury, we suggest that cytochrome P-450 is the critical source of catalytic iron and that additional iron may be released after cells are irreversibly injured.

In any event, our results suggest that when a cytochrome P-450 inhibitor is bound to the heme group of the cytochrome P-450 (22, 23), it prevents the release of iron. The interference by cytochrome P-450 inhibitors in the cytochrome assay system used prevented direct confirmation of protection of cytochrome P-450 in renal epithelial cells during hypoxia/reoxygenation when inhibitors were present. However, the extensive loss of cytochrome P-450 content during hypoxia/reoxygenation *in vitro* and during ischemia/reperfusion *in vivo* suggests that protection of the heme moiety from oxidative injury by inhibitor binding may reasonably be the operative mechanism here. Such oxidative injury results in the release of iron from heme under diverse conditions (37, 38). Alternatively, cytochrome P-450-released iron might be derived from the usual turnover of cytochrome P-450 in the absence of new cytochrome P-450 synthesis, a consequence of ATP depletion during hypoxia. In this case, iron would not be reincorporated into newly synthesized cytochrome P-450 and would thus be available to serve as a Fenton reagent.

Other far less likely explanations for a beneficial effect of cytochrome P-450 inhibition also deserve brief mention. Since cytochrome P-450 is one of several routes for arachidonic acid metabolism in the kidney, inhibition of this pathway could conceivably shunt arachidonate to the cyclooxygenase pathway, increasing the rate of synthesis of prostaglandin E and prostacyclin (39). Because these prostaglandins have a cytoprotective function in renal ischemic injury, the net effect of cytochrome P-450 inhibition would be renal protection (40). In addition, in nephrotoxic renal injury caused by hemoglobin and myoglobin, pretreatment with heme oxygenase inducers was found to be highly protective (41). The presumed explanation was that an increased quantity of heme oxygenase was able to degrade heme proteins more rapidly and deposit released iron in the storage protein ferritin, where it would be innocuous (42). However, the current studies suggest an additional beneficial effect of heme oxygenase induction: namely, that because heme oxygenase is responsible for cytochrome P-450 degradation (43), increased heme oxygenase activity might diminish cytochrome P-450 content. Thus, inducers of heme oxygenase could have an effect similar to inhibitors of cytochrome P-450 in that during oxidative stress there would be less iron release from a smaller cytochrome P-450 pool. This possibility remains to be specifically tested in renal ischemia.

The observation that cimetidine provided modest protection against the renal dysfunction that occurs after 40 min of renal artery occlusion suggests that a cytochrome P-450-accentuated mechanism of renal damage may be clinically important. Cimetidine is known to inhibit creatinine secretion, thus elevating plasma creatinine (44). Hence, the previous failure to have noted a clinically apparent protective effect of cimetidine in patients developing acute renal failure could be due to this masking effect, since it is quite unusual to measure glomerular filtration rate directly in the clinical setting. Alternatively, drugs that induce cytochrome P-450 could have potentially adverse consequences by increasing the supply of readily available iron for renal ischemic injury. Such a phenomenon may occur during ischemia/reperfusion of the liver where phenobarbital treatment aggravated LDH release and spontaneous chemiluminescence of the liver (5).

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