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Nutrition and Health Sciences, Department of

2006

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Renal Damage in Experimentally-Induced Cirrhosis in Rats: Role of Oxygen Free Radicals

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Cirrhosis with ascites is associated with impaired renal function accompanied by sodium and water retention. Although it has been suggested that mediators such as nitric oxide play a role in the development of renal failure in this situation, other mechanisms underlying the process are not well understood. This study examined the role of oxidative stress in mediating renal damage during the development of cirrhosis in order to understand mechanisms involved in the process. It was shown that carbon tetrachloride– or thioacetamide-induced cirrhosis in rats results in oxidative stress in the kidney as seen by increased lipid peroxidation and protein oxidation, accompanied by altered antioxidant status. Cirrhosis was also found to affect renal mitochondrial function, as assessed by measurement of the respiratory control ratio, the swelling of mitochondria, and calcium flux across mitochondrial membranes. Increased lipid peroxidation and changes in lipid composition were evident in the renal brush border membranes, with compromised transport of ^{14}C glucose across these membranes. <u>In conclusion</u>, renal alterations produced as a result of cirrhosis in the rat are possibly mediated by oxidative stress. (HEPATOLOGY 2006;43:1248-1256.)

End-stage cirrhosis results in marked alterations in systemic circulation and renal function. The altered systemic circulation is characterized by increased cardiac output, plasma volume, and splanchnic blood flow and by decreased peripheral resistance leading to decreased mean arterial pressure.[1,2] The progressive reduction in renal blood flow and glomerular filtration rate and the impaired ability to excrete sodium and water lead to ascites.[3] Oxidative stress has been implicated in kidney damage in several conditions, including treatment with indomethacin,[4] ischemia reperfusion injury,[5] and diabetic nephropathy.[6] Treatment with antioxidants has also been shown to be effective in ameliorating renal injury in renal artery stenosis[7] and experimentally in renovascular

disease.[8] Our earlier work showed that cirrhosis results in oxidative damage in distal organs such as the intestine.[9] In patients with cirrhosis, renal function deteriorates as liver function worsens, indicating a link between these two organ systems.[10] Apoptotic cell death and increases in adenosine production (potentiating the vascular effects of angiotensin-II) have been proposed to play a role in the development of kidney damage during cirrhosis.[11] Inhibition of nitric oxide synthases significantly improves renal function in cirrhotic animals, suggesting a role for nitric oxide in renal pathophysiological events induced by decompensated cirrhosis.[12] It is now recognized that a number of effects of NO are mediated through formation of peroxynitrite.[13] Generation of peroxynitrite from NO requires the simultaneous presence of superoxide, which is seen during oxidative stress. This led us to hypothesize that the generation of free radicals and oxidative stress during cirrhosis may result in oxidative damage in the kidney. This hypothesis was tested in two experimental models of cirrhosis using carbon tetrachloride (CCl_4) or thioacetamide (TAA) as the hepatotoxin.

Materials and Methods

Adenosine diphosphate (ADP), 3-[4.5-dimethyl thiazol-2-y;]-2,5-diphenyltetrazolium bromide (MTT), 1,1′,3,3′-tetramethoxy propane, Tris(hydroxymethyl) aminomethane (Tris), N-[2-hydroxyethyl]peperazine-n′-

[2-ethanesulfonic acid] (HEPES), thiobarbituric acid (TBA), dithio-bis-(2-nitrobenzoic acid) (DTNB), arsenazo III, succinic acid, iodo-nitro tetrazolium salt (INT), xanthine, xanthine oxidase, *O*-dianisidine dihydrochloride, nicotinamide adenine dinucleotide (NAD), reduced nicotinamide adenine dinucleotide phosphate (NADPH), and hydroxy proline were obtained from Sigma Chemical Co. (St. Louis, MO). Polyethylene glycol (PEG) 4000 was obtained from Fluka AG (Switzerland). ^{14}C-labeled glucose was obtained from Bhaba Atomic Research Center (Mumbai, India). Millipore membranes (pore size 0.45 μ) were obtained from Millipore (India). Carbon tetrachloride and thioacetamide were obtained from Qualigen Fine Chemicals, Ltd. (Mumbai, India), and Spectrochem (Mumbai, India), respectively. All other chemicals and solvents used were of analytical grade.

Animals. Adult Wistar rats of both the sexes (150-200 g), exposed to a daily 12-hour light/dark cycle and fed water and rat chow *ad libitum,* were used for this study. Rats were divided into four groups: Group I—control (n = 6); Group II—phenobarbitone controls (n = 6 for each of the five time periods, 1, 2, 3, 4, and 5 months, total = 30); Group III—phenobarbitone + carbon tetrachloride treated (n = 6 for each time period, total = 30); and Group IV—thioacetamide treated (n = 6 for each time period, total = 30). This study was approved by the Institutional Animal Ethics Committee (IAEC).

Induction of Cirrhosis in Rats. For rats in whom cirrhosis was CCl_4 induced, the CCl_4 was administered intragastrically. Control animals received no treatment. Phenobarbitone controls received 35 mg/dL of tap water, which was the only source of drinking water. The initial dose of CCl_4 was 40 μL/rat, and subsequent doses were adjusted on the basis of change in body weight as described previously.[9] For TAA-induced cirrhosis, rats were administered intraperitoneal injections of TAA (200 mg/kg ip) in saline twice a week for 1, 2, 3, 4, or 5 months. Control rats received the vehicle alone.[15] Animals were sacrificed after 1, 2, 3, 4, or 5 months of CCl_4 or TAA treatment. For each treatment period, TAA-treated animals were sacrificed 1 week after stopping treatment, and CCl_4-treated animals were sacrificed 10 days after stopping phenobarbitone.

Light Microscopy. Liver tissue was fixed in 10% buffered formalin and processed. Four-micron sections were cut and stained with hematoxylin and eosin and observed under a light microscope.

Electron Microscopy. Renal tissue was fixed in 2.5% glutaraldehyde, postfixed in osmium tetroxide, and embedded in araldite (epoxy resin). One-micron-thick sections were cut and stained with toluidine blue. Suitable areas for ultrastructural study were chosen after examin-

ing the 1-μ sections under the light microscope. Ultrathin sections of these areas were cut on an LKB UM4 ultramicrotome with a diamond knife (Diatome, Switzerland). The sections were mounted on copper grids and stained with uranyl acetate and lead citrate. The grids were examined under a Philips EM201C electron microscope (Eindhoven, The Netherlands).

Estimation of Hydroxyproline. Hepatic hydroxyproline content was measured as described previously[16] and expressed as micrograms of hydroxyproline per gram wet weight of liver tissue.

Serum Parameters. Serum separated from blood obtained by direct heart puncture was used for the assay of alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, and total bilirubin as previously described.[17] Serum creatinine, serum osmolality, sodium, and potassium were measured as previously described.[18]

Preparation of Mitochondria and Assessment of Mitochondrial Function. Kidneys removed from the experimental rats were washed with ice-cold saline and decapsulated. Renal tissue was homogenized (5% w/v) in buffer containing 220 mmol/L mannitol, 70 mmol/L sucrose, 5 mmol/L Tris, and 1 mmol/L EGTA (pH 7.4). The homogenate was used for the various assays and for isolation of mitochondria by differential centrifugation as described previously.[19] The final mitochondrial pellet was washed twice with a buffer containing 250 mmol/L sucrose and 5 mmol/L HEPES (pH 7.4) and was suspended in the same buffer. Purity of the mitochondria was assessed by measuring the marker enzyme succinate dehydrogenase. Mitochondrial function was assessed by oxygen uptake, degree of swelling of mitochondria, and MTT reduction as described earlier.[4] Calcium flux was measured by quantitating changes in the absorption spectrum of Arsenazo III at 675/685 nm. Arsenazo III is an impermeable dye that indicates calcium concentration outside mitochondria. On the addition of exogenous calcium to a suspension of mitochondria, absorbance increases initially because of the calcium binding to the dye. However, this is a dynamic process, and as the mitochondria take up calcium through the uniporter, external concentrations of calcium decrease, reflected in a decrease in absorbance. The mitochondria were suspended in a medium containing 250 mmol/L sucrose, 5 mmol/L HEPES, 5 mmol/L succinate, and 40 μM Arsenazo III (pH 7.4). Calcium was added to the reaction medium at a concentration of 10 μmol/L in order to initiate the flux studies.

Preparation of Renal Brush Border Membrane Vesicles and Measurement of D-Glucose Uptake. Renal tissue was minced and a 5% homogenate made in buffer containing 2 mmol/L Tris-HCl and 50 mmol/L

mannitol, pH 7.1, using a Porter-Elvelhem homogenizer, following which the brush border membranes (BBMs) were isolated as previously described.[20] Purity of the isolated BBMs was checked by enrichment of the marker enzyme alkaline phosphatase.[21] Isolated BBMs were assessed for their ability to transport glucose by measuring uptake using the rapid filtration technique at room temperature, as previously described.[22]

Parameters of Oxidative Stress. The renal homogenate, isolated mitochondria, and BBMs were used for the assessment of parameters of oxidative stress. Malondialdehyde (MDA) was measured using the thiobarbituric acid method.[23] The amount of MDA formed was calculated from a standard curve prepared using 1,1′,3,3′ tetra methoxypropane, and values were expressed as nanomoles per milligram of protein. For measurement of conjugated diene, total lipids were extracted as described previously,[24] dissolved in 1 mL of heptane, read at 233 nm, and expressed as nanomoles per milligram of protein using a molar absorption coefficient of 2.52×10^4. Protein carbonyl formed was measured using 2,4-dinitrophenyl hydrazine and calculated using a molar extinction coefficient of 22 mM^{-1}cm^{-1}.[25] Total thiol content was measured using DTNB and expressed as nanomoles per milligram of protein.[26] Protein was estimated by Lowry's method using bovine serum albumin as a standard.[27]

Enzyme Assays. The activity of alkaline phosphatase in the renal BBM was assayed using p-nitro phenyl phosphate as the substrate.[21] Catalase,[28] myeloperoxidase,[29] glutathione peroxidase,[30] and glutathione reductase[31] were measured in the renal homogenate as described and expressed as units per milligram of protein.

Analysis of Lipids. Renal BBM lipids were extracted by the method of Bligh and Dyer.[32] The lower organic phase was concentrated using nitrogen, resuspended in a small volume of chloroform/methanol (2:1), and used for lipid analysis. Neutral lipids were separated on silica gel G plates using the solvent system hexane/diethyl ether/acetic acid (80:20:1, v/v). Spots corresponding to the standard were identified by iodine exposure and eluted. Cholesterol, cholesteryl ester,[33] triacylglycerol, and diacylglycerol[34] were quantitated as described. Fatty acid content was quantitated using gas chromatography as described previously.[35] Individual phospholipids were separated on a silica gel H plate using the solvent system chloroform/methanol/acetic acid/water (60:36:9.6:4.8, v/v) and quantitated by phosphate estimation after acid hydrolysis.[36]

Statistical Analysis. Data are expressed as means ± SD. Statistical analysis was performed using the nonparametric Mann-Whitney test. A P value of less than .05 was t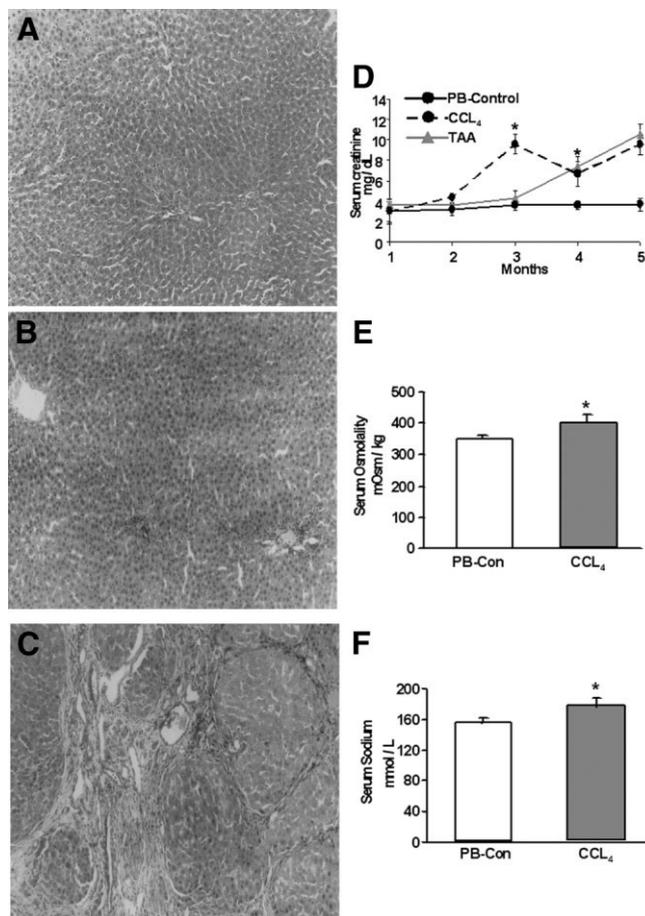aken to indicate statistical significance. Statistical calculations were performed using SPSS software for Windows (version 9.0).

Fig. 1. Light microscopy of liver from (A) control, (B) phenobarbitone control, and (C) CCl$_4$-induced cirrhotic rats after 3 months. (Original magnification ×30.) (D) Serum creatinine in CCl$_4$- or TAA-treated rats at 1, 2, 3, 4, and 5 months compared with that of phenobarbitone-treated controls. (E) Serum osmolality and (F) sodium after 3 months of CCl$_4$ treatment compared with those of the phenobarbitone-treated controls. *$P < .05$ compared to control.

Results

Development of cirrhosis was confirmed by histology and measurement of serum markers of liver injury in animals treated with CCl$_4$ and TAA. Control and phenobarbitone controls showed normal liver architecture after 3 months of treatment (Fig. 1A-B), whereas intragastric administration of CCl$_4$ showed micronodular cirrhosis with extensive fibrosis after 3 months of treatment (Fig. 1C). Serum markers for liver injury such as alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, and total bilirubin were also found to be increased significantly in rats treated with CCl$_4$ after 3 months of treatment (Table 1), accompanied by an increase in liver hydroxyproline content, an indicator of fibrosis (Table 1).

Table 1. Serum Markers for Assessing Liver Function in Carbon Tetrachloride–Treated Rats

	Control	Carbon Tetrachloride Treatment		
		1 Month	3 Months	5 Months
Alanine amino transferase (IU/L)	144 ± 3	158 ± 7	630 ± 46*	689 ± 60*
Aspartate amino transferase (IU/L)	138 ± 2.5	156 ± 6	710 ± 65*	756 ± 52*
Alkaline phosphatase (IU/L)	87 ± 10	97 ± 10	415 ± 44*	410 ± 25*
Total bilirubin (μmol/L)	17 ± 4	18 ± 4	65 ± 3.5*	84 ± 5*
Total protein (g/dL)	9.12 ± 0.24	9.2 ± 0.25	3.4 ± 0.13*	3.2 ± 0.2*
Hepatic hydroxyproline content (μg/g wet weight of liver tissue)	205 ± 14	215 ± 11	913 ± 88*	1160 ± 100*

*$P < .05$ compared with control.

A similar trend was seen in TAA-treated rats.[15] The serum enzymes and liver hydroxyproline remained elevated even after 5 months of treatment, indicating that in this model, frank cirrhosis was established by 3 months of treatment and sustained at 5 months of treatment.

Animals treated with CCl_4 developed ascites after 3 months of treatment, which was observed in 20%, 60%, and 100% of the animal treated with CCl_4 after 3, 4, and 5 months of treatment, respectively, whereas the infection of ascitic fluid was only seen in 60% of the animals after 5 months of CCl_4 treatment. To check for renal function, we looked at the osmolality and the levels of creatinine, sodium, and potassium in the rat serum. Serum creatinine was found to be increased by 3 and 5 months of CCl_4 treatment and by 5 months of TAA treatment. Because the maximum increase in creatinine was observed after 3 months of treatment, we measured serum osmolality, sodium, and potassium in CCl_4-treated rats only for a 3-month period. Serum osmolality and sodium were found to be increased significantly after 3 months of CCl_4 treatment (Fig. 1D-F), but no difference in serum potassium levels was observed (data not shown).

The ultrastructural appearance of renal tissue from control rats treated with phenobarbitone alone appeared to be within normal limits except for minimal swelling of endothelial cells and occasional platelet or fibrin strands in some of the capillary lumina in the glomeruli. After 3 months of phenobarbitone treatment alone, epithelial and mesangial cells appeared to be within normal limits (Fig. 2A). Proximal tubular epithelial cells showed focal dilatation of mitochondria and endoplasmic reticulum, associated with nuclear condensation and chromatin clumping, which is suggestive of focal degenerative change. Some of the tubular epithelial cells showed lipid bodies (Fig. 2B) and reticulated material in their cytoplasm.

Ultrathin sections of kidneys from rats administered CCl_4 for 1-5 months showed more extensive tubular and glomerular changes than did those of controls. After 3 months of CCl_4 treatment, proximal convoluted tubules showed patchy degenerative changes with mitochondrial dilatation (Fig. 2C), nuclear crenation, and chromatic condensation. Lipid, reticulated material, and mixed lipid-reticulated bodies were seen in many of the proximal tubules of rats treated with CCl_4. Additionally, some of
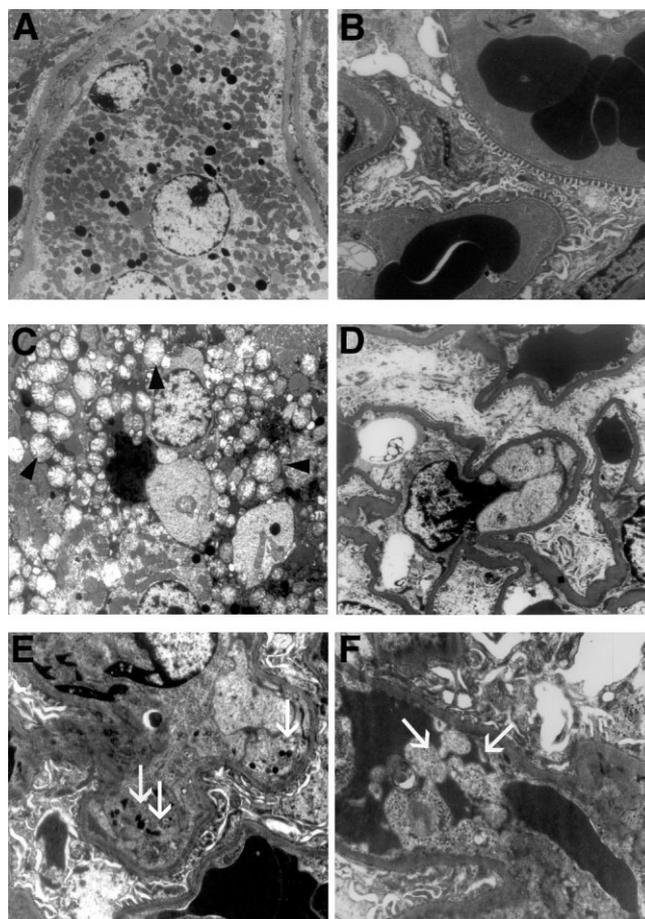
Fig. 2. Electron micrographs of kidney from rats treated with phenobarbitone alone and with CCl_4. Rats treated with phenobarbitone alone for 3 months showing (A) mild mitochondrial dilatation and (B) normal glomeruli. Renal tissue after 3 months of CCl_4 treatment showing (C) mitochondrial dilation (arrow heads), (D) glomeruli changes with endothelial cell swelling, (E) platelets (arrows), and (F) cytoplasmic bleb formation (arrows).
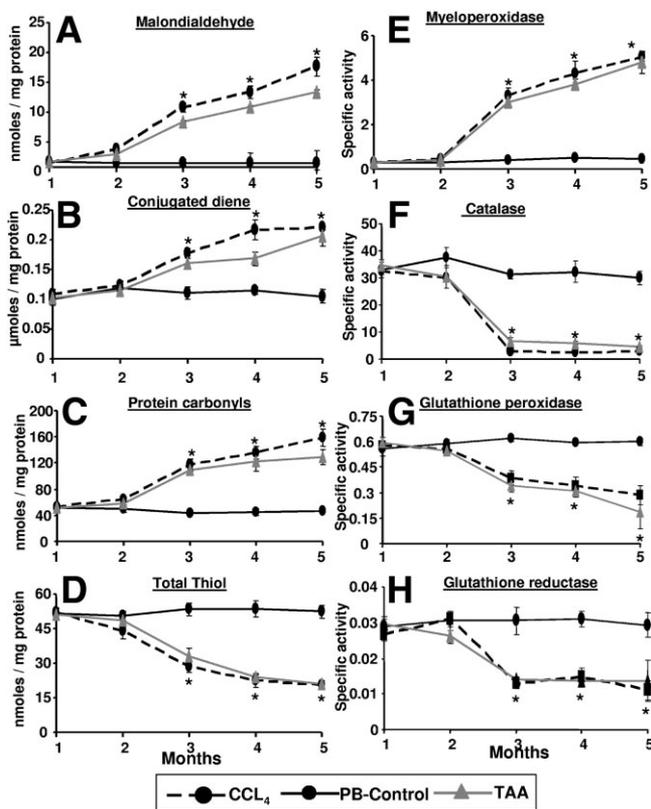
Fig. 3. Oxidative stress parameters—(A) malonaldehyde, (B) conjugated diene, (C) protein carbonyl content, (D) total thiol, (E) activity of myeloperoxidase, (F) activity of catalase, (G) activity of glutathione peroxidase, and (H) activity of glutathione reductase—in renal homogenate from CCl₄- or TAA-treated rats at 1, 2, 3, 4, and 5 months compared with those of the phenobarbitone-treated controls. The assays were done as described in the text. Each value represents the mean ± SD of six separate experiments. *$P < .05$ compared to control.

increase in the kidney after 2 months of CCL₄ or TAA treatment and continued to increase until 5 months of treatment (Fig. 3E). It was seen that catalase, glutathione peroxidase, and glutathione reductase enzyme activities were all decreased significantly after 3 months of CCl₄ or TAA treatment. These changes were apparent up to 5 months of treatment (Fig. 3F-H).

Mitochondria are another important source and target of free radicals in the cell, and mitochondria in kidneys from animals with cirrhosis were studied. There was functional impairment of renal mitochondria in cirrhotic rats, with a decreased respiratory control ratio, increased superoxide production (measured by MTT reduction), altered calcium flux across the mitochondrial membrane, and mitochondrial swelling after 3 months of treatment. These changes were apparent at 5 months as well (Fig. 4A-D). These biochemical measurements complement the electron microscopy results, in which swollen mitochondria were evident in kidneys from animals with cirrhosis (Fig. 2C). Oxidative damage was also seen in renal

these tubules showed cytoplasmic edema. Glomeruli were within normal limits in the first 2 months of treatment but showed focal, segmental endothelial swelling in capillaries at 3 months (Fig. 2D) and later. Visceral epithelial cells were also swollen in some glomeruli. Platelets were seen in some capillary lumina after 3 months of CCl₄ treatment (Fig. 2E). Also, occasional platelet clusters were seen in the peritubular capillaries. Some glomeruli showed focal loss of endothelial fenestration and formation of cytoplasmic blebs (Fig. 2F) as well as intracytoplasmic myelin figures.

Oxidative stress was evident in the kidney, as seen by increases in the parameters of oxidative stress, such as malondialdehyde, conjugated diene, and protein carbonyls, and a decrease in thiol content in cirrhotic animals after 3 months, compared with those in the phenobarbitone controls. These changes were minimal at 1 and 2 months of treatment and were elevated until 5 months of treatment with CCl₄ or TAA (Fig. 3A-D). Myeloperoxidase activity, a marker for neurophil infiltration, began to

Fig. 4. Renal mitochondrial function and oxidative stress parameters—(A) respiratory control ratio, (B) mitochondrial swelling, (C) MTT reduction, (D) calcium flux, (E) malonaldehyde, (F) conjugated diene, (G) protein carbonyl content, and (H) total thiol—from CCl₄-treated rats at 1, 2, 3, 4, and 5 months compared with those of the phenobarbitone-treated controls. The assays were done as described in the text. Each value represents the mean ± SD of six separate experiments. *$P < .05$ compared to control.

Fig. 5.   (A) Alkaline phosphatase activity and oxidative stress parameters (B) malonaldehyde, (C) conjugated diene, (D) protein carbonyl content, and (E) glucose transport in renal brush border membranes isolated from CCl₄- or TAA-treated rats at 1, 2, 3, 4, and 5 months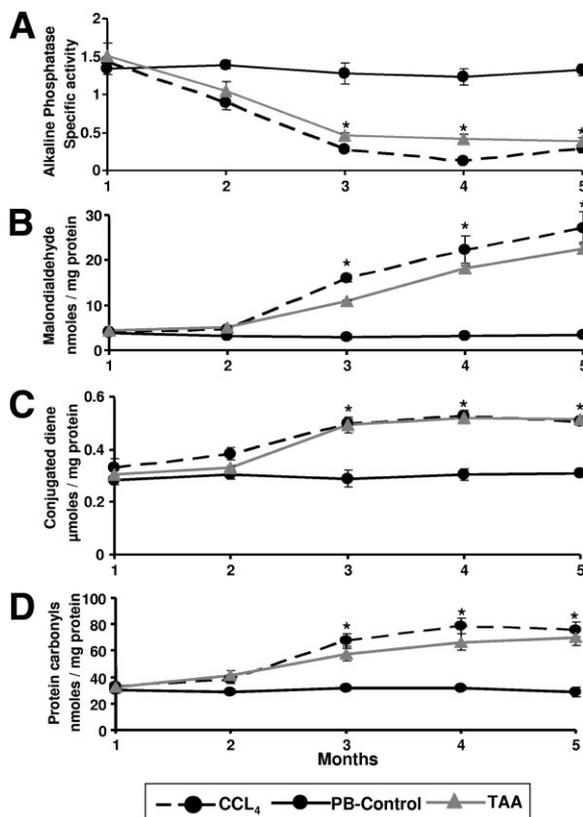 compared with those of phenobarbitone-treated controls. The assays were done as described in the text. Each value represents the mean ± SD of six separate experiments. *$P < .05$ compared to control.

mitochondria from cirrhotic rats, with an increase in malonaldehyde, conjugated diene, and protein carbonyls content along with a decrease in thiol content compared with those in the phenobarbitone controls (Fig. 5E-H).

All these changes were significant in rats treated for 3 months and in those that received CCl_4 treatment for 5 months. All mitochondrial changes were also seen in TAA-treated rats after 3 and 5 months (data not shown). A decrease in alkaline phosphatase activity was seen in renal BBMs after 3 months of CCl_4 or TAA treatment compared with that in the controls. This was accompanied by oxidative stress, as evidenced by an increase in peroxidation parameters (Fig. 5A-D). Renal BBM lipids isolated from cirrhotic rats showed a decrease in triacylglycerol and cholesterol content along with an increase in diacylglycerol after 3 months of treatment compared with those in the controls (Table 2). The levels of total free fatty acids in the isolated renal BBMs of cirrhotic rats were increased after 3 months of treatment as compared with those in the controls (Table 2). Total phospholipids levels were decreased in renal BBMs in cirrhotic animals after 3 and 5 months of CCl_4 treatment compared with those in the controls. Among the individual phospholipids, a decrease in phosphatidylethanolamine and phosphatidylcholine accompanied by an increase in lysophosphatidylethanolamine and lysophosphatidylcholine was seen (Table 2). The ratio of total cholesterol to total phospholipids was lower in the BBMs isolated from cirrhotic rats compared with that in the controls (Table 2). All the lipid changes were still observed even after 5 months of CCl_4 administration. The functional integrity of the renal BBM is also affected in cirrhosis, because the results showed the ability to transport ^{14}C glucose was impaired significantly at 3 months of CCl_4 administration compared with that in the control, an effect that was seen even at 5 months of treatment (^{14}C D-glucose uptake at 20 seconds expressed as picomoles per milligram of protein in controls versus CCl_4-treated rats for 1, 3, and 5 months was 155 ± 5.3 versus 140 ± 9.7, 102 ± 8.7, and 95 ±

Table 2. Lipid Composition of Renal Brush Border Membranes after Various Durations of CCl₄ Treatment

| | Control | Carbon Tetrachloride Treatment | | |
		1 Month	3 Months	5 Months
Neutral lipids (nmol/mg protein)				
Triacylglycerol	58 ± 3	58 ± 4	16 ± 4*	7.8 ± 2*
Diacylglycerol	20.5 ± 2	20 ± 3	31.25 ± 3*	29.3 ± 3*
Cholesterol	205 ± 10	200 ± 11	32.5 ± 4*	25.94 ± 2.9*
Cholesterol esters	76.7 ± 4	64 ± 2	70 ± 3	69.36 ± 5
Total free fatty acids	73.36 ± 4.1	86.27 ± 17.3	184 ± 17*	256.3 ± 17*
Phospholipids (nmol/mg protein)				
Total phospholipids	422 ± 7.2	426 ± 5	380 ± 6.5*	353 ± 7*
Phosphatidylcholine	144 ± 8	146 ± 9	121 ± 7*	82.6 ± 6*
Phosphatidyl-ethanolamine	118 ± 8	123.8 ± 7	89.6 ± 5.6*	77.5 ± 4.5*
Lysophosphatidyl-choline	6.25 ± 1	6.4 ± 1	10.49 ± 1.4*	13.9 ± 1.2*
Lysophosphatidyl-ethanolamine	5.25 ± 1	6.58 ± 1.1	10.8 ± 1.5*	18.4 ± 1.3*
Cholesterol:phospholipid ratio	0.67 ± 0.05	0.619 ± 0.04	0.269 ± 0.03*	0.26 ± 0.03*

*$P < .05$ when compared to control

4.5, respectively, $P < .05$). The lipid alterations as well as functional changes seen in the BBMs were also seen in TAA-treated rats (data not shown). All these changes were not prominent in the early stages of cirrhosis after 1 and 2 months of treatment. There was no significant difference between the control and phenobarbitone controls in all the assessed biochemical parameters .

Discussion

Renal failure has been observed in end-stage cirrhosis because of intense renal vasoconstriction that frequently develops in patients with cirrhosis and ascites.[1] The worsening of renal function and sodium and water retention in cirrhosis not only correlates with the elevation in plasma vasopressin, renin, aldosterone, and norepinephrine, but also with the degree of portal hypertension.[1,10] Nitric oxide and prostacyclin have been suggested to play roles in renal damage during end-stage cirrhosis.[37,38] In addition, oxygen free radicals have been implicated in a wide variety of renal diseases, including cyclosporine-induced nephrotoxicity[39] and diabetic nephropathy.[6] Though the administration of antioxidants ameliorated renal dysfunction and increased survival rates in patients with hepatorenal syndrome,[4] the role of oxygen free radicals in mediating renal failure during cirrhosis has not been well examined. This is important because these active species can initiate a cascade of events that can have far-reaching consequences. In fact, we previously demonstrated in an animal model that cirrhosis results in oxidative stress in distant organs such as the intestine,[9] and this may also affect bacterial translocation from the lumen of the gut (unpublished observation). Oxidative stress gains additional importance in the context of renal failure in cirrhosis, because radicals such as superoxide can interact with nitric oxide to generate peroxynitrite, which is an oxidant and a nitrosating agent.[14,41] Studies have shown that nitric oxide synthase is increased in renal glomeruli of rats with cirrhosis.[42,43]

Intragastric and intraperitoneal administration of CCl_4 and TAA, respectively, resulted in development of cirrhosis by 3 months of treatment, with disruption of normal liver architecture and fibrosis and fatty changes, accompanied by elevation in serum markers of liver function. Significant oxidative stress was evident in the kidney from cirrhotic animals, as seen by the increase in lipid peroxidation parameters, evident by 3 months, when frank cirrhosis was established. It has been demonstrated that experimental cholestatic liver disease is associated with increased lipid peroxidation in the kidney, brain, and heart, suggesting that oxidative stress in cholestatic liver disease is a systemic phenomenon, probably encompassing all tissues and organs, even those separated by the

blood-brain barrier.[44] Our data now suggest that this may also hold true in cirrhosis, at least in the intestine[9] and kidney.

Circulatory dysfunction in cirrhosis is predominantly a result of arterial vasodilation in the splanchnic circulation secondary to portal hypertension.[10] Activation of the vasoconstrictor system such as the renin-angiotensin-aldosterone system, the sympathetic nervous system, vasopressin, and endothelin has also been associated with renal damage in cirrhosis.[45] It has been suggested that oxidative stress can have profound effects on hemodynamics and renal function and markers of oxidative injury such as F2-isoprostanes, and thiobarbituric acid reactive substances are increased in renal venous circulation of bile duct–ligated rats.[46] Treatment with antioxidants such as vitamin E prevents the renal dysfunction and normalized mean arterial pressure, renal blood flow, glomerular filtration rate, and sodium and water retention in these animals,[46] suggesting that oxidative stress functioned upstream of all these alterations.

In the present study, the increase in serum creatinine after CCl_4 or TAA treatment suggests compromised renal function in cirrhotic rats. Interestingly, the elevation in serum creatinine was accompanied by an increase in serum sodium and serum osmolality. Though hyponatremia from sodium and water retention is common in end-stage cirrhosis in humans, hypernatremia has been reported in patients with hepatic failure.[47]

Ultrastructural studies of the kidneys of animals treated with CCl_4 showed mild mitochondrial dilatation as well as tubular and glomerular changes in the first 2 months of treatment. From 3 months on swelling of endothelial cells in the glomeruli was observed, which persisted until 5 months. Endothelial cytoplasmic blebs in the glomeruli suggest damage of these cells after 3 months of CCl_4 treatment. Platelets and occasional platelet clusters were also seen in the capillary lumina after 3 months of CCl_4 treatment, suggesting damage to the vascular endothelial cells in the glomeruli.

The kidney has a number of sources of free radicals, including neutrophil infiltration and mitochondrial dysfunction. These were then examined in the kidney during cirrhosis. Estimation of the activity of myeloperoxidase indicates that neutrophil infiltration occurs in the kidney by 3 months of treatment. These neutrophils may be one source of free radicals, which ultimately lead to the lipid peroxidation observed, because activated neutrophils generate oxygen free radicals and have been proposed to be a major cause of the cell damage associated with many chronic inflammatory diseases.[48] Another important source, as well as target for free radicals, is the renal mitochondria, which are also affected in cirrhosis. The uncou-

pling of mitochondrial respiration and oxidative phosphorylation in the kidney, resulting in the generation of superoxide anions, has been observed in cirrhotic animals, suggesting this may be an alternate source of free radicals in the kidney during the development of cirrhosis. A shift in the balance between free-radical production and scavenging ability in tissues is a mechanism of oxidative injury. The decreased levels of free-radical scavenging enzymes such as catalase, glutathione reductase, and glutathione peroxidase in the kidney in the cirrhotic rats is further evidence of oxidative stress produced in the tissue.

The renal BBM is involved in excretion and absorption of water, electrolytes, and small molecules across the physiochemical gradient, and damage to this important component could have far-reaching consequences for renal function. Lipids are important components of biomembranes, and any alteration in their composition may bring about structural and functional changes in the membrane. Biomembranes are major targets of oxidative stress, and it was found that the renal BBMs of cirrhotic rats showed increased levels of the products of peroxidation, again indicative of oxidative damage to the kidney during cirrhosis. This damage to the BBMs was associated with altered transport function and lipid composition in cirrhotic animals by 3 months of treatment that persisted until 5 months of treatment. The decreases in the levels of phosphatidylcholine and phosphatidyethanolamine, along with the increases in the levels of the corresponding lysophospholipids and free fatty acids in the renal BBMs of cirrhotic animals, suggest the activation of the enzyme phospholipase A_2. It was previously demonstrated that oxidative stress in the kidney can result in activation of phospholipase A_2.[4] The decreased cholesterol/phospholipids ratio seen in the BBMs isolated from cirrhotic rats suggests increased fluidity of the membranes. Previous studies from our laboratory have shown that oxygen free radicals generated from ischemia/reperfusion injury increased the fluidity of the intestinal brush border membrane.[49] It is also known that oxidative stress has been suggested as being involved in renal BBM damage in diabetes[50] and indomethacin toxicity,[4] and our data indicate that cirrhosis-induced renal oxidative stress may have functional consequences of compromising transport function across renal BBMs. The renal oxidative stress observed in the CCl_4- and the TAA-treated rats after 3 months of treatment was significant, and renal complications of cirrhosis such as hepatorenal syndrome develop only during the end stage of the disease. This suggests that oxidative damage in the kidney may occur prior to development of complications such as hepatorenal syndrome.

All the changes seen in the kidney *per se*, as well as in renal mitochondria and renal BBM were minimal after the first and second months of treatment with this hepatotoxin and only became evident after the development of frank cirrhosis, which occurred at 3 months. This suggests that initiation of kidney damage is a later event linked to the progression of cirrhosis and rules out the possibility that the changes seen in the kidney are a direct effect of the hepatotoxin used in the model.

In conclusion, this study has shown that cirrhosis has far-reaching consequences, resulting in the generation of free radicals and oxidative damage in the kidney. Neutrophil infiltration and mitochondrial dysfunction probably contribute to the generation of free radicals locally, which in turn result in extensive lipid peroxidation. This affects functional components of the kidney such as the brush border membrane, which could have severe consequences on organ function. These results suggest that oxygen free radicals might play an important role in the development of complications of cirrhosis such as renal damage.

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