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HEPATOLOGY

Oxidative stress in experimental liver microvesicular steatosis: Role of mitochondria and peroxisomes

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Abstract

Background: Hepatic microvesicular steatosis is a clinical manifestation seen in a number of liver diseases. Although the role of mitochondrial β-oxidation in the development of the disease has been well studied, information on lipid peroxidative damage in liver subcellular organelles is scarce. The present study looked at oxidative stress in hepatic peroxisomes and microsomes in microvesicular steatosis, using an animal model of the disease.

Methods: Rats were given i.p. injections of sodium valproate (700 mg/kg bodyweight) to induce microvesicular steatosis, which was confirmed by histology.

Results: Oxidative stress was evident in liver in steatosis, accompanied by structural and functional alterations in hepatic mitochondria. Alterations in lipid composition, with decreased phosphatidyl choline and ethanolamine and increased lysophosphatidyl choline and ethanolamine, were seen. An increase in triglyceride content was also seen. In addition, increased lipid peroxidation was also evident in peroxisomes and microsomes from steatotic rats. Pretreatment with clofibrate results in partial reversal of changes produced by valproate.

Conclusions: These results suggest that in addition to impaired mitochondrial β-oxidation, oxidative stress is also seen in the hepatic peroxisomes and microsomes during microvesicular steatosis.

Introduction

Hepatic microvesicular steatosis is a pathologic hallmark of disorders such as acute fatty liver of pregnancy, Reye's syndrome, congenital urea cycle enzyme defects and Jamaican vomiting sickness.[1] Microvesicular steatosis occurs as a result of impaired mitochondrial β-oxidation, which in turn leads to accumulation of fatty acids in the form of triglycerides and formation of small lipid droplets in the cytosol of hepatocytes.[2] Fatty acid oxidation in the liver can occur both in mitochondria and peroxisomes. However, unlike mitochondria, peroxisomal β-oxidation generates hydrogen peroxide and is not coupled with phosphorylating systems.[3] The changes in mitochondrial β-oxidation during microvesicular steatosis have been well studied.[4,5] Deficiency of long chain 3-hydroxyl-acyl-coenzyme A-dehydrogenase (3-hydroxyl-acyl-CoA-dehydrogenase) has been reported in patients with acute fatty liver of pregnancy,[6] and ultrastructural studies have shown enlarged mitochondria in patients with microvesicular steatosis.[7] In addition, mitochondrial DNA deletions have also been demonstrated in these patients.[8] The genes encoding peroxisomal and microsomal fatty acid-metabolizing enzymes in the liver are transcriptionally regulated by peroxisome proliferator-activated receptor-α (PPAR-α) and deficiencies of the enzymes of peroxisomal β-oxidation have been recognized as important causes of disease.[9] Clofibrate, a peroxisome proliferator that is commonly used as a hypolipidemic drug, is known to induce PPAR-α.[10] However, peroxisomal and microsomal changes have not been examined in detail in this clinical condition. This is important, because peroxisomal and microsomal oxidation can result in generation of free radicals. Increased oxidation of the accumulated fat as seen in steatosis might possibly lead to oxidative stress. The present study looked at the oxidative stress in liver subcellular organelles such as peroxisomes and microsomes in experimental microvesicular steatosis and the effect of pretreatment with clofibrate on this.

Methods

The following were obtained from Sigma Chemical (St Louis, MO, USA): adenosine diphosphate (ADP); sodium valproate (VPA); ethyl 2(4-chloro-phenoxy isobutyrate (clofibrate); dimethyl sulfoxide (DMSO); 3-(4.5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT); 1,1′,3,3′-tetramethoxy propane; Tris(hydroxymethyl) aminomethane (Tris); *N*-(2-

hydroxyethyl) piperazine-N′-(2-ethanesulfonic acid) (HEPES); bovine serum albumin (BSA); thiobarbituric acid (TBA); 5,5′dithio-bis-(2-nitrobenzoic acid) (DTNB); 2,4-dinitrophenyl hydrazine (DNPH); Arsenazo III; succinic acid; glucose 6-phosphate monosodium salt; iodonitro tetrazolium salt (INT); xylenol orange; and lipid standards. All other chemicals used were of analytical grade.

Animals

Adult female Wistar rats (175–225 g) exposed to a daily 12-h light–dark cycle and fed water and rat chow ad libitum were used. This study was approved by the Animal Experimentation Ethics Committee of the institution. Rats were divided into four groups (I–IV) and each group consisted of six animals. Group I, control; group II, clofibrate alone; group III, valproate alone; group IV, valproate plus clofibrate.

Induction of hepatic microvesicular steatosis

Rats were given i.p. injection of sodium valproate, at a concentration of 700 mg/kg bodyweight in saline for 5 consecutive days.[11] This duration and dose was standardized at our laboratory at the Department of Gastrointestinal Sciences, Christian Medical College, Vellore, India. Control animals were injected with the vehicle alone for 5 days. On the sixth day, animals were killed and liver was used for histology and biochemical studies.

Clofibrate administration

Rats were treated i.p. with 200 mg/kg bodyweight per day clofibrate in coconut oil for 5 days. From the 6th to the 10th day, sodium valproate was also given along with clofibrate, while controls received clofibrate alone for 10 days.

Histology

For light microscopy, liver tissue obtained from the right lobe 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. For ultrastructural studies, liver tissue was fixed in 2.5% glutaraldehyde, post-fixed 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 examining the 1-μm 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).

Isolation of liver mitochondria, peroxisomes and microsomes

The liver was excised, washed and homogenized with 8 volumes of buffer containing 230 mmol/L mannitol, 70 mmol/L sucrose, 3 mmol/L HEPES and 1 mmol/L ethylenediamine tetra-acetic acid (EDTA) pH 7.4. Mitochondria, peroxisomes and microsomes were isolated by differential centrifugation. Briefly, homogenate was first centrifuged at 600 *g* for 10 min to remove cell debris and mitochondria were pelleted at 15 000 *g* for 5 min and washed twice with mitochondrial suspension buffer containing 230 mmol/L mannitol, 70 mmol/L sucrose, and 3 mmol/L HEPES pH 7.4.[12] The post-mitochondrial supernatant was then centrifuged at 39 000 *g* for 10 min to isolate the fraction including peroxisomes, which was resuspended in 250 mmol/L sucrose containing 1 mmol/L EDTA and 10 mmol/L Tris HCl (pH 7.3). This suspension was again centrifuged at 15 000 *g* for 10 min to remove mitochondrial contamination, following which, the supernatant was again centrifuged at 39 000 *g* for isolating peroxisomes.[13] The post-peroxisomal supernatant was centrifuged at 105 000 *g* for 1 h to pellet the fraction containing microsomes, which was finally suspended in 1.15% isotonic KCl solution.[14] Purity of the isolated subcellular fractions such as mitochondria, peroxisomes and microsomes was checked by enrichment of the marker enzymes such as succinate dehydrogenase,[15] catalase[16] and glucose 6 phosphatase,[17] respectively.

Assessment of mitochondrial function

Mitochondrial function was assessed by oxygen uptake, mitochondrial swelling, MTT reduction, and calcium flux measurements. Oxygen uptake was determined polarographically using a Clark-type electrode in 3 mL respiration medium (150 mmol/L sucrose, 1 mmol/L KH_2PO_4, 10 mmol/L Tris, 5 mmol/L $MgCl_2$, 20 mmol/L KCl, pH 7.4) containing 5 mmol/L succinate as respiratory substrate. A mitochondrial protein of 1–2 mg/mL was used. Oxygen uptake was stimulated with 0.3 mmol/L ADP. Oxygen uptake during both state 3 (in presence of ADP and succinate) and state 4 (in presence of succinate alone) respiration was measured and the ratio of state 3/state 4 respiratory rate was used to calculate the respiratory control ratio.[18] A mitochondrial protein corresponding to 100–200 μg in suspension buffer without the addition of calcium, was used to determine mitochondrial swelling by measuring the decrease in absorbance at 540 nm up to 7 min and change in absorbance expressed per mg protein.[19] The MTT reduction was performed using a microplate reader as described.[20] Briefly, in a total volume of 150 μL in each well, mitochondria corresponding to 150–200 μg protein were taken, 6 μL of 1.25 mmol/L MTT was added, and the volume was made up with 25 mmol/L phosphate-buffered saline. Plates were incubated at 37°C for 20 min followed by addition of 150 μL dimethyl sulfoxide and mixed thoroughly to dissolve the formazan formed. The plates were read on a multiwell scanning spectrophotometer at 570 nm. The amount of MTT formazan formed was calculated from the standard curve prepared using authentic MTT formazan. Calcium flux measurement was done by quantifying 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 addition of exogenous calcium to a suspension of mitochondria, there is an initial increase in absorbance due to binding of calcium to the dye. However, this is a dynamic process and, as the mitochondria take up calcium through the uniporter, there is a decrease in external concentrations of calcium, reflected in a decrease in absorbance. Mitochondria were suspended in a medium containing 230 mmol/L mannitol, 70mmol/L sucrose, 5 mmol/L HEPES, 5 mmol/L succinate and

40 μmol/L Arsenazo III, pH 7.4. A concentration of 10 μmol/L calcium was added to the reaction medium to initiate the flux studies.[21]

Oxidative stress parameters

The liver homogenate, peroxisomes and microsomes were assessed for oxidative stress parameters. Malonaldehyde (MDA) was measured using the thiobarbituric acid method.[22] The amount of MDA formed was calculated from the standard curve prepared using 1,1′,3,3′tetramethoxypropane and values were expressed as nmoles/mg protein. For conjugated diene measurements, total lipids were extracted as described,[23] dissolved in 1 mL heptane, read at 233 nm and expressed as nmoles/mg protein using a molar absorption coefficient of 2.52×10^4.[24] Hydrogen peroxide was measured using xylenol orange and expressed as nmoles/mg protein.[25] Protein carbonyl content formed was measured using 2,4-dinitrophenyl hydrazine and calculated using an extinction coefficient of 22/mmol·cm.[26] Protein thiol and total thiol content were measured using DTNB and expressed as nmoles/mg protein.[27] Protein was estimated by Lowry's method using bovine serum albumin as a standard.[28]

Enzyme assays

Catalase activity was estimated by measuring the change in absorbance at 240 nm using hydrogen peroxide as substrate and expressed as units per mg protein[16] (units are expressed as μmol/min).

Lipid analysis

Mitochondrial, peroxisomal and microsomal lipids were extracted by the method of Bligh and Dyer[23] and 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 esters,[29] triacyl glycerol and

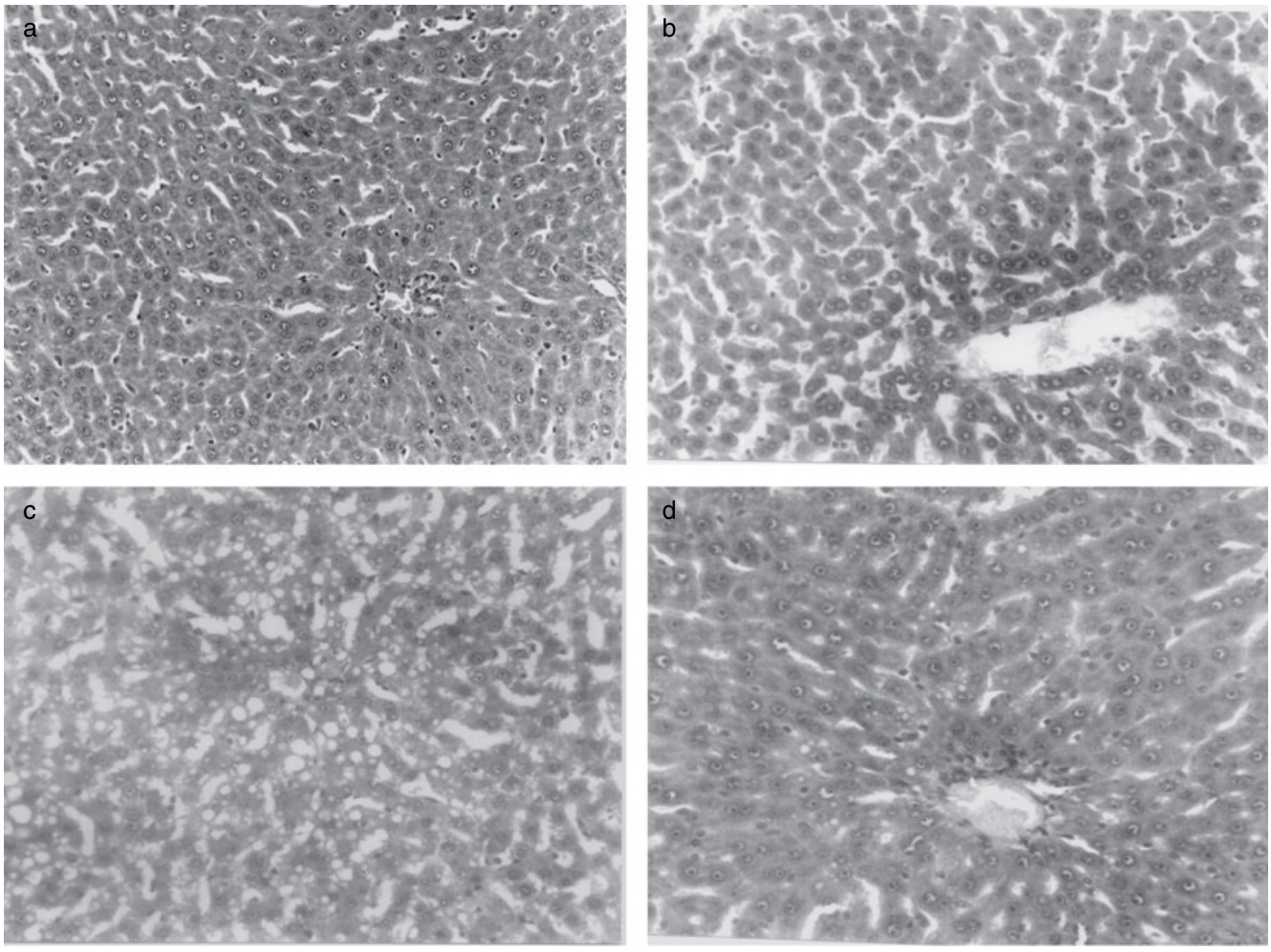

Figure 1 Light microscopy of liver (HE) from control rats treated with (a) vehicle alone, (b) clofibrate alone, (c) sodium valproate alone, showing extensive microvesicular steatosis and (d) clofibrate with valproate. Original magnification ×65.

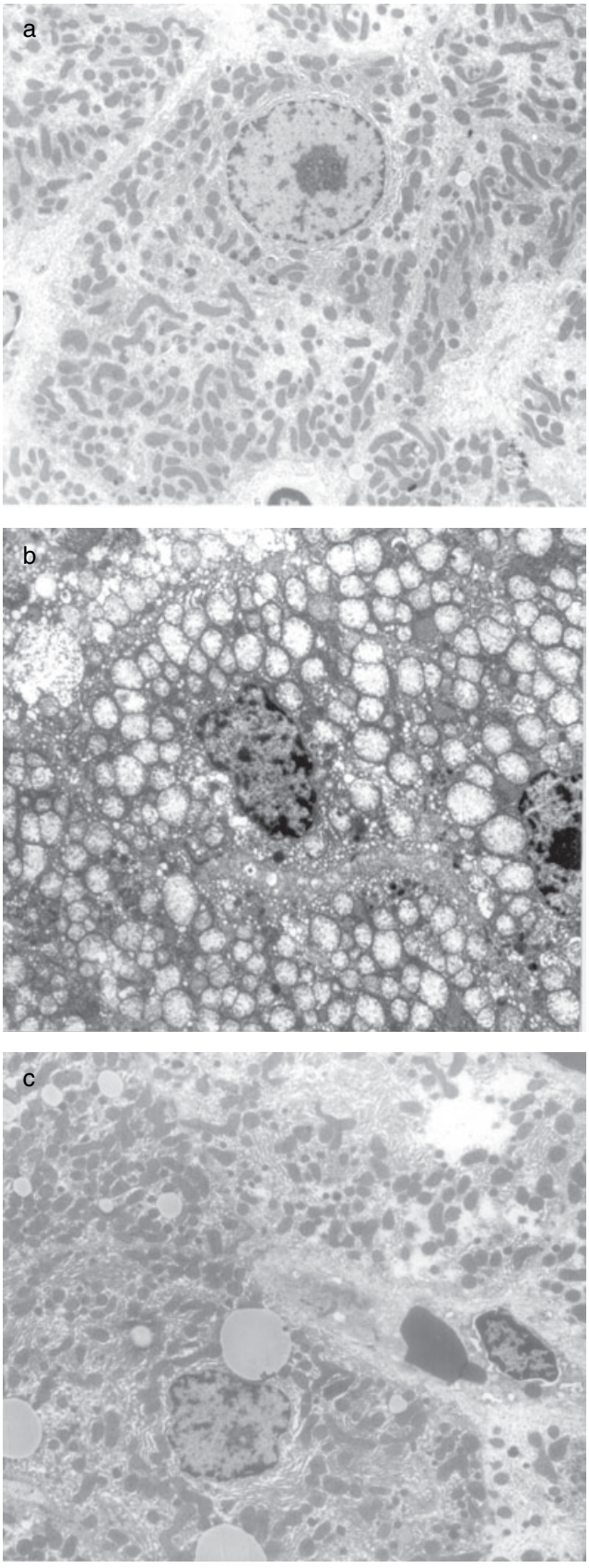

Figure 2 Electron micrographs of liver from clofibrate alone showing (a) normal mitochondria, (b) sodium valproate, showing mitochondrial dilatation and (c) clofibrate with valproate illustrating protection against mitochondrial dilatation. Original magnification ×975.

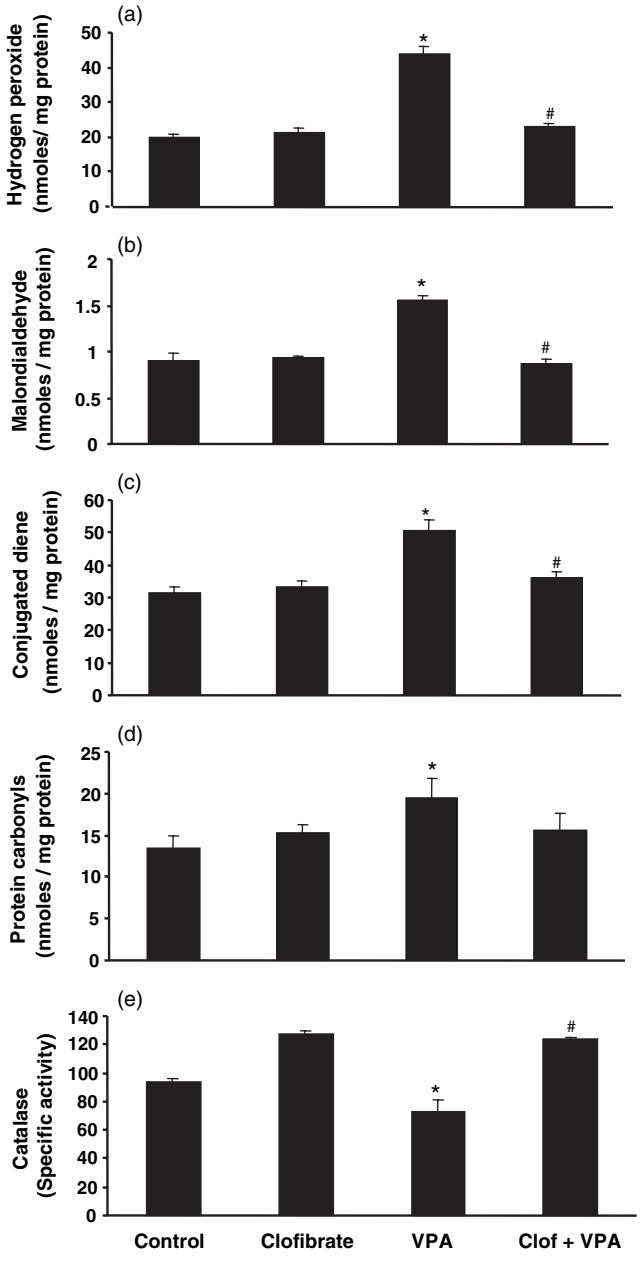

Figure 3 Oxidative stress parameters: (a)hydrogen peroxide, (b) malonaldehyde, (c) conjugated diene, (d) protein carbonyl content and (e) catalase in liver homogenate after various treatments. Each value represents mean ± SD from six separate experiments. *$P < 0.05$, when compared to control, #$P < 0.05$, when compared to valproate-treated rats. Clof, clofibrate; VPA, valproate.

diacyl glycerol[30] were quantified as described. 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 quantified by phosphate estimation after acid hydrolysis.[31]

Statistical analysis

Data are expressed as mean ± SD. Statistical analysis was performed with the non-parametric Mann–Whitney test. Standard calculations were performed using SPSS version 9.0 (SPSS, Chicago, IL, USA).

Results

Administration of valproate for 5 days resulted in predominantly microvesicular and focal macrovesicular steatosis in the liver. Lipid vesicles are evident in 50–70% of the hepatocytes (Fig. 1c). Lipid vesicle accumulation was prevented by clofibrate administration, where only 10–20% of hepatocytes were affected (Fig. 1d). Livers from control animals and those treated with clofibrate alone showed normal liver architecture with normal hepatocytes (Fig. 1a,b). On ultrastructural studies, the hepatocytes of control rats treated with clofibrate alone showed no significant change in mitochondria or other cellular organelles (Fig. 2a). Scattered hepatocytes in the liver of rats treated with valproate showed extensive mitochondrial dilatation and nuclear shrinking with condensation of chromatin. This change was prominent in periportal hepatocytes. Predominant microvesicular and focal macrovesicular fat droplets were also seen in many of the hepatocytes (Fig. 2b). The hepatocytes in the liver of rats treated with valprotate + clofibrate showed a milder degree of steatotic change than rats treated with valproate alone and did not show any evidence of mitochondrial alteration (Fig. 2c).

The role of oxidative stress in this condition was then examined in liver homogenates and, as seen in Fig. 3, a significant increase

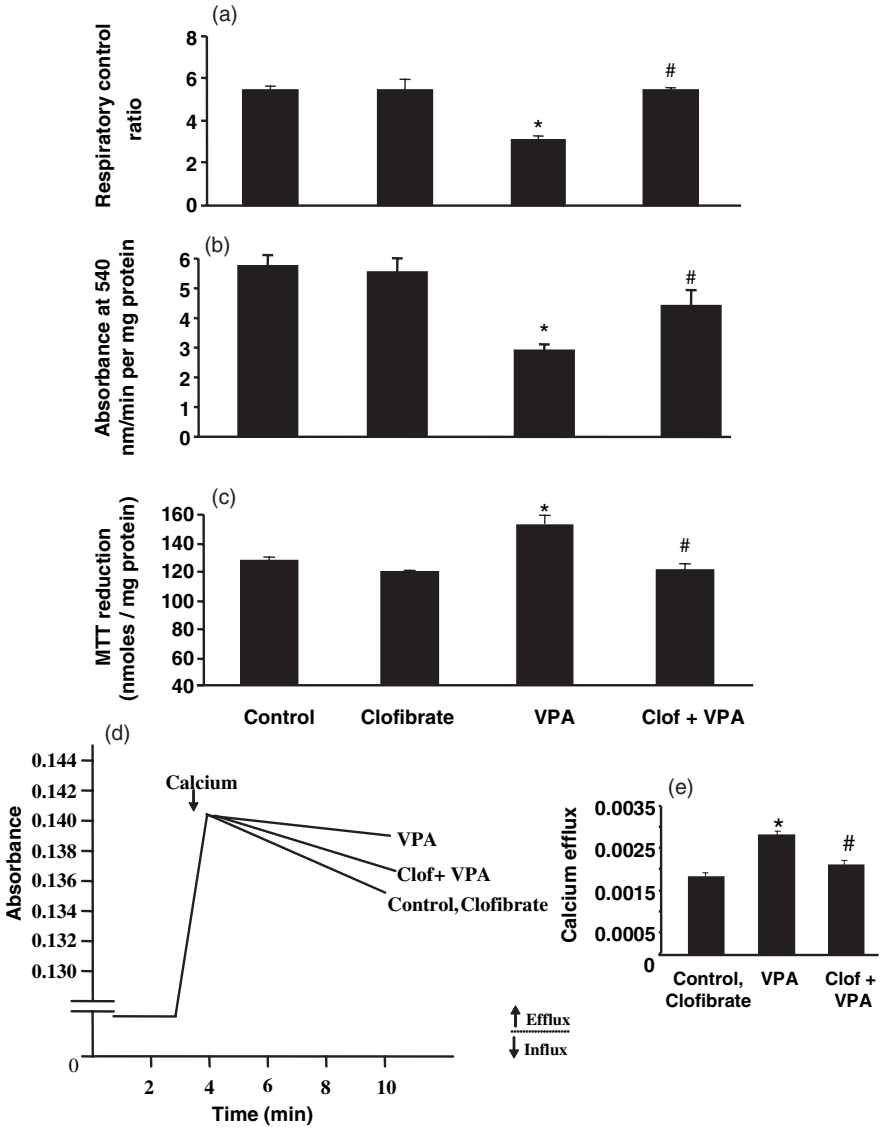

Figure 4 (a) Respiratory control ratio, (b) mitochondrial swelling, (c) MTT reduction, (d) calcium flux and (e) quantitative assessment of calcium efflux in the mitochondria isolated from liver after various treatments. Each value represents mean ± SD from six separate experiments. For calcium flux measurements, the representative trace from six different experiments is illustrated. *$P < 0.05$, when compared to control, #$P < 0.05$, when compared to valproate-treated rats. Clof, clofibrate; MTT, 3-(4.5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide; VPA, valproate.

in hydrogen peroxide (Fig. 3a), malonaldehyde levels (Fig. 3b), conjugated diene (Fig. 3c) and protein carbonyl (Fig. 3d) concentration were evident in animals with steatosis. This was accompanied by a decrease in levels of the antioxidant enzyme, catalase, in rats with steatosis as compared to control (Fig. 3e). Clofibrate pretreatment prevented generation of oxidative stress seen in the animals with steatosis (Fig. 3).

Because mitochondrial β-oxidation is implicated in the etiology of the disease, functional parameters in this organelle were assessed in livers from animals with steatosis and compared to controls. Decreased respiratory control ratio, along with increased mitochondrial swelling and MTT reduction as well as altered calcium flux were seen in mitochondria isolated from liver in steatotic rats compared to control (Fig. 4) and these alterations were again prevented by pretreatment with clofibrate (Fig. 4). These observations were supported by ultrastructural changes as shown in Fig. 2. The calcium flux measurements shown is a representative experiment done in triplicate. Mitochondrial lipid analysis showed increased levels of diacyl glycerol, cholesterol esters and triacyl glycerol, accompanied by a decrease in free cholesterol in microvesicular steatosis. Among phospholipids, phosphatidyl choline and phosphatidyl ethanolamine were decreased, while lysophospholipids were increased. Pretreatment with clofibrate results in the partial reversal of lipid alterations (Table 1).

Impaired β-oxidation in the mitochondria can lead to fatty acid oxidation in peroxisomes and generation of reactive oxygen species. An increase in malonaldehyde, conjugated diene and protein carbonyl levels accompanied by a decrease in thiol content were observed in peroxisomes from rats with steatosis when compared to controls. These changes in lipid and protein oxidation products were prevented by clofibrate pretreatment (Fig. 5). Clofibrate treatment by itself increased activity of the antioxidant enzyme catalase, which is predominantly present in peroxisomes. This is expected, due to the peroxisomal proliferation induced by the drug. However, catalase activity was decreased in the liver from valproate-treated animals as compared to control, although clofibrate pretreatment before valproate administration restored the activity of the enzyme. Analysis of peroxisomal lipid composition in animals with steatosis showed a small increase in diacylglycerol and triacylglycerol along with a twofold increase in free cholesterol. A decrease in phosphatidyl choline and phosphatidyl ethanolamine along with an increase in lysophosphatidyl choline and lysophosphatidyl ethanolamine were also seen. Pretreatment with clofibrate prevented these lipid alterations, although interestingly, treatment with the drug resulted in a threefold increase in triacylglycerol (Table 2).

Fatty acids undergo ω-oxidation in microsomes and these produce toxic metabolites such as long-chain dicarboxylic acids. Measurement of oxidative stress parameters in microsomes showed increased malonaldehyde, conjugated diene, and protein carbonyls in animals with steatosis when compared to control. Clofibrate pretreatment partially prevented these alterations in

Table 1 Mitochondrial lipid composition

	Control	Clofibrate	Valproate	Clofibrate + valproate
Neutral lipids (nmoles/mg protein)				
Diacyl glycerol	6.43 ± 0.36	8.95 ± 1.64	16.43 ± 0.57*	11.25 ± 1.0†
Triacyl glycerol	25.63 ± 2.89	16.73 ± 2.3	45.5 ± 1.92*	31.5 ± 0.73†
Cholesterol	7.07 ± 0.45	8.86 ± 0.32	4.57 ± 0.79*	4.76 ± 0.43
Cholesterol esters	4.08 ± 0.45	4.66 ± 0.32	8.41 ± 0.18*	6.52 ± 0.67
Phospholipids (nmoles/mg protein)				
Phosphatidyl choline	56.44 ± 1.74	56.4 ± 2.0	38.38 ± 0.69*	53.2 ± 0.9†
Lyso phosphatidyl choline	4.98 ± 1.3	5.54 ± 0.26	9.06 ± 2.02*	5.61 ± 0.27†
Phosphatidyl ethanolamine	50.06 ± 0.59	52.1 ± 1.53	39.56 ± 0.82*	48.1 ± 2.0†
Lyso phosphatidyl ethanolamine	4.47 ± 0.09	3.62 ± 0.13	8.52 ± 0.69*	2.8 ± 0.32†

Each value represents mean ± SD of six different experiments.
*$P < 0.05$, when compared to controls; †$P < 0.05$, when compared to valproate-treated rats.

Table 2 Peroxisomal lipid composition

	Control	Clofibrate	Valproate	Clofibrate + valproate
Neutral lipids (nmoles/mg protein)				
Diacyl glycerol	11.89 ± 0.78	9.07 ± 1.8	16.43 ± 0.83*	14.59 ± 0.8
Triacyl glycerol	42.0 ± 2.0	124.5 ± 2.5‡	56.1 ± 2.0	115.2 ± 8.5
Cholesterol	44.82 ± 1.3	42.46 ± 0.79	93.6 ± 7.0*	38.1 ± 6.8†
Cholesterol esters	7.56 ± 0.38	8.79 ± 0.35	3.91 ± 0.48	5.93 ± 0.45
Phospholipids (nmoles/mg protein)				
Phosphatidyl choline	149.06 ± 1.0	146.3 ± 3.0	124.6 ± 3.0*	144.0 ± 3.9†
Lysophosphatidyl choline	3.6 ± 0.3	3.73 ± 0.24	12.23 ± 0.7*	4.21 ± 0.79†
Phosphatidyl ethanolamine	99.28 ± 3.0	101.38 ± 0.9	73.75 ± 1.37*	97.7 ± 1.0†
Lysophosphatidyl ethanolamine	2.49 ± 0.5	2.18 ± 0.49	10.95 ± 0.5*	2.11 ± 0.2†

Each value represents mean ± SD of six different experiments.
*$P < 0.05$, when compared to controls; †$P < 0.05$, when compared to valproate-treated rats; ‡$P < 0.05$, when compared to controls without clofibrate.

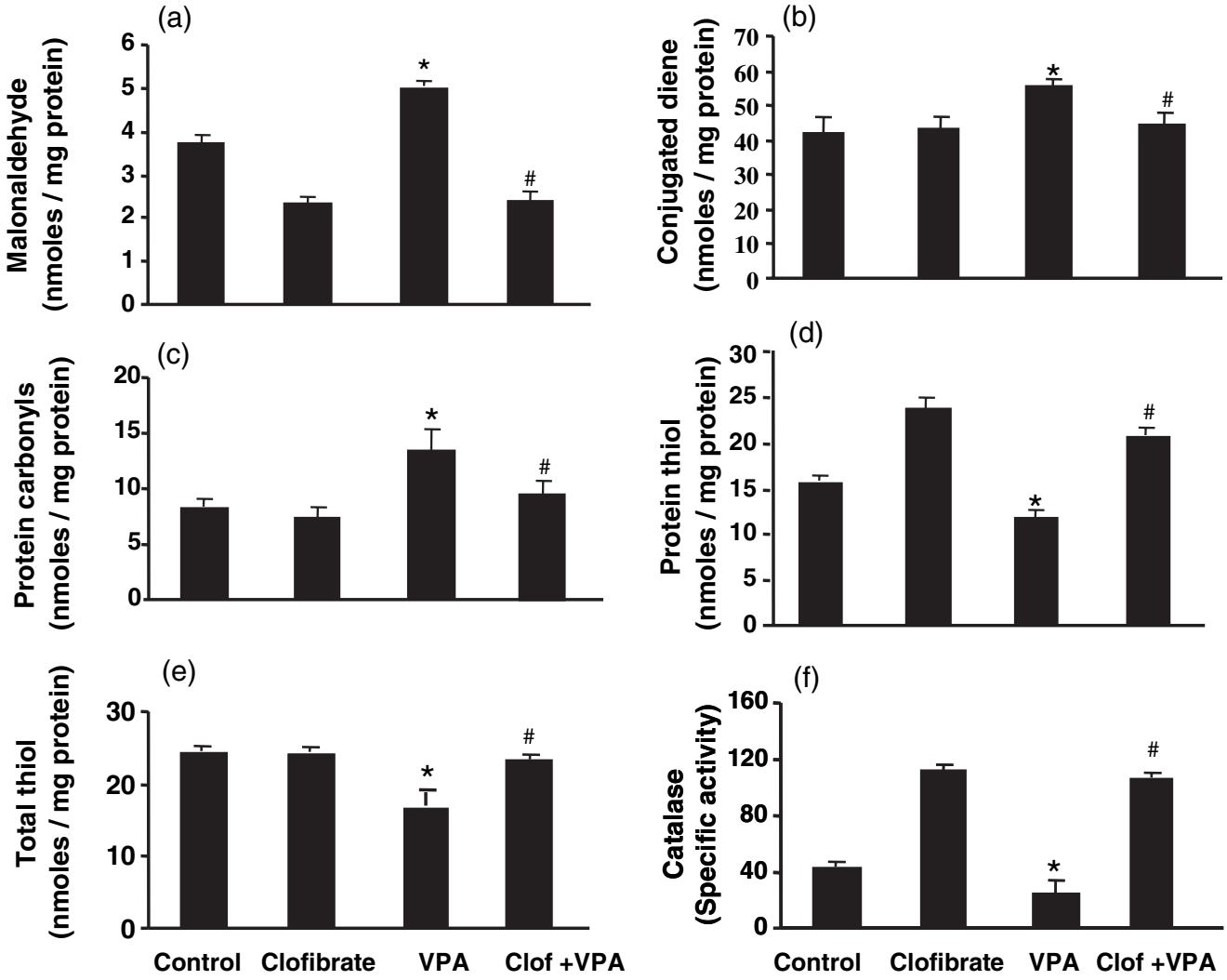

Figure 5 Oxidative stress parameters in peroxisomes. (a) Malonaldehyde, (b) conjugated diene, (c) protein carbonyl content, (d) protein thiol, (e) total thiol and (f) catalase activity in liver peroxisomes of rats after various treatments. Each value represents mean ± SD from six separate experiments. *$P < 0.05$, when compared to control, #$P < 0.05$, when compared to valproate-treated rats. Clof, clofibrate; VPA, valproate.

microsomes (Fig. 6). Microsomal lipid analysis showed an increase in triacylglycerol levels accompanied by decreased cholesterol concentrations in livers from valproate-treated animals. Among the phospholipids, phosphatidyl choline and phosphatidyl ethanolamine were decreased in animals with steatosis when compared to control. These changes in phospholipids were restored to control values by clofibrate pretreatment, although the alterations in neutral lipids were not corrected (Table 3).

Discussion

Hepatic microvesicular steatosis occurs as a result of impaired mitochondrial β-oxidation of fatty acids and is associated with hepatocellular damage leading to fulminant hepatic failure, energy crisis, coma and ultimately death in severe forms of the disease.[32] In clinical conditions such as acute fatty liver of pregnancy, defective mitochondrial β-oxidation of fatty acids due to genetic predisposition results in microvesicular steatosis.[33] The association of mitochondrial dysfunction with microvesicular steatosis has also been shown in experimental models such as orotic acid feeding,[34] methionine-low, choline-deficient diet, tetracycline-induced fatty liver[35] and toxicity due to drugs such as aspirin, ibuprofen or pirfrofen.[5,36,37] The hepatotoxic agent, valproate, can inhibit mitochondrial β-oxidation of fatty acids and produce microvesicular steatosis at a near lethal dose.[38] Valproate affects oxidation of fatty acids in the mitochondria through different mechanisms that include inhibition of the trifunctional enzyme in the β-oxidation pathway[39] and decreasing availability of CoA for fatty acid oxidation.[32] When β-oxidation is affected in the mitochondria, fatty acids are channeled for β-oxidation in peroxisomes and ω-oxidation in microsomes. In the event of defective mitochondrial fatty acid oxidation, peroxisomal β-oxidation is increased. However, unlike mitochondrial fatty acid oxidation this generates hydrogen peroxide, which can lead to oxidative stress.[3]

Table 3 Microsomal lipid composition

	Control	Clofibrate	Valproate	Clofibrate + valproate
Neutral lipids (nmoles/mg protein)				
Diacyl glycerol	15.55 ± 0.14	14.08 ± 1.6	10.8 ± 0.5*	14.37 ± 2.0†
Triacyl glycerol	59.6 ± 4.63	53.43 ± 2.6	83.3 ± 1.94*	82.5 ± 5.1
Cholesterol	96.39 ± 0.99	73.8 ± 1.68	37.2 ± 1.54*	54.58 ± 4.8†
Cholesterol esters	6.7 ± 0.33	5.79 ± 0.35	10.7 ± 0.33*	5.93 ± 0.45†
Phospholipids (nmoles/mg protein)				
Phosphatidyl choline	103.08 ± 5	105.8 ± 2.0	86.2 ± 6*	98.49 ± 2.0†
Lysophosphatidyl choline	4.25 ± 0.5	4.51 ± 0.22	8.9 ± 0.8*	4.86 ± 0.2†
Phosphatidyl ethanolamine	65.4 ± 4	67.7 ± 4.2	49.4 ± 5*	64.8 ± 2.0†
Lysophosphatidyl ethanolamine	3.34 ± 0.5	3.05 ± 0.24	9.4 ± 0.6*	4.8 ± 0.23†

Each value represents mean ± SD of six different experiments.
*$P < 0.05$, when compared to controls; †$P < 0.05$, when compared to valproate-treated rats.

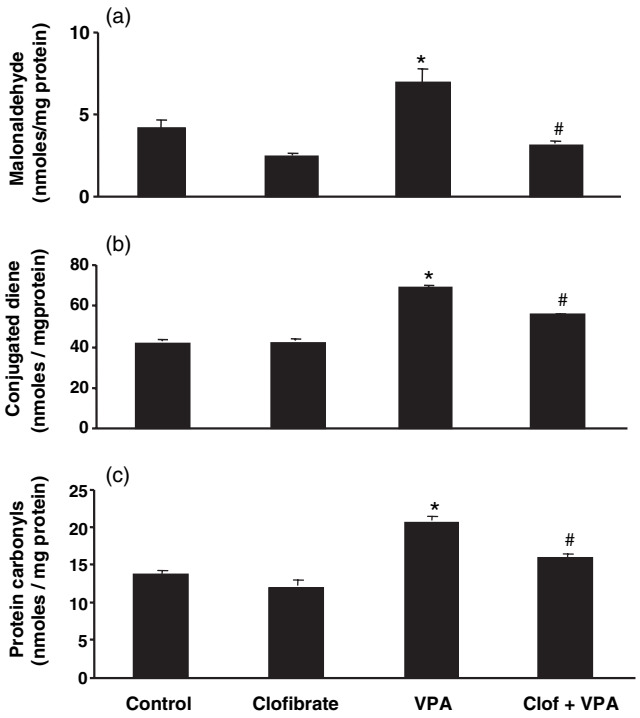

Figure 6 Oxidative stress parameters in microsomes. (a) Malonaldehyde, (b) conjugated diene and (c) protein carbonyl content in liver microsomes after various treatments. Each value represents mean ± SD from six separate experiments. *$P < 0.05$, when compared to control, #$P < 0.05$, when compared to valproate-treated rats. Clof, clofibrate; VPA, valproate.

In addition, ω-oxidation of fatty acids in microsomes form long-chain dicarboxylic acids that are also a substrate for peroxisomal acyl CoA oxidase.[40] Studies have demonstrated increased dicarboxylic acid excretion in patients with long-chain and medium-chain hydroxy acyl CoA dehydrogenase deficiency, where defective mitochondrial β-oxidation is evident.[41] Another possible reason for the oxidative stress could be that the mere presence of oxidizable fat in liver, either in acute or chronic hepatic steatosis, can trigger extensive lipid peroxidation. This has been proven in drug-induced steatosis produced by various agents such as valproate, ethanol, tetracylcline, amiodarone or pirprofen.[42]

In the present study, alterations in the subcellular fractions from the liver were investigated in valproate-induced steatosis, which was confirmed by histology. Rats with steatosis showed oxidative stress in the homogenate as well as in subcellular organelles such as peroxisomes and microsomes. Increased hydrogen peroxide levels seen in the homogenate from steatotic liver is possibly due to defective mitochondrial β-oxidation leading to increased peroxisomal β-oxidation. It has been demonstrated that increased lipid oxidation can result in generation of malonaldehyde, conjugated diene and 4-hydroxynonenal, all of which can trigger hepatic steatosis.[43] All these parameters were increased in peroxisomes and microsomes from steatotic liver, confirming the presence of significant oxidative stress under these conditions. In the present study microvesicular steatosis was associated with lipid alterations in various subcellular organelles. Accumulation of triacyl glycerol in all organelles, accompanied by significant increases in cholesterol in peroxisomes and decrease in microsomes were also seen in valproate-treated rats as compared to control. Phospholipid alterations such as decrease in phosphatidyl choline and phosphatidyl ethanolamine with a corresponding increase in lysophospholipids in the mitochondria, peroxisomes and microsomes suggest the activation of liver phospholipase A_2 during microvesicular steatosis. It has been demonstrated earlier that oxidative stress can result in activation of phospholipase A_2.[44]

Clofibrate, a hypolipidemic drug, is known to offer protection against acetaminophen, and carbon tetrachloride-induced toxicity.[45,46] Studies have also shown that hepatic nicotinamide adenine dinucleotide, nicotinamide adenine dinucleotide (reduced form) and nicotinamide adenine dinucleotide phosphate (reduced form) are increased significantly in rats fed with 0.25% clofibrate in the diet for 10 days.[47] Clofibrate can increase mitochondrial and peroxisomal β-oxidation by two- and six–eightfold, respectively,[3] by activating the enzymes involved in β-oxidation.[48] The drug has also been shown to protect against orotic acid-induced microvesicular steatosis.[49] Studies have shown that clofibrate can induce PPAR-α,[9] which in turn influences genes encoding peroxisomal and microsomal fatty acid oxidation enzymes transcriptionally,[10] supporting our observation of decreased steatosis following treatment with clofibrate along with valproate. The extent of valproate-induced microvesicular steatosis as well as the lipid and protein

oxidation were considerably decreased with clofibrate pretreatment, possibly mediated by increased peroxisomal β-oxidation due to induction of PPAR-α.

In summary, the present study has shown that in experimental microvesicular steatosis, increased oxidation of fat by microsomes and peroxisomes can lead to oxidative stress, which may be augmented by the accumulated lipids leading to hepatocellular damage. Pretreatment with clofibrate results in partial reversal of changes occurring in the subcellular organelles, possibly by increasing microsomal and peroxisomal oxidation of fatty acids and hence decreasing accumulation of fat in the liver.

Acknowledgments

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