The FXR agonist 6ECDCA reduces hepatic steatosis and oxidative stress induced by ethanol and low-protein diet in mice


1. Introduction and background

Ethanol induces deleterious effects in several systems, including the gastrointestinal, nervous, cardiovascular, hematological, and reproductive systems [1]. Among the serious clinical problems triggered by ethanol abuse, liver diseases are prominent, because the liver is the primary site of ethanol metabolism and the target of its own reactive metabolites, particularly acetaldehyde [2]. Lipid accumulation in hepatocytes, namely steatosis, is the most common and immediate hepatic manifestation of excessive ethanol ingestion [3]. While binge drinking typically results in transient steatosis that resolves if drinking ceases, sustained steatosis due...
to chronic ethanol abuse is frequently related to inflammation and liver damage (i.e., hepatitis) [3]. Lipid peroxidation, a component of cellular oxidative stress, is responsible, at least in part, by causing damage to hepatocytes during the process initiated by ethanol. Lipid homeostasis is altered in general by chronic ethanol consumption, inducing not only alcoholic hepatic steatosis (AHS) but also changes in lipids in the blood and other organs [4,5]. Thus, heavy drinkers may develop serious hepatic problems due to changes in lipid metabolism, and this may amplify cardiovascular responses [6].

After decades of research in this field, knowledge of the pathogenesis of the hepatotoxicity of ethanol is still limited [7]. Some progress has been achieving by establishing the role of oxidative stress and the inflammatory response [7]. It is known that dietary deficiency is a factor predisposing liver diseases in alcoholics. A low-carbohydrate diet associated with ethanol induces more severe hepatotoxicity with intense steatosis [8]. Protein deficiency or malnutrition has also reported to cause or contribute to steatosis [9,10]. Liver plays a central role in lipid metabolism, and both dietary deficiency and ethanol metabolism perturb metabolic homeostasis which may be relevant for the pathogenesis of alcoholic liver disease. However, the mechanisms by which the interaction between ethanol consumption and protein intake deficiency contribute to development of liver injuries are not known.

Inspite of being one of the most public health problems around the world [11] effective drugs for the treatment of alcoholic liver diseases are still not available [2]. Although some drugs have been tested, such as steroids [12] and ursodeoxycholic acid (UDCA) or its conjugate (TUDCA) [13], studies in patients have not demonstrated convincing effects [2]. There is increasing interest in the development of drugs that modulate the activity of nuclear receptors involved in the metabolic regulation. The metabolism of lipids in the liver is regulated by several pathways, which are mediated by the farnesoid X receptor (FXR), a nuclear receptor highly expressed in the liver and that function as a transcription factor. FXR regulates important genes involved in the synthesis and transport of lipoproteins, cholesterol, triglycerides, and bile acids, such as SHP, SREBPI, Apo-AI and -CII, CYPLA1, and BSEP [14,15]. FXR is activated by physiological concentrations of bile acids or by potent agonists, such as 6x-ethyl-chenodeoxycholic acid (6ECDCA) [14]. Due to this critical gene regulation, FXR agonists have been suggested as therapy for cholestasis and liver fibrosis [8] and also as potential drugs for the treatment of insulin resistance and non-alcoholic fatty liver disease (NAFLD) [16]. Studies are lacking, however, on the potential use of FXR agonists on animals with alcoholic fatty disease, particularly under a condition of low protein diet.

Taken all these considerations in account, this work was planned to evaluate whether the FXR agonist 6ECDCA can prevent the effects of ethanol in inducing steatosis and oxidative stress in livers of mice under a condition of low-protein diet.

2. Material and methods

2.1. Animals, diets, and treatments

Swiss male mice (8–10 weeks old), weighting 25–35 g, were kept at a controlled temperature (22 ± 2 °C), under 12 h light–dark cycle and fed ad libitum. All the protocols were approved by the institutional ethics committee for animal research (CEEA) and received the certificate number 438. Fig. 1 shows the experimental design and animal groups. Briefly, the animals were separated into individual cages, and food intake, liquid consumption, and the body weight of the animals were controlled weekly. During the six experimental weeks mice received a low-protein diet (chow with 6% protein) or normal-protein diet (chow with 23% protein) and liquids containing either 10% ethanol or water. In the two last weeks, the animals were redistributed into 5 groups (n = 6) for the initiation of oral treatments (gavage) with 6ECDCA (3 mg kg−1) or 1% tween 80 (vehicle), once a day for 14 days. Thus, the final groups were: G1 (basal group): normal-protein diet + water + vehicle; G2 (negative control): low-protein diet + water + vehicle; G3: low-protein diet + water + 6ECDCA; G4 (positive control): low-protein diet + ethanol + vehicle; G5: low-protein diet + ethanol + 6ECDCA. The 6ECDCA dose (3 mg kg-1) was based on the experiments performed by Fiorucci et al. (2005) [17].

2.2. Sample collection

At the end of 6th experimental week, the animals were fasted for 12 h, and then anesthetized with ketamine (80 mg kg−1) and xylazine (10 mg kg−1), intraperitoneally. Laparotomy was performed for collection of blood and liver samples. Blood was drawn from the abdominal cava vein with heparinized syringes, and plasma was separated by centrifugation (3000 rpm for 10 min) and stored at −70 °C for further analysis. The liver was rapidly collected, frozen in liquid nitrogen and also stored at −70 °C. Another section (major lobe) was appropriately stored for histological analysis.

1  Ain-93G 6% Diet Pelleted, Rhoster Industry and Commerce Ltda. São Paulo – SP - Brazil.
2  Nuvilab CR1, Nuvital Nutrientes S/A, Colombo – PR - Brazil.
2.3. Plasma biochemistry analysis

Plasma was used for determination of the enzymatic activity of aspartate aminotransferase (AST) and alanine aminotransferase (ALT), as well as to determine cholesterol and triglyceride (TG) levels. All of the measurements were performed with commercial kits (Labtest®, Lagoa Santa, MG – Brazil) in an automated system (COBAS 1500®).

2.4. Measurement of hepatic cholesterol and triglycerides

Liver samples were subjected to lipid extraction by the gravimetric technique according to the proposed by Folch et al. (1957) [18], with modifications, using hexane (98.5% pure) as the solvent. Then, the lipids were resuspended in chloroform and isopropanol for later determination of cholesterol and triglycerides with commercial kits (Labtest®, Lagoa Santa, MG – Brazil) in an automated system (COBAS 1500®).

2.5. Hepatic oxidative stress measurements

To measure the enzyme activities of catalase (Cat), glutathione-S-transferase (GST), and superoxide dismutase (SOD), which are indicators of the redox state of hepatocytes, liver samples were homogenized and centrifuged at 13,000 rpm for 20 min. The homogenization was performed on ice and with centrifuge at 4°C. The enzymes were analyzed from the supernatant with established methods: Cat activity was measured according to Aebi [19], in spectrophotometric reaction monitored for 60 s at 240 nm; SOD activity was measured by the ability of this enzyme to inhibit pirogallol self-oxidation, in microplate at 440 nm [20]; and the activity of GST was measured following the method of Habig and colleagues [21], which is based on the capacity of this enzyme to conjugate the substrate 2,4-dinitrochlorobenzene (DNCB) with glutathione in its reduced form, forming a toether that can be measured by the increase in absorbance at 340 nm. In addition to the enzyme activity measurements, the rate of lipid peroxidation (LPO) was measured by the ferrous oxidation in xylene orange (FOX) method following a described protocol [22]. This technique quantifies the formation of hydroperoxides in lipid phase during lipid peroxidation (FOX2). GSH levels were measured using the method described by Sedlak [23] which quantifies the formation of 5,5′-dithiobis-(2-nitrobenzoic acid)-sulfidyl groups by a colorimetric assay. The total reactive oxygen species (ROS) content was quantified via the 2′-7′-dichlorofluorescein-diacetate (DCFH-DA) assay, as proposed by Driver and colleagues [24]. Acetate groups of DCFH-DA allow it to enter the organelles. These groups are removed by esterases producing the reduced DCFH within the organelle, which can be oxidized by peroxides to the fluorescent oxidized dichlorofluorescein (DCF). The formation of DCF was measured immediately after stopping the reaction on ice with a spectrofluorimeter RF-5301 (Shimadzu), in which the excitation and emission wavelengths were set at 504 and 529 nm, respectively. A standard curve with oxidized DCF was used to express the results as nmol mg of protein⁻¹. Most of the results were expressed by the amount of protein in the homogenates, determined by the Bradford method [25]. This reaction was examined at 595 nm in a microplate reader, using bovine serum albumin (BSA) as protein standard [25].

2.6. Liver histology

Cross-sections of the right lobe of the liver were quickly harvested, stored in buffered 10% formalin for fixation, and stained with Hematoxylin and Eosin (HE). A further liver sample was stored in buffered formalin for 3 days and then transferred to a
10%, 20%, and 30% sucrose solution, for 24 h at each concentration. After saturation in sucrose, sections were stored in Tissue-Tek® (O.C.T., Sakura®) and rapidly frozen at -80°C, where they remained until the processing of slides for staining with Sudan Black and Nile Blue. These histological techniques were used to confirm the accumulation of lipids in the tissue. The slides were examined under an optical microscope (LeicaDM2500®) to evaluate cellular changes resulting from ethanol treatment or diet. Scores of steatosis and other lesions, such as inflammation, ballooning and presence of Mallory’s hyaline bodies, were analyzed according to the protocol described by Kleiner and colleagues [26], with slight modifications. The steatosis grade was based on parenchymal involvement (%) by lipid drops, in which less than 5% involved was considered grade 0, 5–33% was considered grade 1, 34–66% was grade 2, and more than 66% was considered grade 3. The ballooning grade was classified according to the number of balloon cells: none, grade 0; few balloon cell, grade 1; and predominant balloon cells, grade 2. Also, the steatosis location predominance (zone 3, zone 1, azonal and panacinar) was considered.

2.7. Gene expression

Measurement of the expression of target genes involved in lipogenesis and apoptosis was performed for each of the liver samples. The mRNA levels were determined for the following genes: Srebp1f [Sterol regulatory element-binding protein 1f], FAS [Fatty acid synthase], CYP7a1 [Cholesterol 7-alpha-monooxygenase], HMGCoA reductase [3-hydroxy-3-methyl-glutaryl-CoA reductase], ApoB [Apolipoprotein B], Scd1 [Stearoyl-CoA desaturase], p53 [protein 53], and Bax [Bcl-2-associated X protein]. The complementary DNA (cDNA) was synthesized from 2.0 μg of RNA, while following all of the reaction steps in a specific program for the PCR-thermocycler. The quality of the synthesized cDNA was evaluated by quantification of the housekeeping gene 36b4, using LightCycler 480 System (Roche®). The expression of the above genes was described subsequently as mRNA relative expression. For this purpose, specific primers for murine genes were used, and the sequences (5’ → 3’) were prepared by Biolegio® Company (Nijmegen, The Netherlands).

2.8. Statistical analysis

Data were analyzed for homogeneity of variance and normal distribution. One-way or two-way ANOVA followed by Bonferroni post hoc tests were used. The parameters considered in the two-way ANOVA were time (week) and treatment. The level of significance was set at 95% (p < 0.05). Statistical analysis and the preparation of graphs were performed with Graphpad Prism version 5.0. The results were expressed as mean ± standard error of the mean.

3. Results

3.1. Chow intake, liquid consumption, and body weight of the mice

The group that showed the most expressive body weight gain was that composed by mice fed a low-protein diet associated with alcoholic liquid diet (ethanol + vehicle). In comparison, these mice

Fig. 3. Plasma concentrations of (A) cholesterol (mg dL⁻¹) and (B) triglycerides (mg dL⁻¹), and activities of (C) aspartate aminotransferase (AST) (U L⁻¹) and (D) alanine aminotransferase (ALT) (U L⁻¹) in mice fed a low- or normal-protein diet, liquid diet (water or 10% ethanol), and treated with 6ECDCA or vehicle. Values are expressed as mean ± standard error of the mean, analyzed by one-way ANOVA and Bonferroni tests. Symbols: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.
gained more body weight than the “basal group”, fed by normal-protein diet (23%) and water. In addition, we observed a decrease in ethanol consumption relative to water consumption in both groups that receive 10% ethanol as the liquid diet (ethanol + vehicle; ethanol + 6ECDCA). However, the treatment with 6ECDCA over the last 15 days of the experiment did not influence the liquid or chow consumption. Fig. 2 shows body weight gain (2A), liquid consumption (2B) and food intake (2C) measured over 6 weeks.

3.2. Plasma biochemistry

The agonist 6ECDCA significantly decreased cholesterol levels when compared with the positive and negative control groups, and maintained plasma level of cholesterol equal to those mice of basal group (Fig. 3A). Interestingly, similar results were also observed for TG. The FXR agonist decreased the plasma TG levels in the mice independent of the presence of ethanol when compared with basal group, negative and positive control (Fig. 3B). Regarding the activity of transaminases (Fig. 3C and D), ethanol increased the plasma AST and ALT in mice. This increase was in the order of 50% and 350% for AST and ALT, respectively, when compared with the negative control (low protein diet + water + vehicle). However, these elevations were reversed by administration of the FXR agonist.

3.3. Hepatic oxidative stress

Ethanol decreased the activity of catalase (Cat) when compared with mice that received equal diet and water (Fig. 4A). This effect
was reversed by the administration of the FXR agonist. There was a decrease of SOD in the animals fed a low protein diet and ethanol when compared with basal and negative control groups. The test drug was able to normalize hepatic SOD to basal activity levels (Fig. 4B). No significant difference was observed in the activity of hepatic GST among the groups of mice (Fig. 4C). While the presence of the FXR agonist reduced them, as shown in Fig. 4D. Ethanol elevated the rate of hepatic lipoperoxidation (LPO) in 48%. However, the FXR agonist treatment reversed this effect, and further reduced the LPO (22%) when compared with negative control (water + vehicle) under the same diet condition (Fig. 4E). A considerable evaluation of the total quantity of hepatic ROS (4F) was found in the animals that received ethanol and vehicle. Corroborating other results, the FXR agonist was also able to reverse the increased ROS induced by the ethanol.

3.4. Hepatic cholesterol and triglycerides

The lipids accumulated in the liver of mice treated with ethanol and vehicle expressively increased when compared with the group of animals treated with water and vehicle. This increase was potentially reversed by the administration of 6ECDCA in the animals which received ethanol (Fig. 5A). Similar results were found for the amount of hepatic triglycerides (5B) and cholesterol (5C).

3.5. Liver histology

Considering the alterations observed in plasma biochemistry, oxidative stress biomarkers, and the macroscopic aspect of the liver in mice that received ethanol (yellow dots suggestive of lipid storages), significant histological alterations were expected in the hepatocytes. Cellular changes were observed mainly in the group that was treated with ethanol. No alterations were observed in mice of the basal group, thus the changes described refer specifically to the group of animals treated with a low-protein diet. Absence of steatosis (grade 0) and hepatocyte ballooning code 1, localized mainly in zone 3, were observed in the negative control (water and vehicle). The positive control (ethanol and vehicle) mice presented with micro- and macro-steatosis grade 2 and 3, ballooning code 0 and 1, and Mallory’s hyaline bodies, both diffuse or predominant in zone 3 (data not shown). In addition, multiple necrotic foci were observed (data not shown). The FXR agonist 6ECDCA was able to reverse these alterations, as no changes were observed in histological sections of mice treated with this drug. The accumulation of lipids revealed by the HE technique (vacuoles in white) was confirmed by Sudan Black (lipids in black) and Nile Blue (TG in pink) stains. These histological findings are shown in Fig. 6.

3.6. Gene expression

Since ethanol consumption induced hepatic steatosis, the expression of genes that regulate hepatic lipid metabolism was investigated, as well as genes that participate in apoptosis. The most significant alterations were observed in the expression of Srebp1f and FAS. Both genes showed the same pattern of expression. Ethanol induced the expression of Srebp1f and FAS when compared with basal group, and 6ECDCA markedly decreased the expression of both genes, even in animals that did not receive ethanol. These data are shown in Fig. 7, normalized by the housekeeping gene 36b4. In addition, the relative expression of other genes linked to lipid metabolism was evaluated: the enzymes Cyp7a1, Scd1, HmgCoA reductase, and the lipoprotein ApoB. However, no significant alteration was observed in these genes, regardless of ethanol or FXR agonist treatment. Absence of significance was also found for expression of the apoptosis-linked genes Bax and p53. These data are shown in the Supplementary material (Table S1).

4. Discussion

The influence of a low-protein diet in aggravating the hepatic injuries induced by ethanol is very clear in the data of the present work. Ethanol induced the prominent biochemical and genetic alterations when it was consumed together with 6% protein chow. The combination of ethanol + low-protein diet increased the hepatic accumulation of triglycerides, plasma ALT and AST activity, and the hepatic levels of LPO and ROS, also decreased Cat and SOD.
activity, as well as elevated the gene expression of Srebp1f and FAS.

Our data are similar to other studies recently conducted on non-alcoholic steatosis, in which protein malnutrition induced hepatic steatosis [27].

The drug tested in this study, 6ECDCA, reversed the increase in both transaminases that was induced by ethanol, and decreased the plasma levels of TG and cholesterol. Moreover, the FXR agonist was effective in normalizing biomarkers of oxidative stress, such as...
In our study, the relative gene expression of Activation of FXR down-regulates SREBPs, which inhibits the synthesis of GSH are controversial comparing published data. Hassing showed that in FXR-null mice, even in those treated with FXR agonist, down-regulation of GST expression was previously reported in FXR-null mice, spontaneous generates oxidative stress [28], so it was expected that an FXR agonist would reduce oxidative stress, as we observed in 6ECDCA-treated animals.

The production of ROS is a naturally occurring process, and a variety of enzymatic and non-enzymatic mechanisms are involved in cellular protection against ROS [29]. Importantly, some of these mechanisms are broken after long periods of exposure to ethanol. In this research we found an increase in the overall amount of ROS induced by the association of ethanol and low-protein diet. Furthermore, chronic ethanol ingestion causes an up-regulation of mitochondrial manganese superoxide dismutase (Mn-SOD) at the mRNA level, which seems to be a protective mechanism [30]. With repeated ethanol administration the increased level of Mn-SOD is gradually diminished. Thus, the adaptive response of Mn-SOD is blunted, leading to increased toxicity with prolonged ethanol exposure [31]. In agreement, our results indicate a reduction in the hepatic activity of SOD in animals that received only ethanol and vehicle when compared with the basal and negative control groups. In the group fed the same diet and ethanol, but treated with 6ECDCA, SOD activity returned to basal levels. Catalase is another important antioxidant and cytoprotective enzyme in hepatocytes that are exposed to ethanol [32]. Cat activity was decreased in the group of mice that received ethanol, compared with the negative control group. However, this effect was reversed by the administration of 6ECDCA. We believe that the present work is the first to report the influence of this FXR agonist in hepatic Cat and SOD activities. These data suggest the recognition of 6ECDCA as an antioxidant drug.

Ethanol has been shown to diminish the levels of GSH, particularly in the mitochondria, which usually require high levels of GSH to eliminate ROS generated during mitochondrial respiratory chain activity [33]. Nevertheless, the effects of ethanol on the levels of GSH are controversial comparing published data. Hassing and colleagues [34] found an increase in GSH levels in rats fed with ethanol, while other authors reported decreases in GSH levels after ethanol intake for prolonged periods and reported no change in hepatic GSH levels after 7 weeks of ethanol consumption [35]. In our data an increase in GSH concentration was found only in the negative control group, possibly indicating a diet-linked effect, independent of ethanol or 6ECDCA treatment. From the data presented, the exact mechanism for this effect cannot be inferred. GSH is an essential component of the antioxidant system and acts as a substrate for GST, which is important in the protection against oxidative stress [33,36]. The enzyme GST did not present significant differences of activity among the groups of mice, even in those treated with FXR agonist. However, down-regulation of GST expression was previously reported in FXR-null mice [28].

Steatosis due to chronic ethanol abuse is largely attributed to two main causes. First, as mentioned above, ethanol metabolism leads to oxidative stress, which results in cellular damage and lipid peroxidation. Second, lipid synthesis is induced by the activation of the sterol response element binding proteins (SREBPs) [37–39]. Activation of FXR down-regulates SREBPs, which inhibits the synthesis and promotes the degradation of TG, and fatty acid oxidation [39]. In our study, the relative gene expression of Srebf1 was decreased with administration of the FXR agonist 6ECDCA, which also reduced the accumulation of lipids in the liver. The SREBP1 protein regulates genes involved in the synthesis of TG, such as FAS, which catalyzes the final step in the biosynthesis of fatty acids. Thus, the presented results of gene expression are complementary with previous data, since 6ECDCA reduced the expression of the genes of both the factor SREBP1 and the enzyme FAS. This suppressed gene expression may have affected the hepatic lipids accumulation induced by the ethanol, corroborating the data of Yang and colleagues [40].

It should be mentioned that although the administration of alcohol in drinking-water is not considered the best model to study alcoholic liver disease, this consumption over several weeks mimics human behavior patterns of intermittent alcohol use and changes in dietary intake [41]. The intake of ethanol for prolonged periods has demonstrated to also affect lipid metabolism in extrahepatic tissues, leading to the development of hypertriglyceridemia and hypercholesterolemia [4,42]. Interestingly, in agreement with previously reported studies using the FXR agonist GW40064 [43] and WAY-362450 [44], 6ECDCA was able to decrease plasma and hepatic levels of cholesterol and TG in mice that received ethanol. The decreased levels of circulating lipids and of accumulation of hepatic triglycerides and cholesterol are also reflected in the lipids observed by liver histology, mainly in samples stained with Sudan Black (specific for lipids evidence) and Nile Blue (specific for TG evidence) techniques. Additionally, ethanol consumption increased the body weight of animals. The increase in body weight gain was not concomitant with an increment in food intake, which confirms that body weight gain was due to the intake of ethanol, an important caloric source, and not the result of diet. However, body weight gain was not observed in mice that received a combination of ethanol + 6ECDCA.

The whole of the results allow concluding that ethanol is able to induce hepatic oxidative stress and the accumulation of lipids in the livers of mice under a condition of low protein-diet. This damage is reversed by the oral administration of 3 mg kg⁻¹ 6ECDCA, an FXR agonist. Importantly, 6ECDCA administration is effective in ameliorating hepatic lesions and metabolism disturbances induced by ethanol, and it also protects hepatocytes from oxidative stress. These data corroborate those recently published with another FXR agonist, namely WAY-362450 [44]. The FXR agonist 6ECDCA is also promising as a potential therapy for alcoholic liver steatosis. Further studies are necessary to validate these results, including long-term 6ECDCA treatment and a toxicity investigation.

Transparency Document

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

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.cbi.2014.03.014.