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Role of cytosolic liver fatty acid binding protein in hepatocellular oxidative stress: effect of dexamethasone and clofibrate treatment

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Abstract

The presence of cysteine and methionine groups together with an ability to bind long-chain fatty acid (LCFA) oxidation products makes liver fatty acid binding protein (L-FABP) an attractive candidate against hepatocellular oxidative stress. In this report, we show that pharmacological treatment directed at modulating L-FABP level affected hepatocellular oxidant status. L-FABP expressing 1548-hepatoma cells, treated with dexamethasone or clofibrate, decreased and increased intracellular L-FABP levels, respectively. Oxidative stress was induced by H_2O_2 incubation or hypoxia–reoxygenation. The fluorescent marker, dichlorofluorescein (DCF), was employed to measure intracellular reactive oxygen species (ROS). Hepatocellular damage was assessed by lactate dehydrogenase (LDH) level. Dexamethasone treatment resulted in a significant increase in DCF fluorescence with higher LDH release compared to control cells. Clofibrate treatment, however, resulted in a significant decrease in both parameters ($p < 0.05$). Drug treatments did not affect cytosolic activites of glutathione peroxidase (GPx), superoxide dismutase (SOD), or catalase suggesting that the differences between treated and control cells may likely be associated with varying L-FABP levels. We conclude that L-FABP may act as an effective endogenous cytoprotectant against hepatocellular oxidative stress.

Key words: dexamethasone, clofibrate, L-FABP, hepatoma, oxidative stress, hepatocyte

Introduction

Hepatocellular oxidative stress results from excessive liberation of oxygen-derived free radicals leading to cellular damage. The natural intracellular antioxidant defense system is largely provided by superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase. These enzymes offer protection against oxygen-derived radicals such as superoxide anions, hydroxyl radicals, hydrogen peroxide, etc. However, cellular oxidative stress may not only deplete these antioxidants, but also initiate a chain reaction involving membrane structural lipids resulting in the generation of lipid peroxidation products. In severe cases cells must have the ability to protect themselves against various forms of reactive oxygen species (ROS).

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Liver fatty acid binding protein (L-FABP) belongs to a multigene family of intracellular lipid binding proteins. The primary biological function of L-FABP is thought to be the binding and intracellular transport of long-chain fatty acids (LCFA) [1–4] as well as other ligands [5–7]. L-FABP also possesses all the characteristics necessary to be an effective endogenous protectant. The intracellular concentration of L-FABP is high, making up 2–5% of cytosolic protein [8]. The primary protein structure of L-FABP contains seven methionine and one cysteine amino acids [9]. Proteins containing cysteine and methionine amino acids are known to be effective antioxidant agents [10, 11]. Furthermore, L-FABP avidly binds with polyunsaturated fatty acid oxidation products such as 13-hydroxy octadecadienoic acid (HODE), 15-hydroxytetraenoic acid (HETE) [12], and the arachidonic acid metabolite hydroperoxyeicosatetraenoic acid (HPETE) [13]. These products have the potential to initiate free radical chain propagation processes leading to membrane damage. Thus, L-FABP's high intracellular concentration together with its possession of the antioxidant amino acid groups and high binding affinity for oxidized fatty acid products lead us to investigate its role in hepatocellular oxidative stress.

We previously reported that Chang liver cells, a type of human hepatoma cell line (ATCC, Rockville, MD), have undetectable levels of L-FABP. We transfected those cells with the L-FABP gene to produce a stable L-FABP transfected model [14]. Using this model we reported that the transfected cells were associated with much less ROS than their vector transfected control cells. We concluded that the reduced ROS levels were associated with increased L-FABP level in transfected cells. In the present study we investigated whether reducing L-FABP levels with dexamethasone was associated with a greater amount of ROS, and conversely whether increasing L-FABP levels with clofibrate enhances cellular defences against ROS in an L-FABP expressing 1548 rat hepatoma cell line. The fluorescent dye dichlorofluorescein (DCF) was employed to assay for intracellular ROS levels in hepatocellular oxidative stress induced by both H_2O_2 pre-treatment and hypoxia–reoxygenation injury. We also investigated whether pharmacological treatment altered activities of the naturally present intracellular antioxidants SOD, GPx, and catalase. Identification of L-FABP as an endogenous protectant in the liver could offer novel therapeutic possibilities in treating liver diseases involving oxidative stress.

Materials and methods

Electrophoretic reagents were purchased from FisherBiotech (Pittsburgh, PA) while trypsin-EDTA was purchased from Acros Organics (Ottawa, ON). All other chemicals were purchased from Sigma Chemical (St. Louis, MO). The aqueous buffer was phosphate buffered saline (PBS), which had a composition of (in mM): 137 NaCl, 2.68 KCl, 1.65 KH_2PO_4, and 8.92 Na_2HPO_4, pH adjusted to 7.4 using 0.1 N NaOH.

Cell culture and drug treatment

Liver fatty acid binding protein expressing CRL-1548 hepatoma cells were obtained from American Type Culture Collection (ATCC, Rockville, MD) and maintained in 25 cm^2 culture flasks supplemented with DMEM containing 10% FBS (DF-10), penicillin and streptomycin (50 units/ml) at 37 °C in a humidified atmosphere of 5% CO_2. Dexamethasone (0.5 μM) and clofibrate (500 μM) were dissolved in dimethyl sulfoxide (DMSO) and later added to DF-10 media containing 300 μM bovine serum albumin (BSA) (DMSO $< 0.1\%$ v/v in final solution). Control cells were incubated with DMSO (same final volume). Cells were exposed to DF-10 media containing dexamethasone or clofibrate for 4 days to increase or decrease L-FABP expression [15, 16]. Equal amounts of control and drug-treated cells were subcultured onto 24-well & 96-well black plates (Corning, NY, USA) and allowed to attach for 8 h following which fluorescence experiments were performed.

H_2O_2 treatment

Epifluorescent microscopy

Cells were grown in 24-well plates with DF-10 (control and treated cells) and employed for measuring oxidant production as previously described [17]. Briefly, cells were washed twice with Ca/Mg-PBS and incubated with $DCFH_2$-DA solution at room temperature in the dark for 30 min. Oxidative stress was induced by treating the $DCFH_2$-DA loaded cells (after washing to remove extracellular $DCFH_2$-DA) with 200 μM hydrogen peroxide (H_2O_2) for 10 min followed by washing cells with Ca/Mg-PBS. Image analysis (488 nm excitation wavelength/515 nm emission wavelength) was conducted immediately using an inverted Nikon microscope with an Axon Integrated Imaging System (Molecular Devices, Sunnyvale, CA). Mean fluorescence intensity was calculated by averaging 20 cells (randomly selected) per field. Results are expressed as a percent ratio of average fluorescence obtained from treated cells (dexamethasone or clofibrate) to control cells.

Fluorescence plate reader

The 1548-cells grown in black plastic 96-well plates were used in the fluorescence plate reader studies. Experiments were conducted exactly as outlined above. Fluorescence

intensity of emitted DCF fluorescence was measured using a BMG (Durham, NC, USA) Fluostar Galaxy fluorescence plate reader (485 nm excitation wavelength/520 nm emission wavelength) equipped with excitation and emission probes directed to the bottom of the plate. Mean fluorescence intensity was calculated from triplicate cultures of control and drug-treated cells from six separate experiments.

Hypoxia–reoxygenation injury

The CRL-1548 hepatoma cells (control and treated), grown in 24-well plates, were employed for studying the hypoxia–reoxygenation injury as previously described [18]. A special air- and temperature- controlled chamber (maintained at 37 °C) with inflow and outflow valves was constructed to induce hypoxia–regeneration injury. The flow of gas was always directed onto the top surface of liquid in the culture dish. The gas was humidified by bubbling it through water in a gas-washing tower to prevent evaporative loss of water from the culture dish. The culture media was initially saturated by bubbling the respective gases (Hypoxia: 95% N_2–5% CO_2; Reoxygenation: 95% O_2–5% CO_2) for 15 mins and exchanged in the plate. Following 6 h of hypoxia and 90 min of reoxygenation, the supernatant media from each well was collected and stored at −80 °C for later assessment of cellular damage using lactate dehydrogenase (LDH) release assay. Cells were incubated with 100 μM diluted $DCFH_2$-DA solutions for the final 30 min of the 2 h re-oxygenation period in which the same flow rate was maintained as before. The emitted DCF fluorescence was quantitated using BMG (Durham, NC, USA) Fluostar Galaxy fluorescence plate reader. As a control for the hypoxia–reoxygenation studies, 1548-cells (control and treated) were incubated in normoxic conditions in a humidified atmosphere of 5% CO_2 at 37 °C. Cell viability was assessed by trypan blue exclusion.

LDH release assay

Lactate dehydrogenase release from both normoxic and hypoxic hepatoma cells (control and treated) was measured as an index of cytotoxicity. The amount of LDH released into the supernatant medium was measured in 96-well plates in a Molecular Devices (Menlo Park, CA, USA) plate reader. The LDH catalyzed reaction of NAD^+ with lactate to produce pyruvate and NADH was determined at 340 nm at 25 °C [19]. Change in absorbance with time (ΔA/min) was calculated for each treatment from two separate experiments of triplicate cultures and results were expressed as ΔA/min/cell $\times$ 10^6.

Flourescence measurements using cell lysate

Control and drug-treated cell pellets were obtained by centrifugation at 2000 $\times$ *g* for 10 min. Pellets were sonicated in ice-cold PBS and centrifuged at 13,000 $\times$ *g* for 30 min at 4 °C. The harvested supernatant was employed to investigate the possibility of DCF signal quenching by intracellular L-FABP. The active intracellular 2′,7′-dichlorodihydrofluorescein (DCFH) form of $DCFH_2$-DA was prepared as described by Tollefson *et al.* [20]. The DCFH was then oxidized to the highly fluorescent DCF by incubating with 500 μM H_2O_2 and 10 μM $FeSO_4$ (10 min at room temperature). DCF fluorescence in the presence of 100 μg of lysate (control, dexamethasone and clofibrate treated cells), was monitored and recorded by a 96 well-plate BMG (Durham, NC, USA) Fluostar Galaxy fluorescence plate reader using 470 nm excitation/530 nm emission settings. Results were expressed in arbitrary fluorescence units (AFU). As a positive control the experiment was repeated in the presence of BSA.

Assay of SOD, GPx, and catalase

Total cytosolic activity of the intracellular antioxidant enzymes (SOD, GPx, and catalase) was measured using their respective biochemical spectrophotometric assays. Total cytosolic SOD activity was determined as described by Oberley and Spitz [21]. Activity was measured in units where one unit of activity was defined as the amount of enzyme that decreased the initial rate of nitroblue tetrazolium reduction to half its maximal value. Total cytosolic GPx activity was measured by the Lawrence and Burk procedure [22]. Units for GPx enzyme activity were in μM NADPH oxidized/mg protein/sec. The catalase assay directly measured the catalysis of H_2O_2 [23]. Activity was measured in μM H_2O_2 consumed/μg protein/min. Data represent mean ± SEM from two separate experiments of triplicates.

Western blot analyses

Control and drug-treated (dexamethasone and clofibrate) 1548-hepatoma cells were grown to confluence in 75 cm^2 culture flasks following which the cells were trypsinized, harvested, and centrifuged. The resulting cell pellet was then suspended in 0.5 ml of 0.01 M PBS containing 0.154 M KCl, pH 7.4, 4 °C in 1.5 ml Fisher microcentrifuge tubes and lysed using a Fisher Sonic Dismembrator (Model 300, FisherScientific, USA). After sonication, samples were centrifuged at 13,000 $\times$ *g* (μSpeedFuge, SFR 13K, Savant) for 20 min at 4 °C. Protein concentration of the

supernatant was determined by the Bradford method [24] using a Shimadzu-160 UV spectrophotometer (Shimadzu Corporation, Japan) with BSA as the standard. Equivalent amounts of protein (75 μg) were loaded in each well of a 12% SDS-polyacrylamide gel and transferred onto nitrocellulose membranes after electrophoresis. Membranes were blocked with 5% powdered skimmed milk and incubated overnight at 4 °C with a polyclonal antisera raised against rat L-FABP as described previously [15]. After multiple washes, membranes were subsequently incubated with a peroxidase-conjugated anti-rabbit antibody for L-FABP for 1 h at room temperature. Finally, the antigen-antibody complex was detected by enhanced chemiluminescence (ECL system, Amersham, USA). Western blot bands were quantitated by scanning densitometry using Scion Image Software (Frederick, USA). Data represent mean ± SEM from four cell preparations.

Statistical analyses

Data are presented as mean ± SEM. The n value refers to number of replicates performed for each study. Data were analyzed using one-way analysis of variance with Dunnet's two-sided multiple comparison *post hoc* test taking $p < 0.05$ as the level for significance.

Results

L-FABP levels

Western immunoblot was employed to assay the L-FABP levels in control and treated cells. Cells treated for 4 days with dexamethasone were observed to express a much lower amount of L-FABP compared to control (0.6 ± 0.1-fold, $n = 4$). Conversely, clofibrate significantly increased the intracellular L-FABP content (2.9 ± 0.4-fold, $n = 4$) following a 4-day treatment ($p < 0.05$).

H_2O_2 treatment

The lipophilic diacetate ester of DCFH ($DCFH_2$-DA) was employed as it easily crosses cell membranes and is hydrolyzed by intracellular esterases to DCFH. A rapid laser exposure time of fewer than 2 s in the epifluorescent microscopy experiments ensured minimal photo-oxidation of the internalized DCFH to DCF. Table 1 shows H_2O_2 induced DCF fluorescence was significantly greater ($p < 0.05$) in 0.5 μM dexamethasone-treated cells (138 ± 10%) compared to control (taken as 100%). The high fluorescence intensity observed with dexamethasone was associated with a lower L-fABP level and reflects higher intracellular free ROS. In contrast, DCF fluorescence of 500 μM clofibrate-treated cells (78 ± 6%) was significantly lower ($p < 0.05$) compared to control, suggesting lower intracellular oxidative products were associated with higher L-FABP content. Similar results were obtained of DCF fluorescence for fluorescence plate reader experiments. DCF fluorescence of dexamethasone-treated cells (134 ± 2%) exhibited significantly higher fluorescence compared to control cells. Clofibrate-treated cells (57 ± 3%) exhibited a statistically lower fluorescence intensity ($p < 0.05$) compared to control. These results suggest that modulating L-FABP levels affects the hepatocellular ability in handling excess oxidative stress.

Table 1. H_2O_2 mediated DCF fluorescence from control-, dexamethasone-, and clofibrate-treated 1548 cells recorded by epifluorescent microscopy and fluorescent plate reader

	DCF fluorescence (% of control)	
	Epifluorescent microscopy	Fluorescent plate reader
Control	100 ± 5	100 ± 3
DEX	138 ± 10*	135 ± 2*
CLO	78 ± 6*	57 ± 3*

*Statistically significant from control ($p < 0.05$).
DCF fluorescence from control, dexamethasone- (DEX), and clofibrate- (CLO) treated 1548 cells subjected to H_2O_2 mediated oxidative stress. DCF fluorescence was recorded by both epifluorescent microscopy and 96 well-plate Fluostar Galaxy fluorescence plate reader with 470 nm excitation/530 nm emission settings. Results are expressed as % of control. In both methods, the observed fluorescence was significantly higher (*$p < 0.05$) in DEX-treated cells compared to control (taken as 100 %) whereas CLO-treated cells exhibited a significantly lower fluorescence compared to control (*$p < 0.05$). Data represent mean ± SEM ($n = 6$).

Hypoxia–reoxygenation injury

We conducted hypoxia–reoxygenation studies to investigate whether L-FABP protects hepatoma cells from the burst of oxygen-derived free radicals associated with the reoxygenation process. Table 2 shows that the observed DCF fluorescence pattern of hypoxia–reoxygenation injury was similar to H_2O_2 treatment with dexamethasone treatment (121 ± 1%) resulting in an increased fluorescence intensity compared to control while clofibrate treatment exhibited the reverse effect (82 ± 2%). The emitted fluorescence from normoxic cells (dexamethasone- or clofibrate-treated) was not different from control cells ($p > 0.05$) suggesting that drug treatment by itself did not affect the intracellular DCF fluorescence.

Table 2. Hypoxia–reoxygenation mediated DCF fluorescence and LDH leakage from control-, dexamethasone-, and clofibrate-treated 1548 cells

	DCF fluorescence (% of control)	LDH release (ΔA/min/cell $\times$ 10^6)
Control	100 ± 1	3.9 ± 0.2
DEX	121 ± 1*	5.1 ± 0.1*
CLO	82 ± 2*	3.3 ± 0.1*

*Statistically significant from control ($p < 0.05$).
DCF fluorescence and LDH release from control, dexamethasone- (DEX), and clofibrate- (CLO) treated 1548 cells subjected to hypoxia–reoxygenation treatment. DCF fluorescence was recorded by a 96 well-plate Fluostar Galaxy fluorescence plate reader using 470 nm excitation/530 nm emission settings. The LDH catalyzed reaction of NAD^+ with lactate to pyruvate was employed to measure the supernatant activity of LDH. Results were expressed as % of control. Fluorescence was significantly higher (*$p < 0.05$) in DEX-treated cells compared to control (taken as 100 %), whereas CLO-treated cells exhibited significantly lower fluorescence compared to control (*$p < 0.05$). LDH release from hypoxia–reoxygenation studies exhibited a similar profile with DEX treatment, resulting in statistically significantly higher LDH release compared to control. CLO treatment resulted in a significant reduction in LDH release compared to control ($p < 0.05$). Data are mean ± SEM ($n = 6$).

The measure of LDH release from hepatocytes and myocytes is commonly used to assess the extent of cellular damage. Table 2 shows that preincubation of hepatoma cells with dexamethasone significantly increased the hypoxia–reoxygenation induced LDH release (5.1 ± 0.1) and clofibrate reduced LDH release (3.3 ± 0.1), compared to control (3.9 ± 0.2) ($p < 0.05$).

Quenching studies

To investigate whether L-FABP quenched the DCF signal, we performed fluorescence studies using harvested cell lysates from both control and drug-treated cells. DCF fluorescence, measured in arbitrary fluorescence units (AFU), remained unaffected by the presence of 100 μg cell lysate (DCF: 60,257 ± 989, control: 59,850 ± 335, dexamethasone: 60,719 ± 951, and clofibrate: 61,046 ± 1330). As the harvested cell lysate was primarily composed of cytosol, the results suggest that cytosolic contents, including L-FABP do not contribute to quenching of the intracellular DCF signal and hence the observed fluorescence can be taken to represent the level of free radical species. However, as a positive control we observed that the presence of BSA (50, 100, and 200 μg) significantly quenched the DCF signal (DCF: 60,257 ± 989, DCF + BSA (50 μg): 18,283 ± 258*, DCF + BSA (100 μg): 16,756 ± 198*, DCF + BSA (200 μg): 12,791 ± 316*; *$p < 0.05$), a result consistent with other reports [25]. Thus, these results indicated that varying L-FABP cytosolic levels caused by drug treatment do not affect the DCF signal.

Table 3. Activities of SOD, GPx, and catalase in the cytosolic fraction from control-, dexamethasone-, and clofibrate- treated 1548 cells

	SOD	GPx	Catalase
Control	52.5 ± 14.5	189.6 ± 0.1	34 ± 10
DEX	52 ± 17	189.4 ± 0.2	24 ± 12
CLO	54.5 ± 15.5	189.5 ± 0.1	22 ± 5

Cytosolic enzyme activities of superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase were measured in control-, dexamethasone- (DEX), and clofibrate- (CLO) treated cells. SOD activity was measured in units where one unit of activity was defined as the amount of the enzyme that decreased the initial rate of nitroblue tetrazolium reduction to half its maximal value. GPx enzyme activity was measured in μM NADPH oxidized/mg protein/sec. The activity of catalase was measured in μM H_2O_2 consumed/μg protein/min. Data represent mean ± SEM from two separate experiments of triplicate cultures.

SOD, GPx, and catalase activities

In order to rule out a possibility that dexamethasone and clofibrate affected DCF fluorescence by modulating the endogenous antioxidants, we measured the activities of SOD, GPx, and catalase in the cytosolic fraction of control and treated 1548 cells (Table 3). Results show that neither dexamethasone nor clofibrate treatment affected the activity level of these enzymes. Hence, the reduced (or enhanced) intracellular ROS levels likely resulted from dexamethasone and clofibrate mediated changes in L-FABP level.

Discussion

Hepatocytes are the major site for drug biotransformation and xenobiotic detoxification. Such hepatic biotransformation reactions involve oxidative pathways, primarily by way of the cytochrome P-450 enzyme system. The energy required for these tasks are obtained from intracellular mitochondrial electron transport that utilizes molecular oxygen resulting in a rich source of ROS. Though ROS is essential in physiological concentrations for normal cell function, excessive quantities result in a state of oxidative stress that may ultimately lead to cell death. Herein, we showed that pharmacological treatment aimed at modulating L-FABP levels affected the outcome in ROS mediated hepatocellular damage.

L-FABP is composed of 127 amino acids containing seven methionine and one cysteine groups [26, 27]. The amino acid groups differ in their susceptibility to oxidation by ROS [28]. Of all the amino acids, the sulphur containing cysteine and methionine groups are the most easily oxidizable by ROS [29]. One of the earliest cellular responses to oxidative stress is thought to involve the conversion of protein sulfhydryl (-SH) groups into disulfides and oxyacids

by biological thiols such as cysteine [30, 31]. Proteins containing cysteine amino acid groups participates in ''protein sulfhydryl cycle'' involving S-thiolation/dethiolation reactions where protein sulfhydryls are oxidized to mixed disulfides that are subsequently reduced back to sulfhydryls resulting in the reduction of cellular ROS levels [10]. Methionine residues also have been postulated to function as antioxidant defense system for proteins [11]. The widespread presence of methionine sulfoxide reductases reduces either free or protein-bound methionine sulfoxide formed by the oxidation of methionine [29]. Thus, cysteine and methionine take part in oxidant reactions through redox cycling involving repair mechanisms. This together with a high intracellular concentration of L-FABP makes this protein an attractive candidate against cellular ROS. Our previous work demonstrated a hepatoprotective role for L-FABP using a L-FABP cDNA transfected Chang cell model [14].

In the present study, clofibrate treatment was shown to protect 1548-cells from oxidative stress and resultant cytotoxicity induced by both H_2O_2 treatment and hypoxia–reoxygenation. These results are consistent with previous reports that demonstrated a hepatoprotective role for clofibrate against structurally diverse hepatotoxins such as, acetaminophen, bromobenzene, chloroform, and carbon tetrachloride that ultimately causes cell death involving cellular oxidative stress [32–34]. Manautou et al. showed that peroxisome proliferator activated receptor α (PPARα), a member of nuclear receptor superfamily, is required for clofibrate's hepatoprotective action in acetaminophen hepatotoxicy using PPARα knockout mice [35]. PPARs are ligand-activated transcription factors that regulate the expression of over 100 proteins including intracellular L-FABP [36]. One potential mechanism of clofibrate hepatoprotection could be through the increased expression of cytosolic L-FABP. This protein could directly participate in antioxidative function through its cysteine and methionine groups, or may protect other vital cellular proteins from free radical damage through non-specific interaction with free radical species due to increased intracellular content (i.e., upon clofibrate treatment), or a combination of mechanisms.

The notion of L-FABP mediated clofibrate hepatoprotection is further strengthened by the action of dexamethasone. In both H_2O_2 treatment and hypoxia–reoxygenation models of oxidative stress, dexamethasone-treated cells exhibited much higher oxidative stress and cytotoxicity compared to control, paralleling the lower L-FABP content of cells (Tables 1 and 2). Dexamethasone lowers intracellular L-FABP content through changes in cellular lipid metabolism, a mechanism independent of peroxisome proliferation [15, 16, 37]. Lawrence *et al.* showed that dexamethasone selectively inhibited peroxisome proliferator, WY14,643-induced hepatocellular proliferation independent of other pleitropic effects of peroxisome proliferation [38, 39], suggesting a common molecular target. L-FABP may be that target given that the protein (i) is directly implicated in hepatocellular mitotic activity [40], and (ii) is differentially regulated by these chemically diverse agents. We previously demonstrated that dexamethasone and clofibrate treatment affected hepatocellular proliferation in 1548-cells by possibly modulating L-FABP levels [16]. Thus, the action of clofibrate and dexamethasone in the current study may be attributed to L-FABP. However, we cannot rule out other mechanisms through which clofibrate or dexamethasone could have produced the results observed in the current study. A recent review lists some potential mechanisms by which clofibrate protects the liver from acetaminophen hepatotoxicity including PPARα induced inhibition of 'death proteins', stimulation of cell proliferation through responsive mitogenic genes, expression of multiple proteins, and increased oxyradical quenching enzymes [34].

The total intracellular DCF fluorescence was used as an index to assess the overall oxidative products in 1548-hepatoma cells. Concerns have been raised about the interpretation of DCF fluorescence as a marker of ROS, especially that of H_2O_2 [41, 42]. In our studies, we employed DCF as a qualitative marker of hepatocellular oxidative stress rather than a precise indicator of ROS levels. Also, concerns regarding extracellular DCF efflux and auto-oxidation of DCFH were avoided by completing our DCF studies (incubation and fluorescence measurements) within 30 min. Moreover, results from DCF quenching studies show that the fluorescence signal from DCF was unaffected by any of the cytosolic components including L-FABP.

Collectively, our results support a role for L-FABP as an endogenous protectant. However, the exact mechanism of L-FABP's protective action remains to be elucidated. A recent report on the covalent cysteine-120 modification of E-FABP by 4-hydroxynonenal, a bioactive lipid hydroperoxide both *in vitro* and *in vivo* [43] suggested that E-FABP functions as a reactive lipid hydroperoxide scavenging protein. L-FABP could function similar to E-FABP, as it also possesses the necessary functional groups (cysteine and methionine). The protein could directly counteract ROS through its cysteine and methionine groups, or could effectively reduce the concentration of free radical species through non-specific interactions that would otherwise reach the various membrane fractions. Such a mechanism could have been predominant in the H_2O_2 treatment experiments in the current study as it involved high concentrations of H_2O_2 (200 μM) over a short time period (10 min). Another indirect pathway might involve L-FABP's binding of lipid hydroperoxides including fatty acid hydroperoxides formed by the free radical reaction involving lipid peroxidation of structural membrane lipids. Such a hypothesis for L-FABP's action is supported by its high affinity binding to

lipid hydroperoxides [12, 13]. As hypoxia–reoxygenation experiments involved a longer time frame of exposure to ROS in the present study, both direct and lipid hydroperoxide binding-mediated mechanism could have contributed to the results observed in this case.

In conclusion, pharmacological intervention aimed at modulating L-FABP levels affects hepatocellular susceptibility to oxidative stress. The levels of L-FABP directly correlate with the hepatocellular ability to fight against both hydrogen peroxide and hypoxia–reoxygenation induced oxidative stress. L-FABP warrants further investigation as a potential pharmacological target in understanding hepatocellular oxidative stress mediated liver diseases such as steatosis, steatohepatitis, and drug-induced hepatotoxicity.

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