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Involvement of alcohol and aldehyde dehydrogenase activities on hepatic retinoid metabolism and its possible participation in the progression of rat liver regeneration

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ABSTRACT

Liver alcohol dehydrogenase (ADH) activity is decreased towards exogenous substrates after partial hepatectomy (PH), probably due to putative endogenous substrates acting as ADH inhibitors. Hence, retinoids could be suitable candidates as such endogenous substrates. Therefore, cytosolic ADH kinetic analysis using several substrates, liver cytosolic and mitochondrial aldehyde dehydrogenase (ALDH) activities, retinal and retinol content, as well as expression of proteins for ADH and CRBPI (a retinol carrier protein) were determined in liver samples, at two stages of liver regeneration (one- or two-thirds PH). The effect of inhibiting *in vivo* liver ADH by 4-methylpyrazole (4-MP) was also evaluated after 70%-PH. With 70%-PH, *in vitro* ADH activity towards exogenous alcohols and aldehydes was diminished, but retinol oxidation was increased and retinal reduction was decreased. These activities that be due to the participation of an ADH type which did not correlate with the amount of immunoreactive ADH protein. Cytosolic and mitochondrial ALDH activities oxidized actively retinal, whereas retinol and CBRP-I expression were reduced in these animals. With 30%-PH, these changes were less evident and sometimes opposite to those found with 70%-PH. In addition, retinol readily inhibited ADH-mediated ethanol oxidation. Interestingly, *in vivo* 4-MP administration inhibited ADH activity in a dose-dependent manner correlating with a progressive inhibition of liver regeneration. In conclusion, PH-induced inhibition of ADH (mainly type I) seems to be related to ADH-mediated retinoid metabolism during liver proliferation. Thus, results suggest a role of ADH in retinoid metabolism, which is apparently required during rat liver regeneration.

1. Introduction

The regenerating rat liver induced by partial hepatectomy (PH) is a useful proliferative *in vivo* model that allows for the assessment of the metabolic activity of dividing and quiescent cells [1,2]. Interestingly, this experimental model of liver regeneration is highly sensitive to acute and chronic ethanol, which inhibits DNA synthesis in PH-induced rat regenerating liver and in cultured hepatocytes [3–6]. In addition, one consequence of ethanol intoxication is a reduction in retinoic acid (RA) levels. Indeed, reduced RA synthesis could possibly result from ethanol-induced competitive inhibition of retinol oxidation, catalyzed by alcohol dehydrogenase (ADH), thereby contributing to the adverse retinol/ethanol drug interaction observed after *in vivo* ethanol intoxication [7]. This could be important because RA is involved in regulating gene expression

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of many physiological mechanisms, such as development, regeneration, growth and differentiation of adult epithelial tissues, as well as in apoptosis [8–10]. RA synthesis is mediated by several enzymes pertaining to ADH and aldehyde dehydrogenase (ALDH) families [7,11], which oxidize retinol to retinal followed by oxidation of retinal to RA.

In rats subjected to PH, ethanol elimination efficiently occurs with decreased liver ADH activity, supporting the statement that the NAD+/NADH ratio constitutes the main regulatory factor for ethanol oxidation rather than ADH activity *per se* [12]. In addition, PH rats treated with ethanol produce similar amounts of acetaldehyde, and ALDH activity has been reported to be only slightly affected by PH [13]. We found that rats subjected to PH actively metabolize ethanol *in vivo*, which is mainly attributed to hepatic catabolism in the remnant organ, even in the presence of an *in vitro* decreased ADH activity [14,15]. However, we also found that ethanol administered to PH animals was capable of preserving liver ADH activity in the remnant liver [15]. The clear discrepancy between ADH activity after PH and the enhanced ethanol metabolism presented by the regenerating rat liver, led us to speculate that ADH and, probably, ALDH are actively using endogenous substrates, potentially involved in the progression of the regenerating liver. Among these potential substrates, retinoids are interesting candidates due to their well-known relevance in proliferative processes.

Although it is accepted that retinoid metabolism exerts an important role on epithelial differentiation and regeneration, little is known regarding the possible regulatory role of retinoids on proliferative events that characterize PH-induced rat liver regeneration. After 70%-PH, free liver retinol (not as retinyl esters) decreases, while a cellular retinol-binding protein (CRBP) that regulates cell retinoid levels, mainly those of retinol and RA [16], is actively expressed after PH [17,18]. Indeed, it has been suggested that changes in liver Vitamin A levels would correspond to the proliferative activity of stellate cells, rather than reflect that of hepatocytes [19]. However, participation of ADH and ALDH during retinol metabolism in the progression of PH-promoted rat liver regeneration has not been explored.

The present study explores if the participation of ADH and ALDH activities in metabolizing retinol and retinal is a relevant step to control retinoid levels in the regenerating liver. This was assessed in two stages of liver regeneration: two-thirds PH, which shows the maximal proliferative response as compared with one-third PH (30%), where liver DNA synthesis and rate of mitosis are clearly lower. In addition, the effects of the pharmacological inhibition of ADH *in vivo*, by administering 4-methylpyrazole (4-MP) to 70%-PH, were also assessed regarding progression of liver proliferation under these conditions.

2. Material and methods

2.1. Materials

(All-*trans*)-retinoic acid, -retinol, -retinal, horse liver ADH, NAD+, NADH, 4-MP, and other reagents were purchased from Sigma Chemical Co. (St. Louis, MO). Goat polyclonal antibody against type I ADH, as well as a CRBP-I monoclonal antibody, was from Santa Cruz Biotechnology.

2.2. Animal treatments

Male Wistar rats (240–270 g, body weight) were housed under 12:12 h light/dark conditions with free access to food and water. Two-thirds PH was performed according to the Higgins and Anderson technique [20], while one-third PH was accomplished by removing the left liver lobe (≅30% of liver mass) as described previously [21]. Controls were always sham-operated rats. After surgery (from 12 to 96 h), animals were decapitated under sodium pentobarbital anesthesia. Another set of animals subjected to 70%-PH received 4-MP (from 25 to 200 mg/kg, b.w.) intraperitoneally shortly after surgery, animals were killed 24 h after PH, as described above. Animals were handled according to the Federal Regulations for Animal Experimentation and Care (Ministry of Agriculture, SAGARPA, Mexico).

2.3. Liver sampling and subcellular fractionation

Liver samples were taken for subcellular fractionation. The cytosolic and mitochondrial fractions were obtained by differential centrifugation as described by Aguilar-Delfín et al. [21].

2.4. Analytical procedures

Optimal activity of cytosolic ADH (EC 1.1.1.1) was assayed for ethanol (at pH 9.7, with glycine buffer) and for acetaldehyde (at pH 7.5, with potassium phosphate buffer), as described by Julià et al. [22]. Cytosolic and mitochondrial ALDH activity (EC 1.2.1.3) was determined spectrophotometrically, as described in detail by Ambroziak and Pietruszko [23]. The oxidation of retinol and reduction of retinal through ADH activity (as validated by their inhibition with 1 mmol/L 4-MP) was tested, as well as ALDH-mediated oxidation of retinal. For this, both retinoids were dissolved in acetone and added to a 100 mmol/L sodium phosphate buffer (pH 7.4), containing 20% Tween-80, as described by Parés and Julià [24]. The amount of retinol and retinal was spectrophotometrically determined by their absorbance at 328 and 400 nm, respectively. Thymidine kinase activity (EC 2.7.1.21), as a reliable DNA synthesis marker, was measured according to Sauer and Willmanns [25]. In addition, the mitotic index as indicative of cell proliferation was calculated based upon the conditions described elsewhere [26]. Protein from liver subcellular fractions was determined by the method of Lowry et al. [27].

2.5. Extraction and quantification of retinoids

The levels of free retinol and retinoic acid were quantified in liver samples by HPLC. For this, 2 mL of total liver homogenate (1:4) was mixed with 200 μL of methanol/acetone (v/v), and then centrifuged at 10,000 × *g* for 10 min (4 °C). The organic phase was completely evaporated and suspended in 200 μL of a mixture (1:1 v/v) of methanol/dimethylsulfoxide. Once the samples were extracted, an aliquot was injected into an HPLC equipped with a C-18 column (4.5 mm × 250 mm), flow rate of

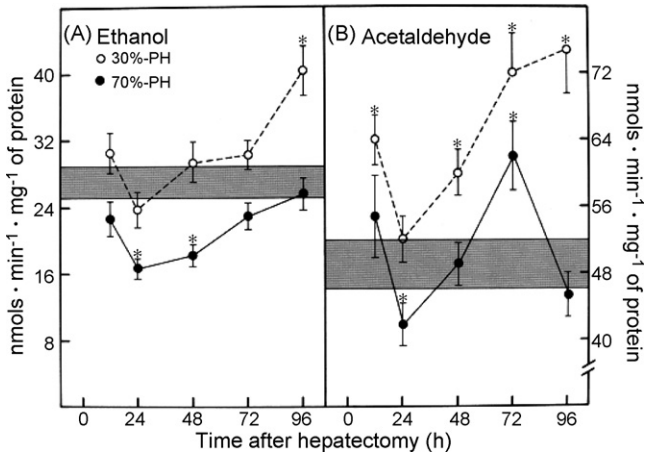

Fig. 1 – Liver ADH activity towards ethanol and acetaldehyde in rats subjected to 30- or 70%-PH. Results are the mean ± S.E.M. of five individual determinations for each experimental point, of animals subjected to 30%-PH (empty symbols) or to 70%-PH (solid symbols). In panel (A) cytosolic ADH was tested against ethanol (20 mmol/L); whereas in panel (B) acetaldehyde (1 mmol/L) was the substrate. Shadowed bars in both panels represent the control range of ADH activity for the substrates tested. Statistical significance was considered at $^{*}p < 0.01$ vs. the control (sham-operated) group.

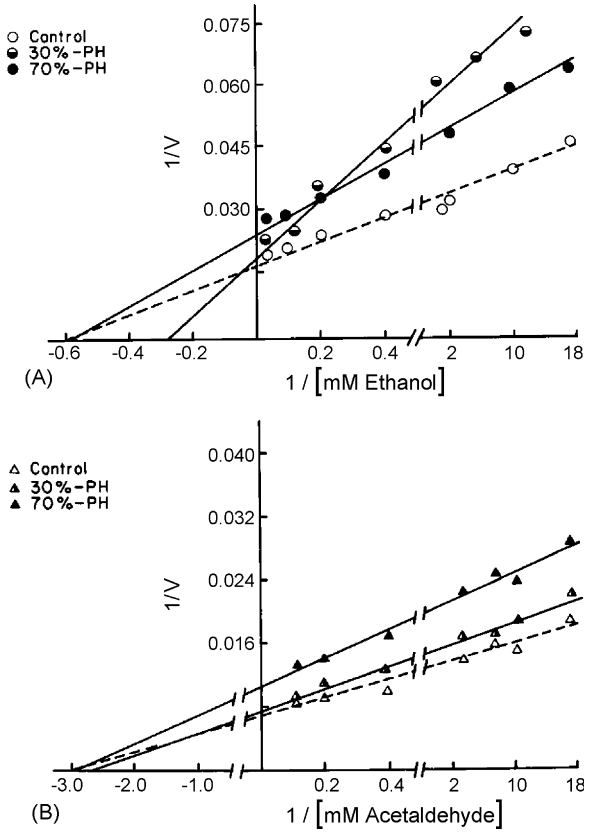

Fig. 2 – Lineweaver–Burk plot analysis of ADH activity towards ethanol and acetaldehyde in control and PH rats. Representative plots resulting from the mean ± S.E. of five individual determinations for each experimental point. Symbols at the top of the figure: control (empty), and animals subjected to 30%-PH (empty/solid), or to 70%-PH (solid symbols), 24 h after PH. The V (rate) is expressed as nmol min^{-1} mg^{-1} of protein. Note the non-competitive-like inhibition presented by preparations from 70%-PH animals, and that corresponding to competitive inhibition in 30%-PH rats.

1 mL/mL of a mobile phase consisting of 0.5 mM ammonium acetate, methanol, and acetonytrile (25:65:10 v/v/v) as solvent A, and acetonytrile alone as solvent B. A/B gradients were determined as described by Molotkov et al. [28]. Retinol was detected at 328 nm and RA at 350 nm.

2.6. *Estimation of retinaldehyde through enzymatic reduction of retinol*

Retinal present in the cytosolic fraction was converted into retinol *in vitro*, using purified liver horse ADH. For this, 200 μg of cytosolic protein were incubated with 0.3 mmol/L NADH in presence of excess horse liver ADH (PBS, pH 7.5). An internal standard of all-*trans*-retinal (50 μg) was included and its reduction to retinol was spectrophotometrically recorded (at 328 nm) in each sample. After 20 min incubation, the additional amount of retinol quantified by HPLC was considered as the net content of retinaldehyde.

2.7. *Western blot analysis*

Cytosolic samples (50 μg) were normalized for protein concentration and Western blot analysis. Samples were applied to a 10% SDS-PAGE; thereafter, proteins were transferred electrophoretically to nitrocellulose filters for 2 h at a constant current of 330 mA/cm^2, and non-specific sites were blocked by overnight (4 °C) incubation with phosphate-buffered saline containing 5% fat-free milk. Membranes were probed with goat monoclonal anti-CRBP-I, or with anti-ADH type I (diluted 1:300 with PBS) overnight and the filters were incubated with anti-goat-HRP antibody. Rabbit anti-mouse β-actin antibody (Calbiochem, La Jolla, San Diego) was used as primary antibody to control the amount of protein loaded on each lane. Proteins in the nitrocellulose membranes were detected using the ECL method and densitometrically quantified.

2.8. *Calculations and statistics*

Kinetic parameters were calculated by means of the non-linear Enzfitter program (1989). Statistical significance of the differences was assessed by two-way ANOVA and by the Newman Keuls' test.

3. Results

We compared ADH activity towards ethanol oxidation and acetaldehyde reduction, under optimal enzymatic conditions, in remnant liver, during two stages of liver proliferation (Fig. 1). Cytosolic ADH activity showed a significantly lower

Table 1 – Apparent cytosolic ADH kinetic constants towards ethanol and acetaldehyde as substrates in animals subjected to 30- or 70%-PH

Time after surgery	Treatment			
	Ethanol		Acetaldehyde	
	K_m	V_{max}	K_m	V_{max}
Controls	0.09 ± 0.02	47.4 ± 2.4	0.012 ± 0.004	110.3 ± 12.1
Rats with 30%-PH				
12 h	0.17 ± 0.05	51.9 ± 3.2	0.034 ± 0.009*	144.1 ± 10.1*
24 h	0.62 ± 0.16*	40.4 ± 3.9	0.018 ± 0.002	94.5 ± 7.6
48 h	0.75 ± 0.23*	49.7 ± 3.3	0.030 ± 0.006*	135.1 ± 12.1
72 h	0.65 ± 0.14*	51.6 ± 4.6	0.016 ± 0.003	162.1 ± 17.8*
96 h	0.11 ± 0.02	67.0 ± 8.1*	0.080 ± 0.040	218.3 ± 28.4*
Rats with 70%-PH				
12 h	0.22 ± 0.06	53.4 ± 4.4	0.032 ± 0.007	129.5 ± 7.8
24 h	0.11 ± 0.03	36.4 ± 2.9*	0.014 ± 0.003	91.7 ± 8.2
48 h	0.10 ± 0.03	38.5 ± 3.5*	0.011 ± 0.003	83.9 ± 9.3*
72 h	0.09 ± 0.03	43.5 ± 3.5	0.010 ± 0.002	109.5 ± 14.2
96 h	0.07 ± 0.02	47.7 ± 4.8	0.028 ± 0.006	107.5 ± 12.2

Results are expressed as mean ± S.E. of five individual determinations per experimental group for K_m (mM) and V_{max} (nmol min^{-1} $mg^{-1}$of protein) values. Statistics: $^*p < 0.01$ as compared to control rats.

capacity to oxidize ethanol (10 mmol/L) during the first 48 h after surgery, as compared with controls, returning to normal 3 days after PH (Fig. 1). In contrast, remnant livers of 30%-PH animals showed no significant modifications of ADH activity (first 72 h after PH), but cytosolic ethanol oxidation was increased thereafter (Fig. 1). Aldehyde reduction (1 mmol/L acetaldehyde) by ADH diminished significantly at 24 h after 70%-PH, and readily augmented at 72 h after surgery (Fig. 1). Interestingly, in 30%-PH animals, ADH reduced acetaldehyde to ethanol, similarly as in 70%-PH, but closer to the higher magnitude (Fig. 1). A classical Michaelis–Menten kinetic analysis revealed an apparently non-competitive inhibition of liver cytosolic ADH activity when animals were subjected to 70%-PH (24 h after surgery) with either ethanol or acetaldehyde as substrates (Fig. 2). In rats subjected to 30%-PH, no significant changes were recorded in ADH activity when tested towards acetaldehyde reduction, but this enzyme seemed to be competitively inhibited to oxidize ethanol, showing an increased K_m value (Fig. 2; Table 1).

In animals subjected to 30%-PH, the K_m of liver ADH towards ethanol oxidation progressively increased, and reached normal values 4 days after surgery. The V_{max} values for ethanol did not change significantly until the fourth day post-PH, when a clear increase in this parameter was observed (Table 1). Interestingly, an opposite pattern of ADH kinetics was recorded in liver from 70%-PH rats. For instance, while the K_m for ethanol oxidation remained practically unchanged, the V_{max} decreased until 48 h after surgery and normalized thereafter (Table 1). Regarding ADH activity in reducing acetaldehyde [24], the K_m was transiently lowered, whereas V_{max} for acetaldehyde reduction progressively increased and remained high until 4 days after 30%-PH (Table 1). In animals with 70%-PH, the V_{max} for acetaldehyde reduction was only significantly decreased at 48 h post-surgery, whereas the K_m values were not significantly modified (Table 1). Optimal pH (9.5–10.0) and kinetic parameters for ADH towards NAD+ (0.32 mM; plots not shown) showed similar values in both, control and PH rats (12–48 h after surgery). In addition, substrate utilization was always inhibited by 4-MP, ruling out the participation of ALDH activity (not shown).

The quantification of immunoreactive ADH protein in cytosolic fractions from experimental animals (Fig. 3) revealed that, in 30% PH, there was an early but not significant decrease in the level of ADH protein (12 h after PH); however, the amount of ADH protein increased progressively thereafter,

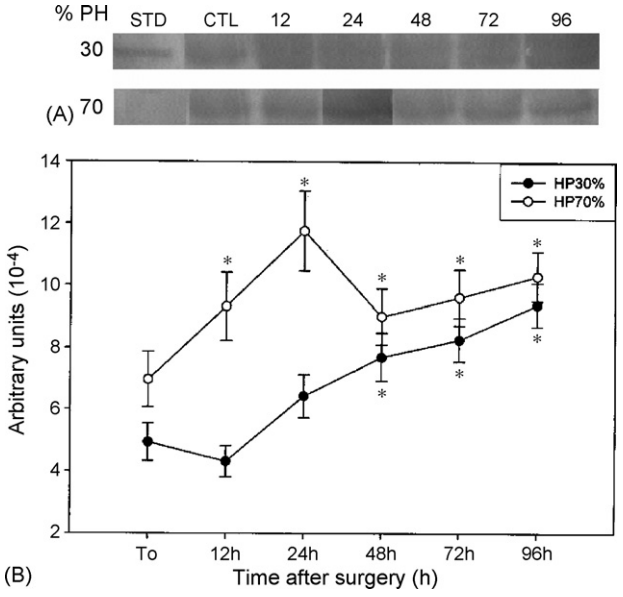

Fig. 3 – Western blot analysis of immunoreactive ADH protein in liver cytosol of control and PH rats. In panel (A) representative analysis by Western blot of ADH protein in control animals and in 30- or 70%-PH rats. In the upper part of this panel, time after surgery is indicated in hours. The respective quantification by densitometric analysis of the Western blot assays is depicted in panel (B); Ctl: control group; Std: the standard used was purified ADH from horse liver. Statistical significance: $^*p < 0.01$ vs. the control (sham-operated; time zero) group.

peaking at 96 h after PH. Contrastingly, after 70%-PH, liver levels of ADH protein increased early after surgery peaking at 24 h. Although levels of immunoreactive ADH decreased thereafter, values remained higher in livers after 70%-PH, as compared with control rats. Therefore, there was no correlation between ADH activity and the liver amount of ADH protein in hepatectomized animals (Fig. 3).

Fig. 4 shows cytosolic ADH activity towards retinol oxidation (with NAD+), as well as to retinal reduction (in the presence of NADH). After 30%-PH, oxidation of 70 μM retinol was practically unaffected, except for a slight but significant decrease (48 h); in addition, reduction of 25 μM retinal diminished at 24 and 72 h after surgery (Fig. 4). In contrast, 70%-PH elicited a progressive increase of ADH-mediated oxidation of retinol, peaking at later times (72–96 h), and accompanied by a constant reduction of retinal, as compared with controls and 30%-PH rats (Fig. 4). Both, retinol oxidation and retinal reduction were strongly inhibited by addition of 4-MP, an inhibitor of liver ADH. For instance, 0.5–2 mM of 4-MP inhibited *in vitro* retinol oxidation in control (up to 60% of inhibition) and in 70%-PH (up to 65% of inhibition) cytosolic samples. Interestingly, cytosolic ADH from 30%-PH animals was even more susceptible to be diminished by the presence of 0.5–2 mM 4-MP (up to 85% of inhibition). Similar data were obtained when considering reduction of retinal, since *in vitro* 4-MP was capable of inhibiting this apparently ADH-mediated reaction by 50–65%.

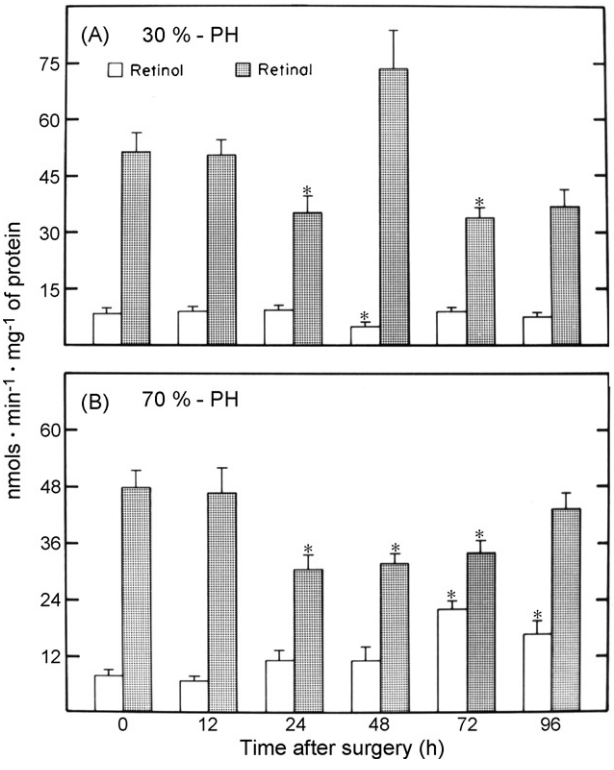

Fig. 4 – Retinol and retinal metabolism by 4-methylpyrazole-sensitive ADH from liver cytosol of control and PH rats. Results are the mean ± S.E.M. of five individual determinations for each experimental point, of animals subjected to 30%-PH (panel A) or to 70%-PH (panel B). Statistics as indicated in Fig. 1.

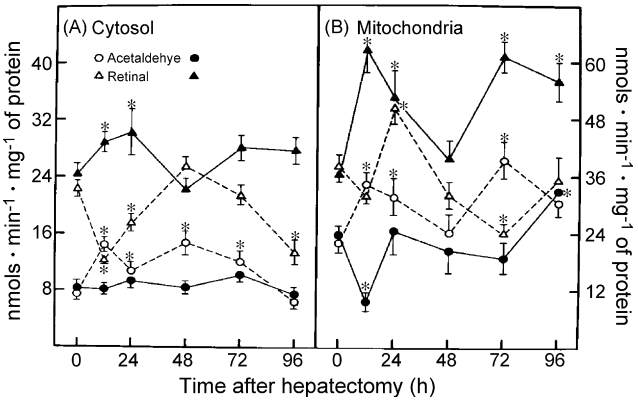

Fig. 5 – Cytosolic and mitochondrial NAD+-dependent ALDH activity towards acetaldehyde and retinaldehyde in control and PH rats. Results are the mean ± S.E.M. of five individual determinations for each experimental point, concerning oxidation of 70 μM acetaldehyde or 70 μM retinal. Controls correspond to time zero, and 30- or 70%-PH to open and solid symbols, respectively. Symbols for the substrates at the top of panel (A). Statistics as indicated in Fig. 1.

Retinaldehyde (retinal) is known to act as substrate for several ALDH isozymes. Therefore, the role of cytosolic and mitochondrial ALDHs in oxidizing retinal and acetaldehyde was explored (Fig. 5). In control livers, when tested with acetaldehyde (70 μM), cytosolic ALDH had low enzymatic activity, but this activity increased three-fold when using 70 μM retinal as substrate (Fig. 5A). Rats subjected to 30%-PH (empty symbols) showed increased cytosolic acetaldehyde oxidation as compared with controls (12–72 h post-PH). The effect of 30%-PH on retinal oxidation was opposite, since cytosolic ALDH oxidized less retinal after it (Fig. 5A). In the case of 70%-PH, cytosolic oxidation of acetaldehyde remained practically unchanged, whereas that of retinal was only slightly increased early after 70%-PH (Fig. 5B). Control mitochondrial ALDH oxidized acetaldehyde and retinal more actively than cytosolic ALDH (Fig. 5B). The 30%-PH rats showed enhanced mitochondrial oxidation of acetaldehyde along the times tested; however, mitochondrial oxidation of retinal did not increase, except at 24 h (Fig. 5B). In contrast, enhanced oxidation of retinal and acetaldehyde was found in liver mitochondria from 70%-PH rats (Fig. 5B).

The levels of free retinol and retinoic acid, as well as those of retinal, enzymatically converted to retinol, were quantified by HPLC. In controls, cytosolic levels of free retinal were higher than those of retinol (Fig. 6), while RA could not be detected. After 30%-PH, liver retinol levels diminished at early stages (12 h), gradually increased, and normalized thereafter, while the amount of retinal decreased continuously after 30%-PH (Fig. 6A). In animals subjected to 70%-PH, retinol always decreased, while an early enhancement of retinal levels (12 h after PH) was noted, which normalized and increased again (4 days post-PH; Fig. 6A). Hence, changes in liver retinoid levels clearly depended on the magnitude of the PH, with the 30%- and 70%-PH showing opposite patterns (Fig. 6A).

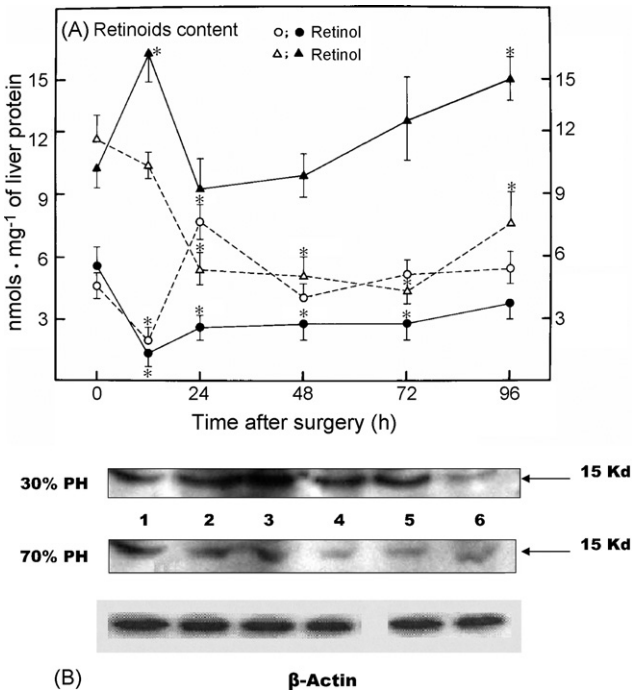

Fig. 6 – Liver cytosolic content of retinoids and Western blot analysis of immunoreactive CRBP-I in control and PH rats. Results are the mean ± S.E.M. of five individual determinations for each experimental point (panel A): controls (time zero), animals subjected to 30- or 70%-PH, symbols for the quantified retinoids, and statistical significance, similar to Fig. 4. In panel B, representative analyses by Western blot of cellular retinal-binding protein-I and β-actin (control for protein load) for the following conditions: (1) control animals; (2) 12 h; (3) 24 h; (4) 48 h; (5) 72 h; (6) 96 h after in animals subjected to PH of either 30 or 70% of the liver mass. Results of the densitometric analysis for panel (B) are shown in Table 3.

We looked for a relationship among free retinoids (retinol plus retinal), retinol/retinal ratio, and the activity of TK, a reliable marker of cell proliferation. Total retinoids were only significantly diminished during 48–72 h after 30%-PH. The retinol/retinal ratio in controls (0.46 ± 0.05) decreased in early stages and increased significantly thereafter (from 24 to 72 h post-PH; Table 2) in animals subjected to 30%-PH. In the case of 70%-PH, the retinol/retinal ratio was markedly lower during all post-PH times tested (Table 2). Maximum DNA synthesis was observed 48 h after 30%-PH, whereas the peak of DNA synthesis was much higher and occurred at 24 h in rats with 70%-PH (Table 2). In any case, DNA synthesis was practically normalized 96 h after surgery (Table 2). In 30%-PH, lower retinoid content, increased retinol/retinal ratio, increased DNA synthesis (Table 2) and mitosis (not shown) were apparently associated, this was not observed after 70%-PH. Hence, accelerated cell proliferation seems to be associated to decreased retinol levels during the first 96 h after PH (Table 2).

The amount of cytosolic immunoreactive CRBP-I was measured and we found, in 30%-PH animals, an early and significant enhancement in CRBP-I concentration that peaked at 24 h after surgery, progressively returned to normal the range at 3 days after PH, and significantly decreased at 96 h post-PH (Fig. 6B; Table 3). When compared with levels of retinol in remnant livers after 70%-PH, the CRBP-I protein was not significantly modified during the first 24 h after surgery, but it markedly diminished thereafter (Fig. 6B; Table 3). Again, an opposite pattern of CRBP-I expression was found depending on the magnitude of the PH.

Fig. 7 is a Dixon plot showing that increasing ethanol concentrations (from 0.01 up to 25 mM) are able to inhibit ADH-mediated retinol oxidation. In controls, retinol oxidation was competitively inhibited by ethanol with an apparent K_i of 90 μM, which was quite similar to that found after 70%-PH (K_i of 130 μM). Unexpectedly, the inhibitory effect of ethanol on cytosolic ADH-mediated oxidation of retinol, in preparations obtained from 30%-PH rats, occurred rather in a non-competitive manner. A very high ethanol K_i value (1.06 mM) was required, in these animals to achieve an inhibition of retinol oxidation.

Since, after PH (mainly with 70%), the cytosolic ADH activity showed a differential management of the substrates tested, including retinoids, we looked for the effect of inhibiting liver ADH activity *in vivo* on the rate of liver regeneration. For this, a set of animals subjected to 70%-PH received 4-MP at a dose ranging from 25 to 200 mg/kg, b.w., and the respective PH controls received saline solution (0.9% NaCl). As shown in Fig. 8, 70%-PH animals injected with 4-MP presented a dose-dependent *in vivo* inhibition of ADH, reaching maximal inhibition of ADH activity (79% of inhibition as compared with controls) when 200 mg/kg of 4-MP were administered. In addition, a similar percentage of inhibition of ADH activity was reached in sham-operated rats receiving 200 mg/kg of 4-MP (Fig. 8). Whereas control animals (sham-operated) did not show alterations in the mitotic index, TK activity (Fig. 8), nor structural alterations (not shown), 4-MP drastically altered parameters indicative of cell proliferation in 70%-PH rats. Here, decreased mitotic index and TK activities were found in a dose-dependent manner after administration of 4-MP; however, reduction of liver regeneration rate was more abrupt after higher 4-MP dosing than that found in the inhibitory effect of 4-MP on liver ADH activity (Fig. 8).

4. Discussion

Liver regeneration must undergo changes in major metabolic pathways in order to achieve DNA replication, cell division, and restitution of the liver mass [1,2]. This process is well known to be highly susceptible to inhibition by ethanol. ADH constitutes a major pathway for ethanol oxidation, but also possesses broad substrate specificity, including dehydrogenation of steroids [29] and ω-oxidation of fatty acids [30]. Based on previous and current results, we believe that liver ADH activity plays a role in controlling PH-induced rat liver regeneration. For instance, the 70%-PH-induced decrease in ADH activity towards ethanol is prevented by administering alcohol to hepatectomized rats [15], and liver levels of ADH protein are remarkably increased in PH rats (Fig. 3), which would explain the enhanced ethanol oxidation found in the regenerating rat liver [14]. Altogether, this has led us to

Table 2 – Cytosolic content of retinoids, their ratio and thymidine kinase activity in animals subjected to 30- or 70%-PH

Time after surgery	Treatment			
	Retinoids		Activity of thymidine kinase	
	Retinol + retinal	Retinol/retinal	nmol min^{-1} mg^{-1} of protein	Times above controls
Controls	16.1 ± 2.0	0.46 ± 0.05	0.18 ± 0.04	–
Rats with 30%-PH				
12 h	12.4 ± 2.3	0.19 ± 0.03*	0.25 ± 0.04	1.4 ± 0.2
24 h	13.1 ± 1.6	1.43 ± 0.17*	0.68 ± 0.11*	3.8 ± 0.6*
48 h	9.1 ± 1.6*	0.82 ± 0.13*	1.55 ± 0.27*	8.6 ± 1.5*
72 h	9.5 ± 1.5*	1.16 ± 0.17*	0.47 ± 0.10*	2.6 ± 0.6*
96 h	13.0 ± 2.1	0.71 ± 0.13	0.31 ± 0.05	1.7 ± 0.3
Rats with 70%-PH				
12 h	17.2 ± 4.3	0.08 ± 0.02*	0.28 ± 0.04	1.5 ± 0.3
24 h	11.8 ± 2.2	0.28 ± 0.05*	4.68 ± 0.84*	26.0 ± 4.7*
48 h	12.6 ± 2.1	0.29 ± 0.05*	1.32 ± 0.21*	7.3 ± 1.2*
72 h	15.2 ± 3.0	0.23 ± 0.04*	0.77 ± 0.09*	4.3 ± 0.5*
96 h	18.3 ± 2.6	0.26 ± 0.04*	0.35 ± 0.06	1.9 ± 0.3

The results are expressed as mean ± S.E. of five individual determinations per experimental group. The sum of retinol and retinal corresponds to nmol mg^{-1} of cytosolic protein. Statistics: $^{*}p < 0.01$ as compared to control rats.

speculate that ADH and, probably, ALDH could be actively using endogenous substrates involved in PH-induced rat liver regeneration. Hence, the present study focused on retinoid metabolism by regenerating livers, and assessed whether retinoids are endogenous substrates for these enzymes, leading to RA synthesis, which is known to play a significant role in development and cell differentiation [31].

Data presented here clearly show that after 70%-PH, ADH activity towards ethanol and acetaldehyde decreased, while in rats with 30%-PH ADH it diminished only slightly (Fig. 1).

Table 3 – Densitometric analysis of Western blots for CRBP-I and its relationship with liver retinol content in animals subjected to 30- or 70%-PH

Time	Treatment	
	Liver cytosolic CRBP-I	Liver retinal
	Times over control	nmol mg^{-1} of protein
Controls	1.00 ± 0.08	5.1 ± 0.8
With 30%-PH		
12 h	2.21 ± 0.20*	2.0 ± 0.6*
24 h	2.35 ± 0.23*	7.7 ± 0.9
48 h	1.53 ± 0.12*	4.1 ± 0.6
72 h	1.52 ± 0.14*	5.1 ± 0.8
96 h	0.49 ± 0.05*	5.4 ± 0.8
With 70%-PH		
12 h	1.14 ± 0.10	1.4 ± 0.6*
24 h	1.22 ± 0.12	2.6 ± 0.6*
48 h	0.32 ± 0.04*	2.8 ± 0.7*
72 h	0.73 ± 0.09*	2.8 ± 0.7*
96 h	0.88 ± 0.11	3.8 ± 0.8

Results are the mean ± S.E. of three individual Western blot analyses such as that shown in Fig. 6B, once corrected by the amount of β-actin used as control for protein load. Results are expressed as times above the control, which corresponded to 15.3×10^3 arbitrary units. The content of liver retinol was taken from Fig. 6A. Statistics: $^{*}p < 0.01$ as compared to control rats.

Interestingly, 70%-PH induced a non-competitive-like inhibition of ADH towards alcohols and aldehydes, while a competitive-like inhibition was found with 30%-PH (Fig. 2; Table 1), pointing towards a threshold of lost liver mass that affects ADH activity; additionally, a clear discrepancy between ADH activity towards the substrates used and the quantified ADH amount (Fig. 3) was also noted. Expression of ADH-I mRNA substantially increases at early stages in the regenerating liver [32], which disagrees with its decreased activity towards exogenous alcohols, suggesting a probable participation of ADH during liver proliferation. Although a relationship between ADH activity and cell proliferation is unknown, this

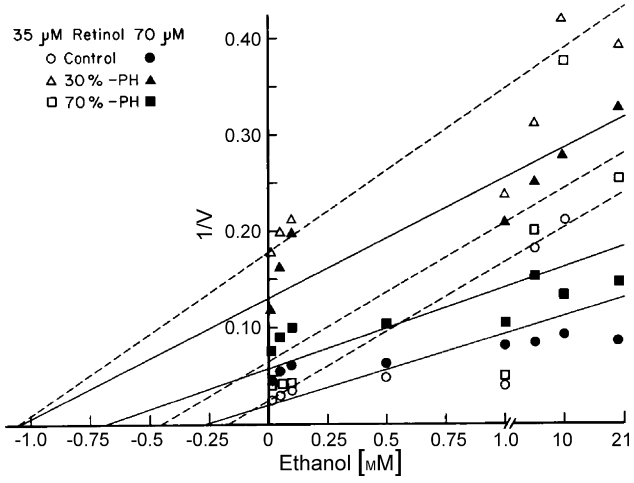

Fig. 7 – Dixon plot analysis of ethanol-induced inhibition of ADH activity towards retinol in control and PH rats. Representative plots resulting of the mean ± S.E. of five individual determinations for each experimental point. Symbols at the top of the figure: control (empty–solid circles), and animals subjected to 30%-PH (empty–solid triangles) or 70%-PH (empty–solid squares), 24 h after PH. ADH activities were determined in the presence of Tween-80, as the vehicle for retinol.

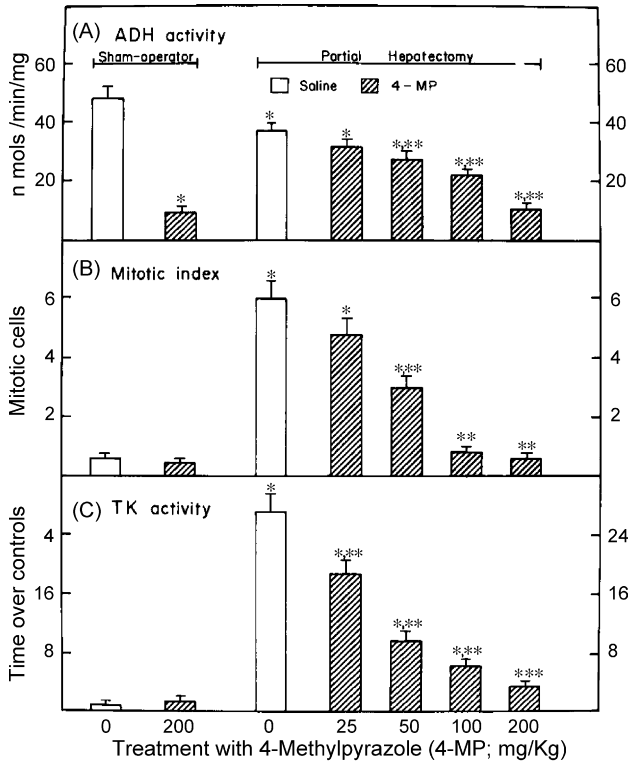

Fig. 8 – Effects of the administration of increasing doses of 4-MP to 70%-PH rats on liver ADH activity and parameters indicative of cell proliferation. Results are the mean ± S.E. of five animals for each experimental group (24 h after treatments). Surgical status and treatments are indicated by the symbols at the top of panel (A). In panel (C), thymidine kinase (TK) activity in controls was of 0.26 nmol h^{-1} mg^{-1} of cytosolic protein. Statistical significance as follows: $^{*}p < 0.01$ vs. the control (sham-operated), and $^{**}p < 0.01$ against the 70%-PH groups.

enzyme has been associated to metabolic characteristics of tumoral tissues [33], similarly to ALDHs, which are related to chemically induced rat hepatomas [34]. In addition, expression of Adh-1 encoding class I ADH in several organs [35] has been suggested to participate during developmental and tissue-specific expression of other genes [36]. Therefore, ADH and/or ALDH could play a role in the control of cell proliferation, at least under specific experimental conditions. All these considerations argue in favor of our postulate that ADH is involved in the management of important endogenous substrates in the regenerating rat liver.

Why could retinoids (mainly retinol and retinal) be suitable candidates as putative endogenous substrates for ADH activity? In this regard, oral administration of 4-methylpyrazole induces a 9–10-fold decrease in RA synthesis from retinol in mice, suggesting the involvement ADH in RA synthesis [37]; however, additional oxidoreductases, other than the cytosolic NAD+-dependent ADH, could be involved in retinol/retinal interconversion [38–40]. Here, our results show that the cytosolic fraction from normal rat liver possessed *in vitro* a discrete capacity for oxidizing retinol in the presence of NAD+, while it efficiently reduced retinal in the presence of NADH, as coenzyme (Fig. 4). Both reactions were inhibited by 4-methylpyrazole (not shown), suggesting a participation of liver ADH class I in the increase of retinal levels in the regenerating liver, mainly after 70%-PH. However, retinol oxidation has been attributed to a cytosolic retinol dehydrogenase activity, which is identical to a medium-chain ADH (class IV ADH) but is practically absent in the adult liver [31]; thus, ADH-I could play a key role in liver retinoid metabolism after PH.

The retinal conversion into RA is performed by some ALDH isozymes that are active using retinal and many other aldehydes [41,42]. ALDH-I functions as a retinal dehydrogenase, overlapping roles in RA synthesis with other enzymes [43]. In our conditions, cytosolic and mitochondrial rates for retinal oxidation were higher than those for acetaldehyde, mitochondrial oxidation of retinal being even more effective (Fig. 5). The magnitude of PH affected cytosolic and mitochondrial oxidation of retinal differentially, compared with that of acetaldehyde (Fig. 5), evidencing a threshold to increase retinal oxidation by the remnant liver. With 70%-PH, ADH- and ALDH-mediated retinoid metabolism seemed to result in a net conversion of retinol into RA. Therefore, retinoid metabolism appears to be efficiently regulated by these enzymatic pathways, and a low free retinol content in the liver and a decreased retinol/retinal ratio could be requisites for the progression of liver proliferation (Fig. 6A; Table 2).

Although retinol may enter target cells via hydrophobic interactions with the plasma membrane or by fluid-phase endocytosis, evidence indicates that retinol is bound to specific carrier proteins, namely CRBPs [8]. The CRBP-I has the function of concentrating retinol at sites where RA is eventually needed, restricting its access to enzymes capable of recognizing the retinol/retinol biding protein "cassette" [8,44]. Thus, holo-CRBP will transfer retinol to enzymes capable of oxidizing it, depending on the cell redox state [45]. Moreover, CRBP-I can also protect cells against amphipathic properties of free retinoids [16]. Our results agree with the aforementioned, showing changes in liver CRBP-I expression that correspond to the liver content of retinol and retinal, and dependent on the magnitude of the remnant liver (Fig. 6B; Table 3). This exquisite regulation of retinol metabolism to RA by the proliferating liver relies on the absence of detectable RA, which is strictly regulated, avoiding RA-induced cell toxicity [16].

In the same context, PH-induced rat liver regeneration is a process highly sensitive to the inhibitory action of a single ethanol administration to these animals, as previously mentioned. Synthesis of retinoic acid is reduced probably due to competitive inhibition of retinol oxidation exerted by ethanol through ADH, as shown *in vitro* [46], rather than being due to an ethanol-induced increase of retinoic acid degradation [7]. Indeed, after alcohol ingestion, tissue ethanol concentration largely exceeds that reported for retinol that is expected to be in the micromolar range [47]; from here, it has been proposed that competitive inhibition by ethanol for retinol oxidation by ADH isoenzymes can potentially block RA synthesis and cause fetal alcohol syndrome [46,48]. Our results strongly agree with this, leading to the suggestion that ethanol oxidation, mediated by cytosolic ADH, can be involved in the ethanol-induced inhibition of rat liver regeneration [3–6]. In

agreement with this statement are the data presented here, which clearly show that after 70%-PH, *in vitro* ADH activity towards ethanol and acetaldehyde decreased, whereas, after 30%-PH, ADH activity only diminished early after surgery (Fig. 1), indicating a relationship between the lost liver mass and ADH activity (Fig. 2; Table 1).

The expression of ADH-I mRNA substantially increased in the regenerating liver, as soon as 36 h after PH [32]. In agreement with these results, we found that the level of liver ADH protein is maintained higher along the regenerative process induced by PH, as assessed by Western blot analysis (Fig. 3). Hence, the present results clearly reflect a discrepancy between enhanced ADH expression and its lower activity found in the rat regenerating liver. The Adh-1 encoding class I ADH is highly expressed in the liver and, at lower levels, in kidney, stomach, and intestine [35]. However, the mechanisms controlling the developmental and tissue-specific expression of Adh-1 are not known [36]. The 4-MP, considered a specific inhibitor of ADH [49,50], inhibits ADH activity *in vivo* and *in vitro*, and blocks many ethanol-induced effects on liver metabolism [51,52]. However, high 4-MP concentrations can also diminish microsomal ethanol oxidation by inhibiting the activity of cytochrome P-4502E1 [53]. *In vivo* administration of 4-MP induced a surprising dose-dependent inhibition of the mitotic index and of the rate of DNA synthesis (TK activity) in the remnant liver, when administered to rats subjected to 70%-PH (Fig. 8). Therefore, these results provide relevance to the discrepancy between ADH protein amount and activity when liver ADH was competitively inhibited *in vivo* (by 4-MP) in the proliferating rat liver induced by PH. The rationale for inhibiting *in vivo* liver type I ADH in animals subjected to 70%-PH was based on our speculation that this is enzyme could use important substrates required for the progression of liver regeneration (i.e., retinoids). Thus, competitive inhibition elicited by 4-MP would blunt ADH activity for any endogenous compound occurring at the onset of PH-induced rat liver regeneration. Data obtained in the present study (Fig. 8) support our hypothesis.

What importance could the ADH-mediated metabolism of retinoids in the regenerating rat liver have? In fact, more than 80% of the total Vitamin A in the whole body is stored in the cytoplasm of hepatic stellate cells, as retinyl esters in lipid droplets [54]. Recently, it has been suggested that hepatic stellate cells play a major role during progression of PH-induced liver regeneration, when they become activated by adhering to proliferating hepatocytes [55], an event that occurred with a remarkable loss of cytoplasmic storage of retinol droplets and the appearance of α-smooth muscle actin, probably as part of the role of hepatic stellate cells of restoring normal sinusoidal structure in the proliferating liver [55]. Although we do not know the meaning of decreasing retinol during rat liver regeneration, we could suggest that hepatocyte ADH and ALDH activity towards retinoid metabolism participates in these interesting interactions among liver cells.

In conclusion, to our knowledge, this is the first report showing that there is a correlation between ADH activity and progression of PH-induced liver compensatory growth. The nature of the "physiological" ADH substrates involved in the regulation of liver regeneration remains unknown but, after 70%-PH, proliferating liver cells display lower ADH activity towards ethanol and acetaldehyde, which could correspond to a non-competitive inhibition, probably due to the participation of increased substrates, such as retinoids. Although it is known that retinoic acid regulates gene expression during development, regeneration, growth and differentiation [10], this seems to be also the first report addressing the importance of ADH-mediated changes in retinoid metabolism, apparently required during PH-induced rat liver regeneration.

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