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Delia Tolosa · Inmaculada Azorín
María Sancho-Tello · Consuelo Guerri
Jaime Renau-Piqueras

Variations in peroxisomal catalase of neonatal rat hepatocyte subpopulations.

Effect of pre- and postnatal exposure to alcohol

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Abstract Alcohol consumption during pregnancy is teratogenic and induces severe alterations in hepatocytes. In the hepatocyte peroxisomal system, ethanol is converted in the presence of H_2O_2 to acetaldehyde and water. Therefore, peroxisomal catalase also acts as an antioxidant defence mechanism by removing H_2O_2 and preventing the formation of hydroxyl radicals in the cell. Alterations in peroxisomal catalase after pre- and pre+postnatal alcohol exposure were investigated in the rat. The effect of pre- and postnatal exposure to ethanol on hepatocyte subpopulations was analysed in isolated hepatocytes originating from periportal, intermediate and perivenous zones. Analysis of catalase revealed that the total activity and content of this enzyme were higher in 12-day-old cells than in cells from newborns and that this increment was more pronounced in treated cells. In controls, the amount of peroxisomal catalase increased mainly in periportal cells, whereas alcohol exposure induced a significant increase in the catalase of perivenous hepatocytes. We conclude that pre- and postnatal alcohol exposure mainly affects the perivenous hepatocyte peroxisomes and that the increase in peroxisomal catalase could constitute a defence mechanism against free radical generation induced by alcohol exposure during the perinatal period.

Key words Peroxisomes · Liver · Hepatocyte subpopulations · Catalase · Alcohol exposure

Introduction

Alcohol consumption can affect almost all organs of the body adversely. However, alcoholic liver disease is the major complication of long-term excessive consumption of ethanol, probably because the liver is the organ responsible for most ethanol oxidation [35, 70]. There are three enzymatic systems in the hepatocyte capable of oxidizing ethanol to acetaldehyde; the cytosolic class I alcohol dehydrogenase system, the microsomal ethanol oxidizing system, and the catalase system mainly located in peroxisomes [32, 70]. In addition, peroxisomes contain oxidases that produce H_2O_2, which is degraded by catalase. The presence of xanthine oxidase and Cu, Zn superoxide dismutase (SOD) reported in peroxisomes [2, 11, 28] indicates that these cell components play a part in the metabolism of oxygen free radicals. Therefore, catalase could act as an antioxidant defence system by eliminating H_2O_2, a precursor of highly oxidizing radicals [22]. Since it has been demonstrated that lipid peroxidation is an important mechanism in alcohol toxicity [32, 41, 68], the catalase system may have a significant role in protecting cells against free radical injury.

Peroxisomes are involved in several important processes, such as gluconeogenesis, catabolism of very-long-chain fatty acids, degradation of prostaglandins and leukotrienes, production of cholesterol and bile acids, and biosynthesis of ether glycerolipids [32, 41, 68]. Marked proliferation and/or functional and ultrastructural alterations of perixosomes have been reported after treatment with several xenobiotics [32]. Although the reports on the effect of chronic alcohol consumption on liver peroxisomes are contradictory [12, 16, 32], an increased number of peroxisomes with reduced size in human liver after chronic alcohol consumption has been reported recently [12, 14].

It has been established that ethanol consumption during pregnancy is teratogenic [66]. Little is known, however, of the effect of prenatal alcohol exposure on fetal liver, which differs from adult liver in many ways including the stage of cell differentiation, functional activity, the supply of nutrients and the response to drugs. We have previously demonstrated that prenatal exposure to ethanol induces severe alterations in newborn hepatocytes [48, 49, 50, 52] and that alcohol exposure during zonal development in the liver has a selective effect on several cell components and enzyme activities, depending on the acinar zone [51,

D. Tolosa · I. Azorín · J. Renau-Piqueras (✉)
Centro de Investigación Hospital La Fe, Avda. Campanar 21; E-46009 Valencia, Spain

M. Sancho-Tello (✉) · C. Guerri
Instituto Investicationes Citológicas, E-46010 Valencia, Spain

61]. In both cases alcohol exposure induces changes in several stereological parameters of the peroxisomes, including a decrease in the mean size and an increase in the total number of these cell components [48]. This effect is more pronounced in perivenous hepatocytes [51, 61].

This study was an attempt to clarify the effect of pre- and postnatal exposure to ethanol on hepatocyte peroxisomal catalase and the distribution of this enzyme in the hepatic acinus.

Material and methods

Animal treatment

Female Wistar rats weighing 150-200 g were used. All animals were maintained under controlled conditions of light and dark (12/12 h), temperature (23° C) and humidity (60%). The rats were fed the Lieber-DeCarli liquid diet, either containing 5% (w/v) ethanol or isocalorically balanced with dextrin-maltose for pair-fed controls [36, 60]. The female rats received the liquid diet (ethanol or control) for a minimum of 40 days prior to exposure to male rats. After mating, the rats were kept on the ethanol or the control liquid diet in separate cages during gestation. The day of parturition was counted as day 0. Since the metabolic zonation of the liver acinus does not occur until the 2nd week of life [3, 26, 27], the litters were culled at birth to 8–10 pups per dam, and all were kept with their own mothers for the next 12 days. During lactation, the mothers were fed the ethanol or the control liquid diet, as above. All the animals were sacrificed by decapitation at the same time of day (9:00 a.m.) to avoid circadian variations in the hepatocyte ultrastructure [21]. Under these conditions we previously found significant variation in the body and hepatic weight of 21-day-old alcohol-exposed fetuses [60]. The variations in the weight of control and alcoholic rats during pregnancy as well as their blood levels of alcohol and acetaldehyde were reported in the same study [60].

Isolation and fractionation of hepatocytes

The method described by Sancho-Tello et al. [61] was followed in detail. Livers from 12-day-old rats were minced and incubated in Ca^{2+}-free Krebs-Henseleit bicarbonate buffer containing 0.5 mM EGTA for 30 min at 37° C. After centrifugation, fragments were resuspended in the same buffer containing Ca^{2+} and collagenase (0.5 mg/ml) for 60 min at 37° C. After washing with Ca^{2+}-free buffer, the cells were filtered through several nylon meshes (pore size 500, 90, 60 and 30 μm) and resuspneded for 15 min in the same buffer containing 1% BSA. Then 2.2 ml of the cell suspension (4×10^6 viable cells/ml) was layered on a discontinuous Percoll isosmotic gradient, and centrifuged for 20 min at 600 *g* at 12° C. During all the steps of this process the cells were gassed with carbogen. After centrifugation, six distinct layers were obtained (F1, buoyant density 1.086 g/cm^3; F2, 1.089 g/cm^3; F3, 1.095 g/cm^3; F4, 1.101 g/cm^3; F5, 1.107 g/cm^3; and F6, 1.112 g/cm^3). Hepatocytes from the different fractions were washed three times, and the cells were resuspended in Ca^{2+}-free buffer containing 0.5 mM EGTA and 1% BSA. To check the reliability of the subfractionation method, rebanding experiments were carried out in some cases. Viability was determined by the trypan blue exclusion test.

Biochemical determinations

Hepatocytes were resuspended in 10 mM HEPES-buffer with 1% Triton X-100 and frozen at −80° C. The next day the cells were disrupted by freezing and thawing three times and were homogenized. The enzymatic activities of alanine aminotransferase (ALAT) [44] and glutamate dehydrogenase (GDH) [63] were measured as periportal and perivenous markers, respectively [59, 62].

Livers from control and alcohol-exposed rats were homogenized in 10 vol of 0.25 M sucrose. The homogenates were centrifuged at 10000 *g* for 10 min and the supernatants were used for determining the protein content and the catalase activity. This was determined spectrophotometrically by measuring the decrease in absorbance of H_2O_2 at 240 nm [1]. Units were expressed as seg^{-1}/mg protein or seg^{-1}/g tissue.

The protein concentration was determined using Coomassie protein assay reagent (Pierce, Rockford, Ill.).

SDS gel electrophoresis and immunoblotting

Liver tissues were homogenized in sample buffer (60 mM Tris-HCl, 10 mM EDTA, 2% SDS, pH 6.8). The protein concentration was determined as above. Proteins were separated by sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis (SDS-PAGE) following the buffer system of Laemmli [31] and with 19% (w/v) polyacrylamide in the separation gel. Proteins from SDS-PAGE were blotted onto Immobilon-P membrane (Serva, Heidelberg, Germany) according to Kyhse-Andersen [30], and the immunoblot was carried out following the ProtoBlot Immunoblotting System (Promega, Madison, Wisc.). A rabbit polyclonal anti-catalase antibody, kindly provided by Drs. H.D. Fahimi and A. Völkl (Heidelberg, Germany), was used as the primary antibody at 1 μg/ml in TBST for 120 min at 22° C. As the secondary antibody, a goat anti-rabbit IgG-alkaline phosphatase conjugate (Sigma), at 1/6500 in TBST, was used. Finally, the colour was developed with Western Blue Stabilised Substrate for Alkaline Phosphatase (Promega).

Electron microscopy and immunocytochemistry

Samples of liver from newborn (day 0) and 12-day-old control and ethanol-exposed rats, and hepatocytes from each fraction were processed for electron microscopy and embedded in Epon as described elsewhere [24, 61].

Immunolocalization of catalase was performed using the immunogold technique [5, 24, 39, 53] and the rabbit anti-catalase polyclonal antibody used in immunoblotting. Ultrathin sections (80 nm) mounted on carbon-formvar-coated nickel grids were floated for 60 min on a saturated aqueous solution of sodium metaperiodate, washed three times with water [7, 24], floated for 30 min on 0.1% BSA-Tris buffer (20 mM Tris-HCl, 0.9% NaCl, pH 7.4, containing 0.1% BSA, type V) supplemented with 5% heat-inactivated serum (FCS) and then transferred to droplets of 0.1% BSA-Tris buffer containing 1% FCS and the antibody (1 μg/ml) for 12 h at 22° C. After three rinses for 10 min each with 0.1% BSA-Tris buffer, the grids were placed on droplets of 0.1% BSA-Tris buffer containing 0.5% Tween 20, 5% FCS, and a goat anti-rabbit IgG-gold complex (Sigma, 10 nm, 1:10 dilution). The incubation time was 60 min at 22° C. After two 10-min rinses with 0.1% BSA-Tris buffer and a rinse in double distilled water, the sections were air-dried and finally counterstained with uranyl acetate. Immunocytochemical controls were incubated without the first antibody or using a nonimmune antiserum (Sigma). Quantitative analysis of gold particle distribution was carried out as described elsehwere [6, 71]. For each sample, a minimum of 100 peroxisomes were analysed and the labelling was expressed either as the mean number (±SD) of gold particles per peroxisome or as the mean number (±SD) of gold particles per square micrometre of peroxisome matrix.

Statistical analysis

The results were expressed as mean±SD. Statistical comparison of data was done using ANOVA and Student's *t*-test with the SOLO 4.0 program (BMDP Statistical Software, Ireland).

Results

Characteristics of subfractionated hepatocytes

The viability, recovery and ultrastructural features of control and alcohol-exposed hepatocytes after separation

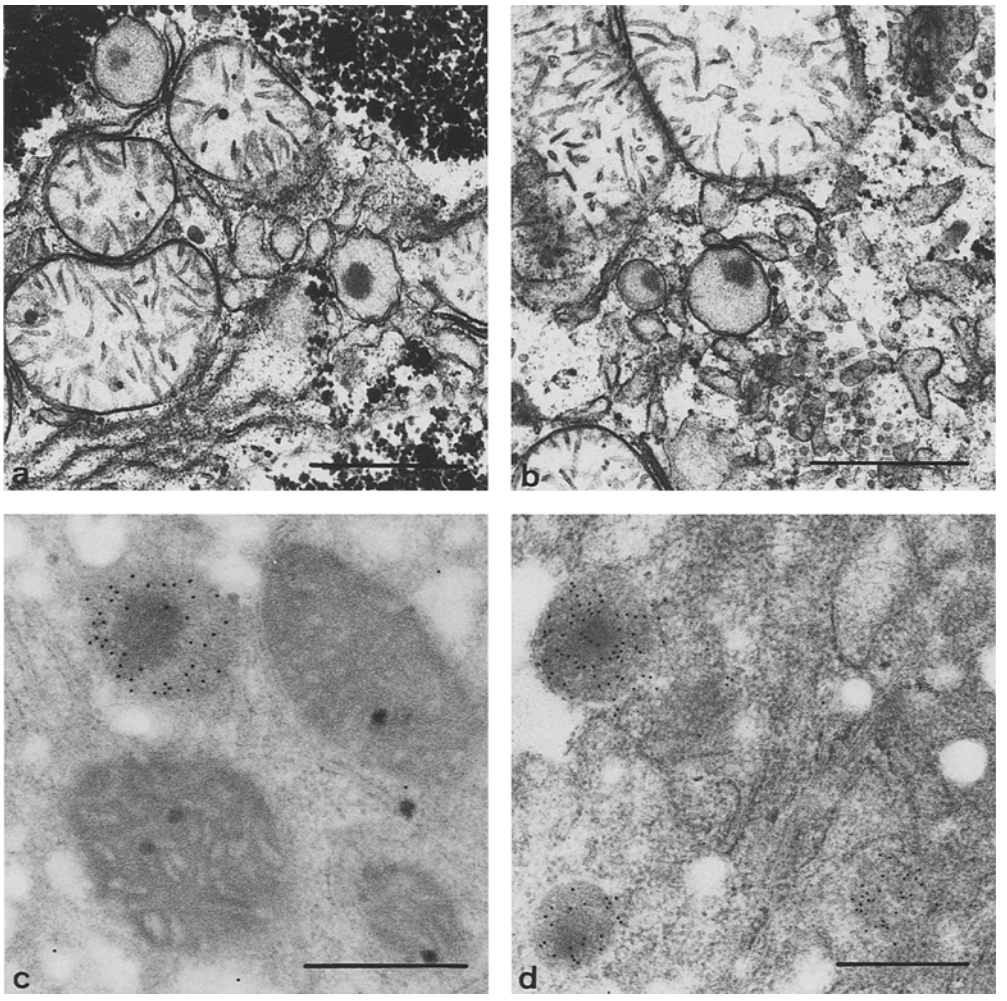

Fig. 1A–D Micrographs showing the ultrastructural features of subfractionated hepatocytes (F5 fraction) from **A** control rats and **B** rats subjected to pre- and postnatal exposure to ethanol. Several cell components, including peroxisomes, display good ultrastructural preservation. Small peroxisomes, as illustrated in **B**, were frequently present in alcohol-exposed cells. **C**, **D** Electron micrographs from control and alcohol-exposed day-0 rat liver sections incubated for immunocytochemical localization of catalase. Labelling appears over peroxisomes. The gold particle density over peroxisomes of alcohol-exposed hepatocytes was greater in cells from experimental rats than in control cells: for example, the gold density in the peroxisome in **D** (416 particles/μm^2) was approximately twice that of the peroxisome in **C** (196 particles/μm^2) **A** ×27300; **B** ×27300; **C** ×56000; **D** ×45000; *bars* **A, B** 1.0 μm; **C, D** 0.5 μm

by discontinuous gradients were similar to those described previously [61]. Figure 1 illustrates some ultrastructural aspects of isolated hepatocytes and shows that the small peroxisomes were frequently present in alcohol-exposed cells. The F1 fraction was not considered because this fraction contained damaged cells and doublets. The specific activity of the periportal marker ALAT, as already reported [61], increased from F2 (7.5±0.83 nmol/min per mg protein) to F6 (11.8±1.07 nmol/min per mg protein). In contrast, the perivenous marker enzyme GDH showed the highest activity in F2 (57.5±6.27 nmol/min per mg protein; F6, 26.1±3.66 nmol/min per mg protein). Although the same distribution of ALAT and GDH activities was obtained for alcohol-treated hepatocytes, a lower GDH-specific activity was found in all the fractions.

Biochemistry and immunobloting in liver tissue

The activity of total catalase in the liver increased significantly ($P<0.005$) from newborn levels to those of 12-day-old control animals (15.7±1.2 U/g tissue and 25.1±2.1 U/g tissue, respectively). A significant ($P<0.005$) increase in catalase activity was also observed in alcohol-exposed rats (day-0 rats: 18.6±2.0 U/g tissue; 12-day-old rats: 33.1±3.2 U/g tissue). These data also indicate that alcohol induces a moderate increment in the activity of hepatic catalase, which is more pronounced in 12-day-old rats.

The immunoblot analysis showed a higher total catalase content in control adult rat liver than in 12-day-old liver (Fig. 2). It also indicated that pre- and postnatal exposure to ethanol slightly increased the catalase content in 12-day-old rats (Fig. 2).

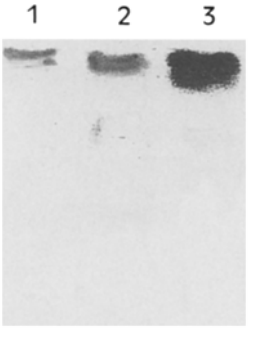

Fig. 2 SDS-PAGE of liver homogenates from 12-day-old control (*1*), 12-day-old ethanol exposed (*2*) and adult control rats (*3*). The same amount of protein was applied on each lane, and blots were incubated with an antibody to catalase

Immunocytochemistry

Day-0 hepatocytes. Liver tissue

In hepatocytes from newborn rats, as in 12-day-old cells and isolated hepatocytes, the anti-catalase antibody labelled mainly peroxisomes (Fig. 1), and the labelling over cytoplasm and mitochondria was scant (Fig. 1). The extracellular space lacked gold particles. This labelling pattern was considered specific when compared with immunocytochemical controls. Quantification of labelling showed no significant differences between control and alcoholic animals (Fig. 3). In both types of animals the intensity of labelling was heterogeneous both between different hepatocytes and within the same cell.

Day-12 hepatocytes. Liver tissue

Peroxisomes in both control and ethanol-exposed hepatocytes from 12-day-old rats showed an increase in labelling compared with newborn rats. Analysis of immunogold labelling showed striking differences between the anti-catalase-binding sites in peroxisomes from control and alcohol-exposed hepatocytes (Fig. 3). When this labelling is expressed as the number of gold particles per peroxisome, the data indicate that alcohol induces a significant increase in the number of anti-catalase binding sites. In both control and alcohol treated hepatocytes, peroxisomes also showed heterogeneous labelling.

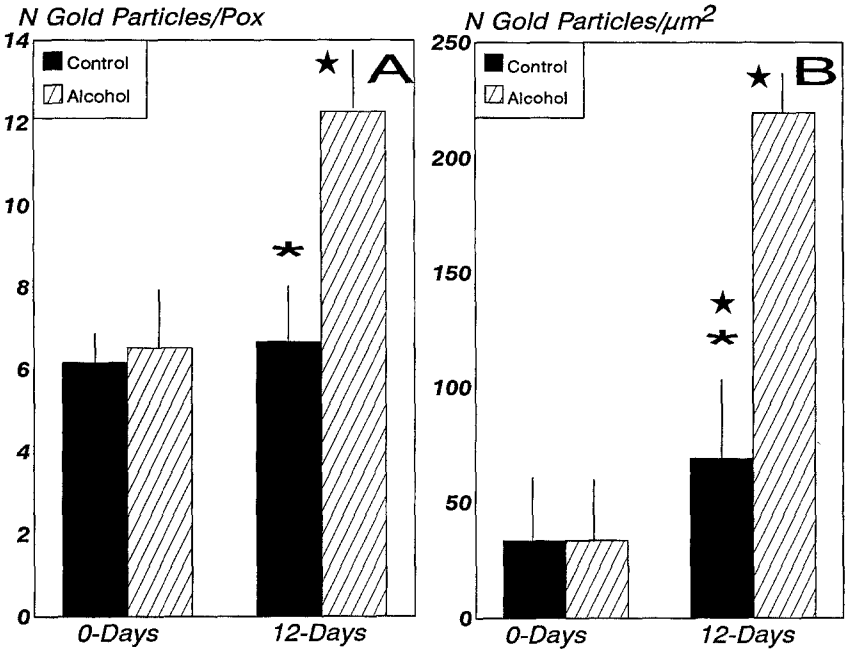

Fig. 3A, B Results of quantitative analysis of immunocytochemical study using anti-catalase in peroxisomes from control and alcohol-exposed animals. The labelling density is expressed as the number (mean±SD) of gold particles per peroxisome (**A**), and as the number (mean±SD) of gold particles per μm^2 of peroxisomal matrix area (**B**). Significant differences ($P<0.05$) between controls and alcohol-exposed rats are indicated with an *asterisk*. *Stars* indicate significant differences ($P<0.05$) between day 0 and 12-day-old controls or between day-0 and 12-day-old treated animals

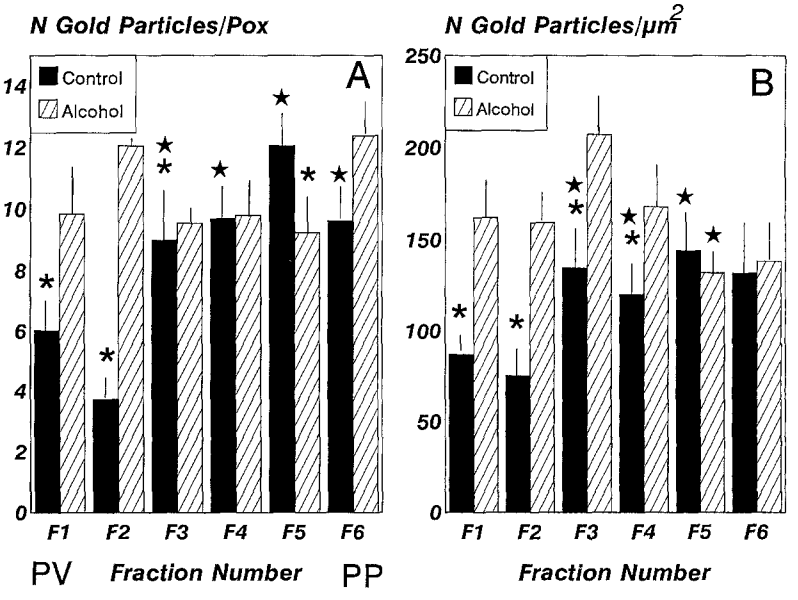

Fig. 4 Quantitative analysis of immunocytochemical labelling with anti-catalase of peroxisomes from subfractionated control and alcohol-exposed hepatocytes. Labelling is expressed as in Fig. 3. Significant differences ($P>0.05$) between each control and treated fraction are indicated by *asterisks*. Statistical differences between F2 and the other fractions are indicated by *stars*

Day-12 hepatocytes. Hepatocyte subpopulations

Quantitative analysis of gold particle distribution in control cells indicated that when the labelling was expressed as the number of gold particles per peroxisome, these organelles exhibited significant increases in F4 and F5 fractions (Fig. 4), and when this density was expressed as the number of gold particles/μm^2, the maximum labelling was found in the F3 to F6 fractions, with a peak corresponding to the F5 fraction (Fig. 4). This variation could be due to differences in the mean peroxisomal size in the different fractions [61]. As in tissue, heterogeneous labelling of the peroxisomes was observed. However, the cells of the different fractions showed a similar heterogeneity.

Figure 4 shows the distribution of anti-catalase binding sites over peroxisomes in the different hepatocyte fractions from alcoholic animals, which clearly differs from that observed in controls. The main finding was a significant increase in the labelling of peroxisomes, which was more pronounced in fractions F1 to F4, with a maximum in F3. Heterogeneous labelling of peroxisomes in all the fractions was also found, although this heterogeneity was similar to that found in controls.

Discussion

The advantages and disadvantages of the hepatocyte fractionation method used here, as well as the correlation between the different cell fractions obtained and the hepatocyte subpopulations in the liver acinus have been discussed previously [38, 61]. Selective isolation of periportal and pericentral hepatocytes from adult rats has recently been used as a new approach to studying the heterogeneity of enzyme peroxisomes [72].

Quantitative immunocytochemistry indicated a gradient of peroxisomal catalase concentrations along the acinus. Previous studies have reported zonal heterogeneity of peroxisomal enzymes in adult rat liver [6, 37, 72], and we show here that catalase labelling increased in hepatocyte subpopulations corresponding to zones I and II. If we take into account the metabolic role of catalase [40, 65], we see that the distribution of this enzyme in the fractions agrees with the model of metabolic zonation of liver parenchyma [25]. In addition, we found a moderate increase in the amount and activity of catalase in 12-day-old animals compared with newborn rats. This increase is in agreement with the results of previous studies [67] showing postnatal development of the activities of several peroxisomal enzymes, such as urate oxidase, catalase and D-amino acid oxidase. In all cases, marked differences were noted in the intensity of labelling of peroxisomes within the same cell. This agrees with previous reports concerning not only catalase but also several peroxisomal oxidases [10, 17, 57]. These differences suggest, as already reported, that peroxisomes do not constitute a homogeneous population [17, 57].

The results presented in our study show that pre- and postnatal exposure to ethanol induces a significant increase in catalase activity and content. These results agree with those of previous studies on adult liver and brain [23, 33, 34, 43, 54, 55] and with those of a recent study reporting that fetal brain cells in culture showed an alcohol-induced increment in the activity and amount of catalase [4]. In addition, it is important to point out that pre- and postnatal exposure to ethanol resulted in a strong negative effect on the activity of class I alcohol dehydrogenase in the liver [8]. The activity of the alcoholic offspring reached only 30% of that of the controls. This decrease in this enzyme and the increase in catalase after alcohol exposure suggest that the catalase system may be a significant pathway of ethanol metabolism during the perinatal period.

It has been reported that catalase plays a role not only in the direct oxidation of ethanol but also in the elimination of the H_2O_2 originating during alcohol metabolism throughout the microsomal NADPH oxidas system [20]. The enzyme, which is present at high concentrations in the peroxisomal matrix, decomposes H_2O_2 either catalytically, when H_2O_2 serves as the hydrogen donor, or by peroxidation, when another substrate, such as ethanol, functions as a hydrogen donor [40, 41, 68]. Although it is generally accepted that ethanol is oxidized predominantly via cytolosic ADH, recent studies suggest that ethanol elimination via catalase may be significant if such substrates as fatty acids are available to generate H_2O_2 inside the peroxisomes [23]. For example, in the fasting state, in which the substrates for peroxisomal β-oxidation increase, there is a greater generation of H_2O_2 [23, 69]. Under these conditions, the rate of methanol oxidation via catalse- H_2O_2 accounts for about 60% of total alcohol oxidation in the presence of fatty acids [23]. Therefore, it appears that in the fasting state, catalase-H_2O_2 could be the predominant pathway of alcohol oxidation.

H_2O_2 can be generated during ethanol metabolism by the microsomal mono-oxygenase system and particularly by the ethanol-inducible form of cytochrome P-450 (CYP2E1) [20]. This isoform has a strong potential for producing both superoxide anion and H_2O_2 through the NADPH oxidase activity [15, 47]. Therefore, the induction of CYP2E1 with the concomitant free radical formation that occurs after chronic alcohol consumption may play an important part in the pathogenesis of alcohol-induced liver damage [18]. In addition, acetaldehyde, the first product of the ethanol degradation, may also stimulate the formation of oxygen-derived free radicals and H_2O_2 through aldehyde oxidase and xanthine oxidase [29, 64]. Finally, H_2O_2 is detoxified by peroxisomal or cytoplasmic catalase [19, 42].

Our results also show that pre- and postnatal alcohol exposure induces changes in the amount of peroxisomal catalase in the different hepatocyte subpopulations. The main effect consists in an increase in this enzyme in the fractions corresponding to perivenous hepatocytes, which could be related to the alcohol-induced increase in the number of undersized peroxisomes within these cells [48, 61]. We have previously demonstrated that pre- and

postnatal exposure to ethanol alters the ultrastructure of hepatocytes corresponding mainly to the perivenous zone (zone III) [51, 61], and it is known that a characteristic feature of alcohol-induced adult liver injury is the predominance of steatosis and other lesions in the perivenous zone of the hepatic acinus [33, 56, 58]. It has been proposed that in these conditions, anoxic injury is produced mainly in perivenous hepatocytes by an increased consumption of oxygen, which exacerbates the hypoxia [35, 56, 70]. In addition, CYP2E1 is located in the perivenous region, which might aggravate the centrilobular lesions induced by alcohol in this zone by generating reactive oxygen radicals [9]. Therfore, the increase in catalase might be an adaptive mechanism against free radical generation in the perivenous zone of the liver.

Pre- and postnatal, exposure to ethanol has been shown to induce significant stereological variations and an increase in catalase, indicating a possible role for this enzyme in reducing free radical toxicity and in metabolizing ethanol during the perinatal period. These alterations are mainly related to perivenous hepatocytes, in which the disposal of xenobiotics, such as ethanol, is catalysed preferentially, leading to free radical generation [25].

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