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ABSTRACT

In recent years, there has been a growing interest to explore the responsiveness to injury in steatotic hepatocyte. VL-17A cells, which express ADH and Cyp2E1 overloaded with free fatty acids (1 mM of oleic and palmitic acid 2:1) showed an increased oxidative damaged after 24 h free fatty acids treatment when exposed to ethanol (100 mM) for 48 h as a second injury. An increment in reactive oxygen species, determined by DCFH-DA, protein oxidation, and apoptosis were observed although an increase in main antioxidant proteins such as superoxide dismutase 1 and glutathione peroxidase were observed, but failed in gamma-glutamylcysteine synthetase, suggesting a decreased capacity of synthesis of glutathione compared with cells treated only with free fatty acids or ethanol. The increased oxidative stress and toxicity in lipid overloaded VL-17A cells subjected to ethanol exposure were accompanied by increases in Cyp2E1 protein expression. Our data show that lipid loaded in an in vitro model, VL-17A cells, is more susceptible to cell damage and oxidative stress when treated with ethanol.

1. Introduction

Non-alcoholic fatty liver disease (NAFLD), a spectrum of liver damage ranging from steatosis to stetohepatitis and cirrhosis, is the most common liver disease in clinical practice (Duvnjak et al., 2009). The mechanisms by which steatosis leads to hepatocyte damage and inflammation observed in non-alcoholic steatohepatitis (NASH) is not well understood although it has been correlated with increased inflammatory cytokine levels, elevated cytochrome P450 (Cyp) activity, specifically the isoforms 2E1 and 4A, impairing mitochondrial function, reducing ATP levels, and promoting oxidative damage (Daly, 2013; Enriquez-Cortina et al., 2013; Gutierrez Ruiz et al., 2012; Robertson et al., 2001). Based on this evidence, some authors consider that steatosis renders hepatocytes susceptible to damage to another aggression (Robertson et al., 2001). It has been generally proposed that the aggression increases the production of reactive oxygen species (ROS) in the steatotic hepatocyte resulting in the progression of cellular damage (Sumida et al., 2013) So, fatty liver may predispose the organ to further injury (hepatitis and fibrosis) by Cyp2E1 activity induction, free radicals generation, lipid peroxidation, nuclear factor-kappa B (NF-κB) activation, and increased transcription of proinflammatory mediators, including tumor necrosis factor-alpha (TNF-α) (Begriche et al., 2013; Robertson et al., 2001). Liver steatosis induces sensitization to damage under ischemia, endotoxin, and alcohol (Soltys et al., 2001). Steatotic livers are particularly vulnerable to ischemia/reperfusion injury, resulting in an increased risk of postoperative morbidity and mortality after liver surgery, including liver transplantation (Tashiro et al., 2014). Fatty overload in the organ has been related to liver transplant failure that could be linked to oxidative stress (Reiniers et al., 2014), and elevated free fatty acid (FFA) levels, especially saturated, such as palmitic acid, may be predisposing factors for cyclosporine A toxicity (Luo et al., 2012), and patients with underlying diseases that would elevate free fatty acids may be susceptible to toxicity that worsens the outcome in transplant patients (Luo et al., 2012). Steatosis negatively influences the rate of response to antiviral hepatitis C treatment, as confirmed by large clinical trials (Negro, 2006). Obesity dramatically increases the risk of having advanced fibrosis in all chronic liver diseases (Rosselli et al., 2014), on par with the well-described risk of alcohol ingestion (Riley et al., 2009). On the other hand, obesity may contribute to the development of alcoholic liver disease by generating free radicals, increasing TNF-α production, inducing insulin resistance, and producing fibrogenic

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agents, such as angiotensin II, norepinephrine, neuropeptide Y, and leptin (Purohit et al., 2004). The wide prevalence of alcohol abuse in society makes it an important cofactor in many other liver diseases, including chronic viral hepatitis, disorders of iron overload, and obesity-related liver disease (Zakhari, 2013).

In vitro models of steatosis have been used to study the hepatocellular consequences of lipid accumulation in hepatic cells (Gómez-Lechón et al., 2007; Tu et al., 2014; Yang et al., 2010). These models are hepatocyte cell lines and primary hepatocytes treated in culture with monounsaturated and saturated fatty acids, which seem to reproduce the key features of NAFLD in humans. Consequently, well-standardized and relevant in vitro hepatic cellular models have been very useful to help experimentally investigate the role and biochemical effects of fat accumulation in the liver due to alcohol consumption, dietary fatty acids or obesity, excluding other factors that could influence hepatocyte behavior. A useful model is the human hepatocyte-derived cell line HepG2. Palmitic (C16:0) and oleic (C18:1) acids are the most abundant FFA in liver triglycerides in both normal subjects and patients with NAFLD (Gómez-Lechón et al., 2007). Gómez-Lechón et al. (2007) have demonstrated that the FFA mixture containing the proportion of oleic acid/palmitic acid (oleate/palmitate, 2:1 ratio, 1 mM) is associated with minor toxic and apoptotic effects, thus representing a cellular model of steatosis in which unsaturated FFAs serve a protective function against lipotoxicity mimicking benign chronic steatosis. Moreover, similar intracellular levels of lipid overaccumulation, as in hepatocytes from human steatotic liver, have been achieved (Gómez-Lechón et al., 2007). We have previously shown that the recombinant HepG2 cells VL-17A, which express Cyp2E1 and alcohol dehydrogenase, presented a decreased cell viability and oxidative damage when they were treated with 100 mM ethanol (EtOH) for 48 h in comparison with untreated VL-17A cells or HepG2 cells that lack Cyp2E1 activity. VL-17A oxidative damage induced by EtOH increased peroxide formation and lipid peroxidation. Also, the ratio GSH/GSSG was decreased (Valdes-Arzate et al., 2009).

The aim in this work was to evaluate if there is an increasing damage susceptibility of steatotic VL-17A cells by EtOH biotransformation. The wide prevalence of obesity and alcohol use in society makes this an important liver health problem. This in vitro model would allow to evaluate the damage induced by FFA, EtOH biotransformation, and the susceptibility that FFA confers to hepatocytes to the EtOH aggression independently.

2. Materials and methods

2.1. Cell culture

The recombinant HepG2 cells (VL-17A), which express Cyp2E1 and alcohol dehydrogenase, were routinely grown in monolayer culture in Williams E medium supplemented with 10% fetal bovine serum (Hyclone Lab Inc., Logan UT, USA), 1% antibiotics in a humidified atmosphere of 5% CO_2/95% air. Medium was replaced twice a week and cells were trypsinized and diluted every 7 days at a ratio of 1:3. All experiments were carried out using 225,000 cells/cm^2. In order to lessen ethanol evaporation all dishes were wrapped with parafilm during time of treatment.

2.2. Experimental design

Twenty-four hours after cells platting, the culture media were replaced with a serum free medium and after 4 h the media were replaced with a serum free medium containing 1 mM of a mix of free fatty acids, such as oleic and palmitic (2:1). After 24 h, cells were treated with 100 mM EtOH final concentration for 48 h. Free fatty acids were present during EtOH treatment. Controls with only free fatty acids or EtOH were seeded and treated at the same time. All chemicals were purchased from Sigma Aldrich Chemical (St Louis MO, USA).

2.3. Oil red O staining

To detect intracellular neutral lipids accumulation, cells were seeded onto 24-well chambers. After treatments, cells were washed with ice-cold phosphate-buffered saline (PBS) and then fixed with 2.5% paraformaldehyde for one hour, then cells were stained with freshly diluted 0.2% Oil red O solution for 4 h. Oil red was then removed, and cells were washed 4 times with tap water. After washing and drying completely, 0.2 ml of isopropanol was the added to each well and incubated for 10 min, followed by gently vibration to release oil red O from steatosis staining. OD measurement was done at wavelength 510. Parallel experiments were performed counterstaining with crystal violet for microphotography.

2.4. Triglycerides determination

Triglycerides (TG) content was determined using the triglyceride determination kit (Sigma-Aldrich, St Louis MO, USA) following manufacturer's instructions.

2.5. Trypan blue exclusion test

Cells were seeded and, after treatments, viability was determined by measuring the ability of living cells to exclude trypan blue dye at 0.2% final concentration. Living cells were counted in a ViCell, Cell Viability Analyzer (Beckman Coulter, Brea CA, USA).

2.6. LDH activity

Medium from cells of the different treatments was decanted and centrifuged at 3000 rpm for 10 min and the supernatant was stored at –70 °C until the time of assay. Cytotoxicity was evaluated by measuring extracellular lactate dehydrogenase (LDH) activity. The assay was carried out as previously described (Moldeus et al., 1978).

2.7. Apoptosis and necrosis determination by flow cytometry

Apoptotic and necrotic cell death were determined using an Annexin V Fluos staining kit (Roche Applied Science, Pleasanton CA, USA) according to manufacturer's instructions. Samples were analyzed with FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes NJ, USA). Apoptotic cells were identified as Annexin V positive/PI negative cells in quadrant 3 (Q3).

2.8. Cell extracts preparation and immunoblotting

Protein content was determined by Western blotting as previously reported (Gomez-Quiroz et al., 2005). Briefly, 1.5×10^6 cells which were seeded and different treatments done. Cells were washed twice with ice-cold PBS. The cell pellet was resuspended in 200 µl of M-Per (Pierce Chemical, Rockford IL, USA); M-Per lysis buffer (Pierce Chemical, Rockford IL, USA) was supplemented with proteases and phosphatases inhibitors (Roche Applied Science, Peniberg, Germany). Lysis was performed on ice for 15 min, and cell debris was removed by centrifugation at 10,000 *g* at 4 °C for 10 min, supernatant was recovered and proteins were separated by electrophoresis using 4–20% Mini-Protean TGX precast gels (Bio-Rad, Hercules CA, USA) and transferred to a polyvinylidene difluoride (PVDF) membranes (Invitrogen, Carlsbad CA, USA). Immunodetection was performed using the indicated primary antibodies: anti-Cyp2E1 (Cell Signaling, Danvers MA, USA, concentration 1:1000), anti superoxide dismutase (SOD) 1 (concentration 1:2000), antiSOD2, (concentration 1:3000), anti-glutathione peroxidase (GSHPx, concentration 1:1000) 1/2, anti-actin (concentration 1:10,000), and gamma-glutamylcysteine synthetase(γ-GCS, concentration 1:1000) (Santa Cruz Biotechnology, Dallas TX, USA), anti-catalase, (Sigma-Aldrich, St Louis, MO, USA concentration 1:3000), followed by horseradish peroxidase-conjugated secondary antibody (Amersham, Pistacaway NJ, USA, concentration 1:8000) and detection by an incubation with SuperSignal West Pico Chemiluminescent Substrate (Pierce Chemical, Rockford, IL, USA).

2.9. Reactive oxygen species (ROS) detection

Intracellular ROS was monitored by 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA), as previously reported (Clavijo-Cornejo et al., 2013). Briefly, cells (2×10^5 cells) were washed with ice-cold PBS and incubated with DCFH-DA for 30 min. Cells were scraped and suspended in ice-cold PBS in the dark and fluorescence intensity was measured with excitation wavelength of 503 nm and emission wavelength of 529 nm using a Multimode Detector (DTX880, Beckman Coulter). A positive control with cells treated with 250 µM H_2O_2 for 3 h was used.

2.10. Protein oxidation

Oxidative modification of proteins generates protein carbonyl groups, which represent a hallmark of oxidation status of all proteins. Carbonyl groups were immunodetected using an Oxyblot kit (Millipore, Dormstadt, Germany) according to the manufacturer's instructions.

2.11. Total protein determination

Protein concentration was determined using a bicinchoninic acid kit (Pierce Chemical, Rockford IL, USA), according to the manufacturer's instructions.

2.12. Statistical analysis

The data are presented as mean ± SEM for at least three independent experiments. Comparison between groups were performed by one-way analysis of variance (ANOVA) with Bonferroni post hoc test using GraphPad Prism 5 for Mac OSX. Differences were considered significant at $^{*}p \leq 0.05$.

3. Results

3.1. FFA treatment results in intracellular lipid accumulation

In order to confirm the FFA uptake by VA-17A cells, oil red staining was performed in all treatments. Microscopic images showed the presence of cytoplasmic lipid droplets in FFA and FFA plus EtOH treated cells (Fig. 1b and c). This pattern was evident with that seen in non-FFA treated cells. Quantitative approach was taken by the extraction and spectrophotometric measurement of oil red. Fig. 1e clearly shows that FFA treated cells increased 4.6-fold the lipid accumulation, in comparison with NT cells. VL-17A cells exposure to one mM oleic and palmitic acid (2:1) for 72 h plus EtOH induced 7.7-fold lipid accumulation compared with control cells.

3.2. FFA overload enhances EtOH metabolism-induced cell damage

VL17A cells with FFA significantly decreased the viability determined by trypan blue exclusion (Fig. 2a). Moreover, this effect is more evident in cells treated with FFA plus EtOH, suggesting that lipid overload enhanced cell damage induced by EtOH metabolism, which in addition decreases by itself, cellular viability. To explore further, LDH activity was measured in culture media. Fig. 2b shows that, although FFA increased LDH leakage, EtOH treatment exacerbated this effect. To address cell apoptosis and necrosis we performed the FACS analysis of Annexin V and propidium iodide (PI). VL17A cells treated with both FFA and EtOH presented 17% cells in apoptosis and 25% in necrosis, while FFA treatment produced 11% apoptosis and 9% necrosis, and EtOH-treated cells presented 10% apoptosis and 6% necrosis (Fig. 2c); this result confirms that FFA enhanced the toxicity induced by EtOH metabolism.

3.3. FFA and EtOH increase CYP2E1 protein content and oxidative damage

In order to explain the increment on EtOH metabolism-induced toxicity we looked at the expression of Cyp2E1 (Fig. 3). EtOH treatment significantly increases Cyp2E1 content (1.6-fold). A similar effect was observed with FFA alone treatment (2.3-fold); however, the FFA pretreatment significantly increased Cyp2E1 content (3.2-fold).

Due to our group's previous report that VL-17A cells treated with EtOH exhibited oxidative damage, judged by lipid and protein oxidation (Valdes-Arzate et al., 2009), we decided to quantify ROS production by using 2′-7′-dichlorodihydrofluorescein diacetate (DCFH), a cell-permeable non-fluorescent probe that is de-esterified intracellularly and converted to the highly fluorescent 2′-7′-dichlorofluorescein upon oxidation by ROS (Clavijo-Cornejo et al., 2013). As Fig. 4a shows FFA pretreatment induced the increment in ROS production due to EtOH metabolism. Interestingly, FFA alone treatment also increased ROS values. To confirm these observations we decided to determine protein oxidation (Fig. 4b); result shows that treatment with only FFA or only EtOH increased the content of protein carbonyl groups in VL17A cells, and the effect was increased in FFA and EtOH treatment cells, which indicates an oxidative damage process.

3.4. FFA and EtOH upregulate the antioxidant defense in VL-17A cells

Then, we decided to explore the status of the main antioxidant enzymes. As Fig. 5 depicts, SOD1, SOD2 and GSHPx were evaluated by Western blot. Interestingly, SOD1 and 2 exhibited an increment with FFA alone, but just SOD1 was enhanced in the other treatment, suggesting a clear compromise in mitochondria when the two insults are present. GSHPx was increased only in FFA + EtOH, proposing a compensatory response initiated by SOD1 increment in the management of reactive oxygen species.

γ-GCS and catalase exhibited no effect by any treatment (Fig. 5).

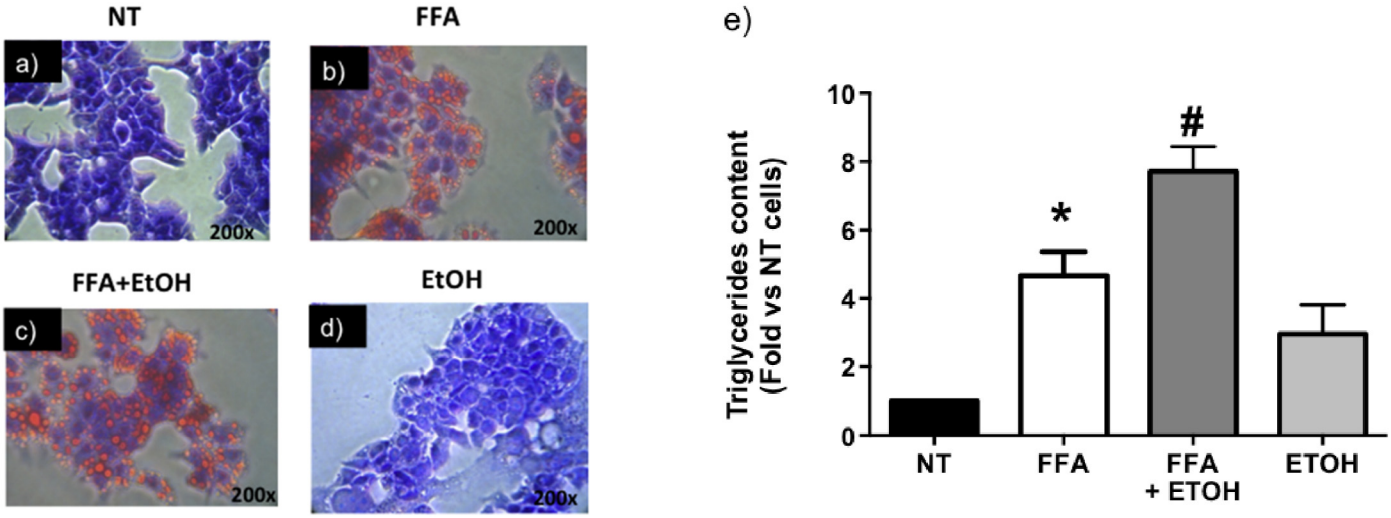

Fig. 1. Free fatty acids treatment induces lipid overload in VL-17A cells. Cells were treated with free fatty acids (FFA) 1 mM and/or ethanol (EtOH, 100 mM) as indicated in Materials and Methods. (a–d) Oil red O staining for detection of neutral lipids. Images are representative of at least three independent experiments. Original magnification 200×. (e) Quantification of triglycerides content. Each column represents mean ± SEM of three independent experiments. Differences were considered significant at * $p \leq 0.05$ vs NT cells, and # $p \leq 0.05$ vs FFA treated cells.

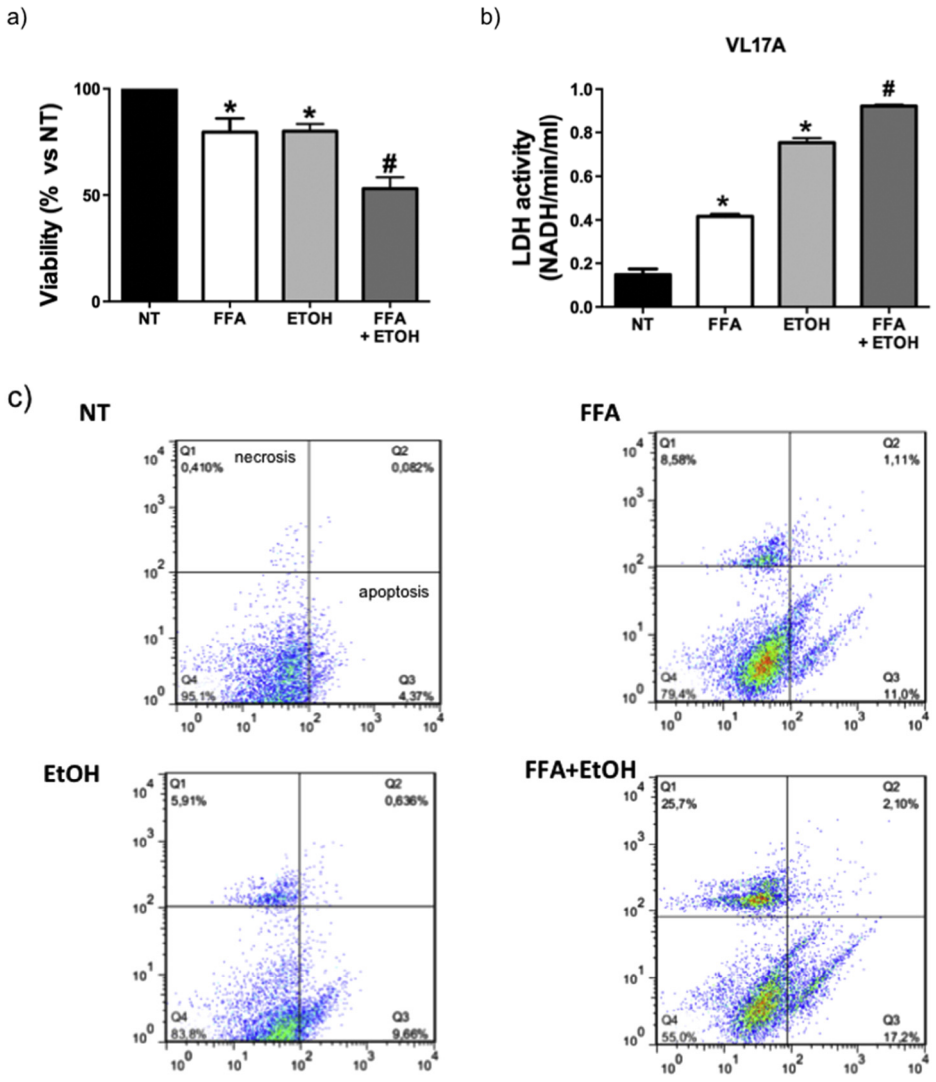

Fig. 2. Free fatty acids induce cell damage and decrease in viability. Cell viability determined by (a) trypan blue exclusion test and (b) by lactate dehydrogenase activity. Each column represents mean ± SEM of three independent experiments. Differences were considered significant at * $p \leq 0.05$ vs NT cells and # $p \leq 0.05$ vs FFA treated cells. Differences were considered significant at * $p \leq 0.05$ vs NT cells and # $p \leq 0.05$ vs FFA treated cells. (c) Cell death assayed by flow cytometry using Annexin-V- FLUOS staining kit following manufacturer's instructions. Quadrant (Q) 1, necrotic; Q3, apoptotic; and Q4 normal cells. 10,000 cells were acquired by event.

4. Discussion

Steatosis is one of the most common liver diseases in Western countries. Important pathophysiological changes, an increased susceptibility of steatotic liver to injury have been reported. (Rolo et al., 2012).

The wide prevalence of alcohol abuse in society makes it an important cofactor in many other liver diseases. In this way, it is

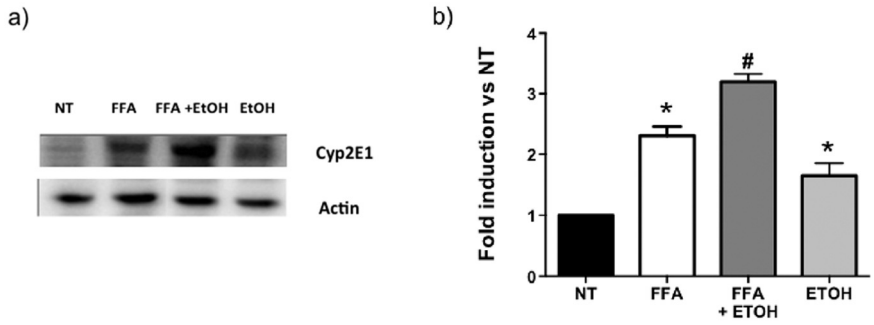

Fig. 3. Cyp2E1 is overexpressed by free fatty acids. Whole cell lysate was obtained and subjected to Western blotting. (a) Representative image of the immunoblot. (b) densitometric analysis of protein content relative to actin used as loading control. Each column represents mean ± SEM of three independent experiments. Differences were considered significant at * $p \leq 0.05$ vs NT cells, and # $p \leq 0.05$ vs FFA treated cells.

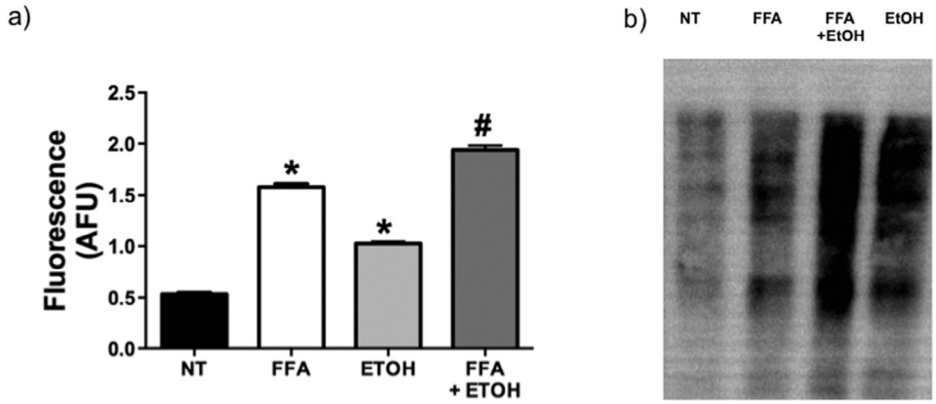

Fig. 4. Lipid overload enhances ROS production and oxidative damage induced by ethanol metabolism. (a) Reactive oxygen species determination by DCFH. Each column represents mean ± SEM of three independent experiments. Differences were considered significant at * $p \leq 0.05$ vs NT cells and # $p \leq 0.05$ vs FFA treated cells. (b) Protein oxidation determined by Oxyblot kit as referred under Materials and Methods. Image is representative of at least 4 independent experiments.

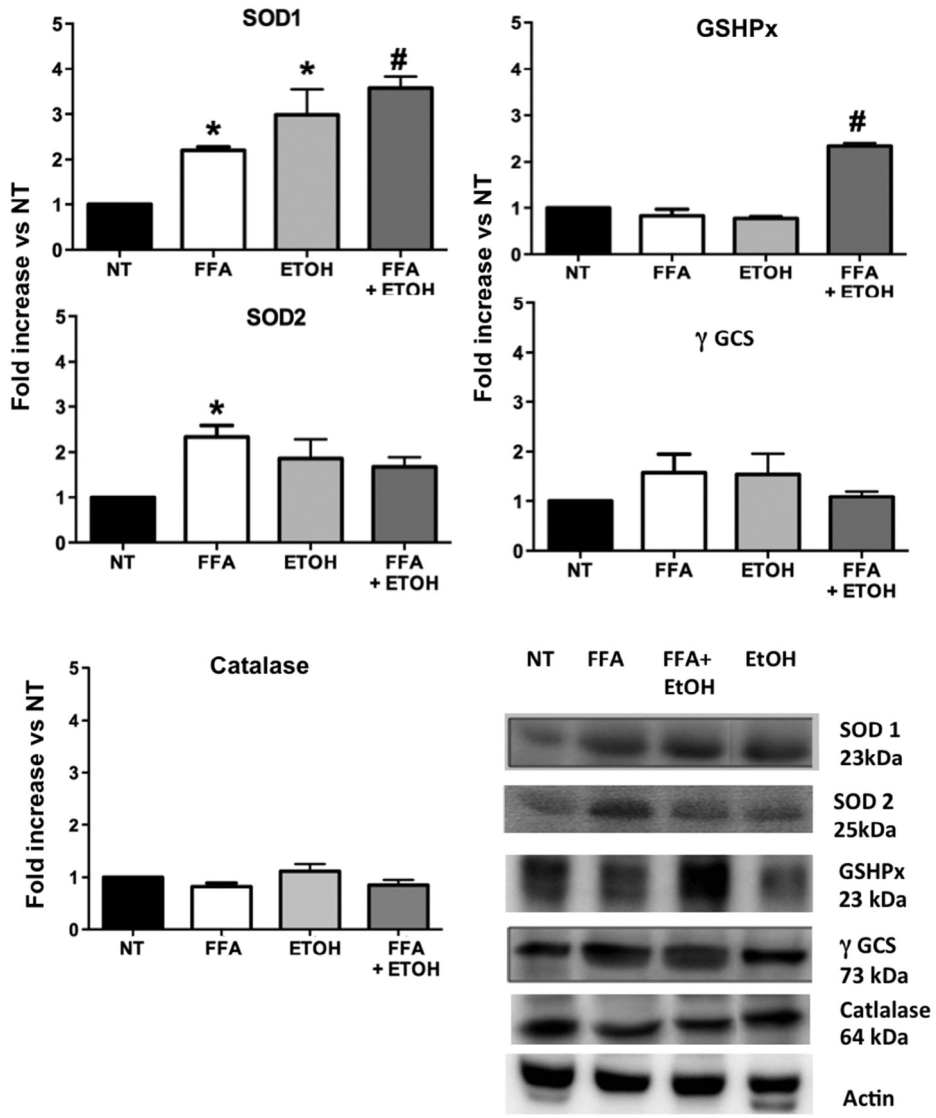

Fig. 5. Antioxidant enzymes expression as a consequence of free fatty acids and ethanol treatment. (a) Representative image of the immunoblot of at least three independent experiments. (b) Densitometric analysis of protein content relative to actin used as loading control. Each column represents mean ± SEM of three independent experiments. Differences were considered significant at * $p \leq 0.05$ vs NT cells, and # $p \leq 0.05$ vs FFA treated cells.

important to address the effect of EtOH on steatotic hepatocytes. FFA overload in VL-17A cells may serve as a good in vitro model for studying fatty liver disease. Consistent with previous reports, we revealed that 1 mM oleic and palmitic acids (2:1) could induce steatosis that can be assessed by oil red O staining (Fig. 1). Our results show that FFA treatment increased triglyceride content in all experimental treatments, with a 7.7-fold increment in FFA plus EtOH treated cells. As VL-17A cells accumulate intracellular lipids there is a significant decrease in cell viability in approximately 50% while LDH activity increased 3.8-fold in cells after the second insult, suggesting that cells are particularly dying by necrosis. VL-17A cells treated with FFA and then EtOH presented 17% cells in apoptosis and 25% in necrosis while FFA treatment produced 11% apoptosis and 9% necrosis, and EtOH treated cells presented 10% apoptosis and 6% necrosis. Percentage of cell death did not present an additive effect when cells were exposed to the two insults, although under this condition, cells presented the highest death.

Free fatty acids not oxidized by mitochondria mainly do by Cyp 2E1 (Lu et al., 2008). Cyp2E1 induction potentiated liver injury in obese mice, and the elevated oxidative stress could be blunted by Cyp2E1 inhibitors (Dey and Cederbaum, 2007). S-adenosyl-L-methionine decreased oxidative stress, steatosis, liver injury, and mitochondrial dysfunction in obese mice. In ethanol fed rats plus high fat diet, serum protein adducts with MDA and 4-hydroxynonenal were significantly increased, accompanied by an increase in Cyp2E1 (French et al., 1993). Thus, Cyp2E1 contributes considerably to the oxidative stress in hepatocytes. Steatotic VL-17A cells treated with EtOH increased 3.5-fold Cyp2E1 content, while treatment with only one of the insults, FFA or EtOH increased 2.1- and 2.3-fold respectively compared with non-treated cells. The Cyp2E1 increment corresponds to an increase in oxidative stress. Steatotic VL-17A cells treated with EtOH increased 3.7-fold peroxide production and 3- and 1.9-fold FFA and EtOH treated cells vs not treated cell value respectively. Protein oxidation, determined by protein carbonyl groups, showed clearly that cells treated with FFA and EtOH presented more oxidized proteins than cells treated only with one of the toxics. These data show that Cyp2E1 increment corresponds to higher oxidative stress damage; thus, cells treated with FFA plus EtOH presented the highest oxidative stress damage. To deal with oxidative stress, hepatocytes have available a wide range of antioxidants and survival factors which are induced by changes in the redox status as an adaptation response.

GSH is the main protector against oxidative stress in the liver as we have previously reported (Enriquez-Cortina et al., 2013; Gomez-Quiroz et al., 2005; Valdes-Arzate et al., 2009) and its relevance in steatosis (Chen et al., 2013; Han et al., 2006) and other liver pathologies has been well documented. GSH is synthesized in the cytosol as a response of many signals, including oxidative stress, and γ-GCS is the rate-limiting enzyme of GSH synthesis. In agreement with our previous report where we showed that one of the main mechanisms of hepatoprotection induced by the hepatocyte growth factor (HGF), in VL-17A cells under EtOH treatments, is the induction of GSH synthesis by a mechanism dependent of γ-GCS expression and NF-kB activation (Valdes-Arzate et al., 2009), in the present work we could not see any change in γ-GCS. These results support the idea that under EtOH toxicity the main antioxidant system is abrogated, possibly by a mechanism of ROS generation in mitochondria that consumes GSH available; even more, it has been reported that HepG2 cells conditioned with a mixture of FFA decreased intracellular GSH and increased lipid peroxidation (Soardo et al., 2011), indicating that GSH could be the main target of ethanol and FFA toxicity. Our data clearly show that, because GSH systems are diminished, cells could thwart the Cyp2E1-derived ROS by GSH recycling mainly driven by GSHPx; as our current data suggest (Fig. 5), this is related to an increment in SOD1 expression.

Hepatocytes are the main cell types that EtOH, and it is herein where more antioxidant protection is needed in addition to GSH system. Our data revealed that some antioxidant proteins are induced in response to the cell insult. Steatotic hepatocytes treated with ethanol increased protein expression of the main antioxidant proteins, such as SOD1 and GSHPx, but failed in SOD2, catalase and γ-GCS.

HepG2 cells devoid of ADH and Cyp2E1 expression did not show significant decrease in viability, and increase in oxidative stress and apoptosis when treated with alcohol. However, VL-17A cells exposed to alcohol were characterized by significant decrease in viability and increase in oxidative stress. The increased oxidative stress and toxicity in VL-17A cells subjected to ethanol exposure were accompanied by increases in Cyp2E1 protein expression. Based on this evidence, we consider that steatosis renders hepatocytes susceptible to damage to another aggression, which increases the production of ROS in the steatotic hepatocyte resulting in a progression in cellular damage.

Our findings indicate that one of the major mechanisms of EtOH-induced hepatocellular damage is the breakdown of GSH system, as we previously reported, by a failure of the activation of NF-kB, and the presence of a steatotic environment worsened the oxidative effects induced by ethanol metabolism. Inducers of the reestablishment of GSH could be useful alternatives for the amelioration of liver damage in the alcoholic liver disease and NASH.

Conflict of interest

The authors declare that there are no conflicts of interest.

Transparency document

The Transparency document associated with this article can be found in the online version.

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