Etomoxir-induced PPARα-modulated enzymes protect during acute renal failure

Portilla, Didier, Gonghe Dai, Jeffrey M. Peters, Frank J. Gonzalez, Mark D. Crew, and Alan D. Proia. Etomoxir-induced PPARα-modulated enzymes protect during acute renal failure. Am J Physiol Renal Physiol 278: F667–F675, 2000.—Regulation of fatty acid β-oxidation (FAO) represents an important mechanism for a sustained balance of energy production/utilization in kidney tissue. To examine the role of stimulated FAO during ischemia, Etomoxir (Eto), clofibrate, and WY-14,643 compounds were given 5 days prior to the induction of ischemia/reperfusion (I/R) injury. Compared with rats administered vehicle, Eto-, clofibrate-, and WY-treated rats had lower blood urea nitrogen and serum creatinine following I/R injury. Histological analysis confirmed a significant amelioration of acute tubular necrosis. I/R injury led to a threefold reduction of mRNA and protein levels of acyl CoA oxidase (AOX) and cytochrome P4A1, as well as twofold inhibition of their enzymatic activities. Eto treatment prevented the reduction of mRNA and protein levels and the inhibition of the enzymatic activities of these two peroxisomal enzymes. Acyl CoA oxidase; fatty acid oxidation; ischemia/reperfusion

THE PROCESS of β-oxidation of long-chain fatty acids (LCFA) occurs predominantly in the S3 segment of the proximal tubule and represents an important mechanism for energy production in the kidney cortex. Fatty acid β-oxidation (FAO) enzymes exist in the mitochondria, peroxisomes, and microsomes of this nephron segment (1, 14, 15). We have recently shown that the inhibition of carnitine palmitoyltransferases (CPTs), mitochondrial enzymes involved in FAO, protects proximal tubules against hypoxia-induced cell death (21).

Recent studies suggest that inhibitors of mitochondrial CPT I activity can work also as direct ligands for a peroxisome proliferator-activated receptor-α (PPARα) target genes (4, 9, 31).

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determining preservation of kidney function during acute ischemic renal failure.

EXPERIMENTAL PROCEDURES

Induction of ATN. Male Sprague-Dawley rats weighing 200–250 g were obtained from Harlan. Rats were fed ad libitum with a Pro Lab diet (Purina) and housed in an animal facility at 21°C. Under anesthesia using Nembutal (50 mg/kg body wt), we exposed the retroperitoneal cavity via a lumbar incision, and both renal arteries were identified and freed by blunt dissection. Microvascular clamps were placed on both renal arteries to effect complete cessation of blood flow. The core temperature of these animals was maintained at 37°C by placing them on a homeothermic table. After 45 min, the clamps were removed with return of blood flow to the kidneys. Visual inspection corroborated successful reperfusion with a change in the color of the kidneys from dark blue to bright red after releasing the clamps. Postsurgery, the animals were kept for at least 3 h in a 37°C incubator to ensure that postoperative recovery was satisfactory.

Administration of Eto, clofibrate, and WY-14,643 compounds. Sham-operated animals and rats subjected to I/R were treated with Eto for 5 days (25 mg/kg body wt). Eto sodium (Research Biochemicals International, Natick, MA) was dissolved in distilled water and administered intraperitoneally. Clofibrate (150 mg/kg body wt, Sigma) and WY-14,643 (45 mg/kg body wt, Chemsyn Science Laboratories) were dissolved in corn oil and given intraperitoneally for 5 days. The last injection was given 4 h before the induction of ischemic injury.

Measurement of renal function. Tail vein blood was obtained before induction of ATN, after 45 min of ischemia, and after 24, and 48 h of reperfusion for measurement of serum creatinine, blood urea nitrogen (BUN), and electrolytes.

Fluorometric assay of palmitoyl CoA oxidase activity. Kidney homogenates were prepared in SET buffer (0.25 M sucrose, 1 mM EDTA, and 10 mM Tris-HCl, pH 7.40). Palmitoyl CoA oxidase activity was assayed fluorometrically with homovanillic acid in the presence of peroxidase to give a fluorescent dimer (32). The assay was performed in a final volume of 500 µl containing 5 mM homovanillate, 1.0 mg peroxidase type II, 0.02 mM FAD, 0.1 mM palmitoyl CoA, and 80 mM glycylglycine, pH 8.30. The reaction was started by adding 50 µl of sample homogenate (0.1–0.2 mg of proteins) into the reaction mixture, which was incubated at 26°C. At 5, 10, 20, and 40 min, 75 µl of the mixture was transferred into 1.5 ml of 0.5 M sodium bicarbonate buffer (pH 10.7) and read immediately, utilizing a fluorescence spectrometer at excitation wavelength of 325 nm and emission wavelength of 425 nm. A blank without the substrate (palmitoyl CoA) was set up for each sample assay. The enzyme activity was expressed as arbitrary units per milligram of protein per minute. The protein content of homogenates was assayed by Bio-Rad reagents as described by the manufacturer.

CYP4A1 enzyme activity assay. Micromolar fractions from kidney cortex homogenates were prepared by differential centrifugation between 10,000 g and 100,000 g, and the final protein concentration was adjusted to 5–10 mg/ml. The preparation was used immediately for the assay. CYP4A4 activities were measured by monitoring the conversion of 14C-labeled lauric acid into ω-hydroxylauric acids in 1 ml of 50 mM Tris-HCl (pH 7.4) in the presence of 0.2 mM NADPH at 37°C for 5 min (8). The reaction was stopped by adding 0.20 ml of 1 N HCl and vortexing. The lipids were extracted with 0.5 ml of ether twice, dried in a stream of nitrogen gas, and then separated by thin-layer chromatography (TLC) using benzene:ethanol:formic acid (90:10:1:0.2) as described previously (22). The bands corresponding to lauric acid and ω-hydroxylauric acids were scraped separately and counted. The enzymatic activity was expressed as nanomoles of ω-hydroxylauric acid produced per minute per milligram of microsomal protein.

Western blot analysis. Rat kidney cortex tissue from every experimental condition was homogenized with glass Teflon homogenizer in ice-cold SET buffer (0.25 M sucrose, 1 mM EDTA, and 10 mM Tris HCl, pH 7.4). Organelles were isolated by differential centrifugation. Mitochondria and microsomal fractions were collected at 10,000 g (10 min) and 40,000 g (60 min). The supernatant of 40,000 g was considered as cytosol in these experiments. All of these fractions were used immediately or kept at −70°C. Eighty micrograms of protein of each microsome fraction or cytosol fraction was precipitated with 70% ice-cold acetone. The pellets were solubilized in 20 µl of SDS sample buffer and heated in boiling water for 3 min. SDS-PAGE was conducted with 10% gels in a Bio-Rad mini-Gel II apparatus at 200 V for 36 min. Protein was transferred to polyvinylidene difluoride (PVDF) membrane in Tris-glycine buffer and rinsed with Tris-buffered saline twice, then blocked in 4% dry milk in Tris-Tween-buffered saline for 1 h at room temperature.

To measure changes in CYP4A1 protein levels by Western blot analysis, the membranes were incubated with goat anti-rat CYP4A1 serum obtained from Daichi Pure Chemicals (1:500 in 4% dry milk) for 2 h at room temperature. Peroxidase-conjugated rabbit anti-goat IgG (1:10,000 dilution with 4% dry milk) was used as the secondary antibody, followed by ECL staining (Amersham). To measure changes in AOX protein, rabbit antiserum raised against rat AOX was used (19). The primary antibody was diluted 1:1,000 in 4% dry milk with incubation was at room temperature for 4 h. Peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad) was used as the secondary antibody (1:5,000 dilution in 4% dry milk), and the AOX protein was visualized by ECL staining. Western blot images were analyzed and quantitated with Adobe Photoshop 3.0 and the NIH imager program.

Northern blots. Rat kidney total RNA was purified by Polytron sonic disruption of frozen kidney tissue in TRIzol reagent as described by the manufacturer (GIBCO-BRL, Life Technologies), and 30 µg was electrophoresed on a 1% agarose gel containing 2.2 M formaldehyde, transferred to a nitrocellulose membrane using 20 % sodium bicarbonate buffer (pH 10.7) and read immediately, utilizing a fluorescence spectrometer at excitation wavelength of 325 nm and emission wavelength of 425 nm. A blank without the substrate (palmitoyl CoA) was set up for each sample assay. The enzyme activity was expressed as arbitrary units per milligram of protein per minute. The protein content of homogenates was assayed by Bio-Rad reagents as described by the manufacturer.

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(presence of necrotic cells, apparently denuded areas of tubular basement membrane, or ruptured tubular basement membranes); tubular regeneration; mitotic figures in tubular cells; dilatation of Bowman's space with retraction of the glomerular tuft ("acute glomerular ischemia"); loss of PAS-positive tubular brush border; "tubularization" of the parietal epithelium of Bowman's capsule; tubular casts; interstitial inflammation; interstitial edema; tubular dilatation; and prominence of the juxtaglomerular apparatus. The morphological changes were graded on a scale from 0 to 2, where 0 = none, 0.5 = minimal, 1 = mild, 1.5 = moderate, and 2 = marked (11).

I/R on PPARα (−/−) and wild-type mice. Male mice, homozygous wild-type (+/+), or PPARα null (−/−), pure Sv129 background, 10−12 wk of age (13), were housed in plastic cages in a temperature- and light-controlled environment. Groups of mice (3 mice/group) were subjected to either sham or I/R injury. Mice were preanesthetized with isoflurane in a bell jar and then anesthetized using isoflurane discharged through a veterinary small animal flowmeter at 1.5% isoflurane mixed with oxygen. Temperature was regulated through a servo-controlled heating pad and light, with use of a rectal temperature probe. The skin corresponding to the abdominal area was shaved and cleaned. A midline incision was made. The left renal hilum was exposed by gentle traction under loop (×3.5) magnification. The left hilum was encircled with a 5-0 silk and polyethylene PE-90 tubing. Ischemia of the left kidney was confirmed by visualization of color change of the kidney parenchyma. The right renal hilum was similarly exposed and occluded. Occlusion was carried out for 45 min while the mouse continued under isoflurane anesthesia and temperature regulation. After 45 min of ischemia, the clamps were removed, and visual confirmation of reperfusion was made. The abdomen was closed with running 4-0 silk, and the mouse was awakened. Animals were kept in metabolic cages for the remaining time of the experiment and killed after 24 h of reperfusion. Serum creatinine levels were measured using tail vein blood to monitor changes in renal function. One kidney from each mouse was placed into buffered fixative for histological examination.

Statistical analysis. All experiments were performed in at least four animals per each condition. Statistical significance was determined by the paired Student’s test. P < 0.05 was considered statistically significant.

RESULTS

Kidney function was evaluated in animals subjected to sham surgery (controls) and to I/R by determining blood urea nitrogen (BUN) and serum creatinine levels. Compared with rats administered vehicle, rats administered Eto prior to I/R had significantly lower serum creatinine and BUN levels (Fig. 1, A and B). Because oxirane compounds like Eto have been shown to be direct ligands for PPARα (5), we next performed studies in animals pretreated for 5 days with fibrate compounds prior to I/R injury. Two known PPARα activators, clofibrate and WY-14,643 had an effect similar to Eto in protecting kidney function during acute ischemic renal failure. Figure 1C shows that serum creatinine levels were much higher in rats administered vehicle

Fig. 1. A: effect of Etomoxir (Eto), 25 mg/kg body wt, on serum blood urea nitrogen (BUN) and creatinine in rats measured over time; levels of BUN in sham-operated control rats, vehicle-treated rats subjected to ischemia/reperfusion (I/R) injury, and Eto-treated rats subjected to I/R injury as described in EXPERIMENTAL PROCEDURES. *P < 0.05 compared with I/R. B: levels of serum creatinine in rats measured over time; levels of sham-operated control rats (C), vehicle-treated rats subjected to I/R ( ), and Eto-treated rats subjected to I/R injury ( ). Data are means ± SE; n = 9 rats in each experimental group. *P < 0.05 compared with I/R. C: serum creatinine levels in sham-operated control rats (Sham), vehicle-treated rats subjected to I/R (I/R), clofibrate-treated rats subjected to I/R (I/R + Clof), and WY-treated rats subjected to I/R injury (I/R + Wy). Data are means ± SE; n = 5 rats in each experimental group. *P < 0.05 compared with I/R.
(corn oil) subjected to I/R compared with clofibrate and WY-treated rats. Because of the marked improvement on kidney function using Eto and the observation of increased mRNA and protein levels of FAO in kidney tissue using clofibrate and WY (results not shown), the next series of experiments was conducted using Eto-treated animals prior to I/R injury.

In addition to biochemical assessment of kidney function, histopathological alterations in the kidneys were evaluated 24 h after renal perfusion was reestablished, as described in EXPERIMENTAL PROCEDURES. The morphological changes were graded on a scale from 0 to 2 where 0 = none, 0.5 = minimal, 1 = mild, 1.5 = moderate, and 2 = marked as described (28). The results, shown in Fig. 2 and Table 1, demonstrate that rats pretreated with Eto when subjected to I/R injury exhibited minimal to mild tubular necrosis compared with rats not receiving Eto, which exhibited marked tubular necrosis (P < 0.005). The number of casts and the degree of tubular dilatation were significantly reduced by pretreatment with Eto (P < 0.05), as would be expected for rats with less ATN. Thus histological changes correlated with preservation of kidney function (lower serum creatinine and BUN) in animals treated with Eto.

To confirm that Eto administered intraperitoneally was capable of inhibiting renal CPT, CPT activity was measured following I/R in vehicle- or Eto-treated animals. CPT I activity did not change significantly during ischemia; baseline CPT I activity went from 1,195 ± 34 to 1,092 ± 40 dpm·min⁻¹·mg protein⁻¹ at the end of I/R. Pretreatment with Eto for 5 days prior to ischemia resulted in marked reduction (65%) in the levels of CPT I activity compared with ischemic animals pretreated with vehicle alone (Fig. 3). Thus the concentration and route of administration of Eto used in these studies effectively inhibited kidney CPT activity.

Previous studies have demonstrated that I/R injury is accompanied by a reduction in the activities of AOX and CYP4A1. Therefore, to examine the potential effect of Eto on stimulating FAO in the kidney during I/R we evaluated mRNA, protein levels and enzymatic activi-
ties of palmitoyl CoA oxidase. Northern blot analyses of AOX and CYP4A1 mRNAs (Fig. 4, A–C) demonstrated that both mRNAs were reduced by I/R and that pretreatment with Eto restored the levels to those of control rats. Western blot analyses of kidney proteins demonstrated similar changes in protein levels of AOX and CYP4A1 (Fig. 5, A–C). Enzymatic activity (Fig. 6) paralleled the changes in mRNA and protein levels.

To more directly determine the role of PPARα in I/R injury, PPARα null mice were subjected to I/R injury and compared with wild-type mice treated in an identical manner. PPARα null mice have been shown to lack the induction of AOX and CYP4A1 gene transcription when administered peroxisome proliferators or CPT inhibitors (3, 13). Compared with wild-type mice, the PPARα null mice exhibited significantly greater kidney dysfunction after I/R injury, as assessed by higher serum creatinine levels and enhanced tubular necrosis at the corticomedullary junction (Fig. 7, A–C). No differences in baseline creatinines were observed between sham-operated wild-type and null mice.

**DISCUSSION**

Previous studies demonstrated the inhibition of peroxisomal enzymes including AOX and CYP4As during renal ischemia. This inhibition was initially attributed to excessive accumulation of hydrogen peroxide (7, 29). Those studies showed that progressive amounts of ischemia from 30 to 90 min led to 40–50% inhibition of AOX enzyme activity. Reperfusion for 24 h following 90 min of ischemia led to a further decrease (37 and 63%, respectively) in the amount of 72- and 52-kDa protein subunits of AOX compared with controls. Similar observations were reported for CYP4A protein and enzyme activity (29). Our studies also showed ∼50% reduction in enzyme activities of both AOX and CYP4A1 after 45 min of ischemia and 24 h of reperfusion, consistent with previous studies (7, 29). Those earlier studies also concluded that inhibition of peroxisomal β-oxidation enzymes was caused by I/R-induced proteolysis. Our data revealed a significant reduction (60%) in the protein levels of AOX, as well as CYP4A1 in the rat kidney. In addition, a concomitant reduction in the mRNA levels of these two genes was detected after I/R in our studies. Therefore, our results suggest that the inhibition of enzymatic activity of AOX and CYP4A1 which accompanies ischemic acute renal failure (ARF) can also occur as a result of reduced mRNA levels and subsequent decreased protein synthesis of these two enzymes in the rat kidney.

**Fig. 4.** A: effect of ischemia and Eto on microsomal cytochrome P-450 (CYP) 4A1 and peroxisomal acyl CoA oxidase (ACoA; AOX) mRNA levels after ischemic/reperfusion injury. Rats were given daily intraperitoneal injections of vehicle or Eto (25 mg/kg body wt) for 5 days (see EXPERIMENTAL PROCEDURES) and subsequently sham operated or subjected to I/R injury as indicated at top of autoradiograms. Total cellular RNA (30 µg/lane) from the rat kidneys of each experimental condition was used to perform Northern blot analysis using 32P-labeled cDNA probes corresponding to AOX, CYP4A1, and glyceroldehyde-3-phosphate dehydrogenase (GAPDH) as indicated at left. B: laser densitometry scanning of Northern blots for AOX from 4 separate experiments. Isch, ischemia. C: laser densitometry scanning of Northern blots for CYP4A1 from 4 separate experiments. An arbitrary number of 1 was assigned to the density measured in the band corresponding to sham-operated animals. *P < 0.05 compared with I/R.
Previous studies have documented changes in gene expression during ischemic and nephrotoxic renal failure (25). Reduced gene expression has been established for prepro-epidermal growth factor (preproEGF), Tamm-Horsfall protein (TH), and Kid-1 during ischemic ARF (18, 24, 37). These genes are expressed in the distal tubule. Our studies are the first to demonstrate ischemia-induced decreased expression of fatty acid oxidation genes, which are preferentially expressed in the proximal tubule (1). A general paradigm which could explain changes in gene expression is that regeneration following posts ischemic injury recapitulates renal development. Thus cells that enter the cell cycle after an ischemic insult express proteins such as vimentin (30, 36) that are expressed in those cell types only in early stages of renal development. With regard to the regulation of expression of genes encoding peroxisomal FAO enzymes, previous studies have demonstrated that mature oxidative enzyme levels are reached progressively over the first weeks of postnatal life in the developing proximal tubule and that thyroid hormones represent an essential factor in the acquisition of these metabolic patterns that characterize the fully differentiated proximal tubule (35). Thus the reduced expression of each of these peroxisomal FAO genes during renal injury may be a necessary component of the regenerative response that characterizes the "undifferentiated state" of the proximal tubule after I/R injury.

The mechanism by which Eto as well as clofibrate and WY compounds restore mRNA, protein, and enzyme activities of palmitoyl CoA oxidase and CYP4A1 close to those levels observed in animals not subjected to I/R is likely mediated through PPARα. Eto, like other oxirane compounds including tetradecylglycidate (TDGA), can directly bind and activate PPARα (5), leading to increased transcription of genes encoding peroxisomal enzymes. We performed additional studies (Fig. 1C) in which we observed significant protection of kidney function during ischemia when animals were pretreated with clofibrate or WY, two compounds known to induce mRNA levels of peroxisomal and microsomal enzymes via activation of a PPARα nuclear receptor (13). Moreover, renal damage following I/R in PPARα null mice was more pronounced than in identically treated wild-type mice. Together these data indicate PPARα activation as the underlying mechanism whereby Eto, clofibrate, and WY treatment results in protection of renal function after ischemia.

One of the most important effects shown in this study is the remarkable protection Eto affords against necrosis of the proximal tubule during ischemia, as shown by the histological data. All kidneys of rats subjected to I/R treated with vehicle (controls) exhibited marked epithelial necrosis of tubules in the inner cortex/outer stripe of the medulla, the zone corresponding to the S3

Fig. 5. A: effect of ischemia and Eto on protein levels of CYP4A1 and AOX. Rats were treated (as described in Fig. 4) with or without Eto prior to sham operation or subject to I/R injury as indicated above each lane. Total kidney protein (80 µg) was analyzed by Western blot analyses using antibodies recognizing AOX or CYP4A1 as described IN EXPERIMENTAL PROCEDURES. B: laser densitometry scanning of Western blots for AOX from 4 separate experiments. C: laser densitometry scanning of Western blots for CYP4A1 protein from 4 separate experiments. An arbitrary number of 1 was assigned to the density measured in the band corresponding to sham-operated animals. *P < 0.05 compared with I/R.
segment of the proximal tubule (34). This confirms previous observations that S3 cells selectively undergo progressive injury and death following I/R in the rat (34). In contrast to the control animals, Eto-treated rats had only mild necrosis of the tubular epithelial cells. This observation confirms previous in vitro experiments that showed protection against cell death during hypoxia in freshly isolated proximal tubules using oxefencine and glyburide, two compounds known to modulate FAO in kidney tissue (21). In those studies, we demonstrated that CPT inhibition was accompanied by inhibition of α-fodrin proteolysis and decreased accumulation of toxic amphiphilic metabolites. The present data do not provide a definitive cellular mechanism by which preservation of peroxisomal and microsomal FAO enzymes during I/R injury will eventually prevent necrotic cell death. However, this cytoprotection could be the result of a complex interaction between FAO

Fig. 6. Effect of ischemia and Eto on enzymatic activities of palmitoyl CoA oxidase (A) and CYP4A (B). Shown are the results obtained with sham-operated rats, vehicle-treated (open bars) rats subjected to I/R injury, vehicle-treated (solid bars) sham-operated rats treated with Eto as described in Fig. 4 (hatched bars), and rats subjected to I/R injury following Eto treatment (cross-hatched bars). Data are means ± SE; n = 5 for each group.

Fig. 7. Histologic changes after 45 min of ischemia/24 h reperfusion injury in peroxisome proliferator-activated receptor-α (PPARα) wild-type (+/+) (A) and knockout (−/−) mice (B). Histology of cortical medullary junction in kidneys from wild-type mice show mild epithelial swelling with minimal cellular sloughing (A) compared with kidneys for PPARα (−/−) mice (B) showing extensive complete cellular necrosis with loss of epithelial cell lining and occlusion of tubular lumens by sloughed epithelial cells. C: levels of serum creatinine in sham-operated PPARα wild-type mice (1), PPARα wild-type mice subjected to I/R (2), and PPARα null mice subjected to I/R injury (3). Data are means ± SE; n = 5 mice in each experimental group. *p < 0.05 compared with I/R in PPAR null mice.
enzymes and cytoskeletal proteins. Recent proposed models for the regulation of CPT activity in the liver suggest an active role of calmodulin-dependent kinases and cytoskeletal proteins (33).

In addition, an increase on acyl CoA activity leads to the increased catabolism of long-chain fatty acyl CoA compounds. The accumulation of this LCFA metabolite along with long-chain acylcarnitines could potentially lead to inhibition of the Na-K-ATPase and ion transport in kidney cells during ischemia (21). On the other hand, inhibition of CYP4A1 ω-oxidation of fatty acids during ischemia could lead to lack of production of 20-hydroxyeicosatetraenoic acid (20-HETE). This compound has been shown to cause vasoconstriction; therefore, having this compound produced by stimulation of PPARα and CYP4A1 enzyme has the potential to improve glomerular filtration rate during ischemia.

Although the effects observed in in vivo organ studies are usually more difficult to study than the effects observed in isolated tubules, we demonstrated that in both models of ischemic injury (hypoxic tubules and bilateral renal artery occlusion), protection of kidney function and preservation of membrane integrity of proximal tubules is associated with the preservation of FAO enzymes. These studies strongly suggest that the induction of peroxisomal FAO during ischemic injury is important to maintain structure and kidney function. This is particularly important since nuclear receptors such as PPARα and PPARγ are abundantly expressed in distinct nephron segments (2, 39). These nuclear receptors could be modulated by tissue-specific coactivators, corepressors (38), or various ligands including fatty acids. Interestingly, fatty acids and prostaglandins have been shown to be ligands for PPARs (12). Isolation and identification of ischemic-induced regulation of levels of activators, corepressors, or native endogenous PPAR ligands in the kidney should help to elucidate the molecular mechanisms by which I/R leads to downregulation of FAO enzymes and help provide therapeutic strategies to overcome renal injury.

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