Volume 331, number 1,2, 119–122 FEBS 13019 September 1993

Chronic ethanol administration enhances retinoic acid and triiodothyronine receptor expression in mouse liver

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Received 29 July 1993

Chronic alcoholism induces perturbations of storage and metabolization of retinol and related compounds. After 6 months of ethanol consumption we have observed in mouse liver an increased expression of Tri-iodothyronine receptors (TR) while the expression of retinoic acid (RA) receptors (RAR) was unaffected. After 10 months of alcoholization the TR expression was strongly increased and the RAR expression was also increased. At this time the activity of aldehyde dehydrogenase and that of alcohol dehydrogenase, two enzymes involved in biosynthesis of RA from retinol, were similar in the liver of alcoholized and pair-fed mice. Thus it can be hypothesized that (i) the change of RAR expression was, at least in part, the result of a change of TR expression (result in agreement with previous data), (ii) the increased expression of RAR could induce apoptosis and subsequently liver necrosis.

Chronic ethanol administration; Triiodothyronine receptor; Retinoic acid receptor; Nuclear receptor; mRNA; Liver

1. INTRODUCTION

Chronic ethanol abuse is known to result in liver damage and associated functional disorders. Liver damage is clearly multifactorial. Metabolism of ethanol generates molecules (as fatty acid ethyl esters) that accumulate in tissues and can be correlated with ethanol-induced damage. Morcover ethanol is a rich source of non-nutritive calories and is able to induce malnutrition status and vitamin deficiency. For some years several observations have been reported suggesting that the mechanism of ethanol-induced fetal anomalies was mediated by the retinoids and particularly retinoic acid (RA) [1,2]. In alcohol-treated rats, a decreased level of liver retinol has been reported resulting from a mobilization of vitamin A from the liver to other organs [3] and/or an increased metabolization [4]. A change in the level of RA in tissues has been suspected, but conflicting experimental results are available on which two alternate hypotheses have been based. Indeed ethanol interference with the alcohol dehydrogenase enzyme system could induce either a lack of RA synthesis or increased levels of RA [5]. Thus the aim of this study was to investigate the effect of chronic ethanol on the binding capacity of RA receptors (RAR) and on the level of RAR mRNA in mouse liver. Moreover, previous studies performed in the laboratory have shown that changes of vitamin A status inducing changes of binding capacity of RAR also induced changes of triiodothyronine receptors (TR) [6,7]. So we have simultaneously studied in the liver the effect of chronic ethanol on the binding capacity of TR and the level of TR mRNA (c-*erb-A* mRNA). It was expected that the results of this study could be related to the liver hypermetabolic state observed after chronic ethanol consumption [8] and related to hyperthyroidism [9,10].

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2. MATERIALS AND METHODS

2.1. *Experimental animals and ethanol administration*

The official French regulation Number 87848 for the care and use of laboratory animals were followed.

Pathogen-free male mice (C57BL/6) were obtained from IFFA-CREDO (L'Arbresle, France). Animals were raised on a standard laboratory food diet, and randomly assigned to one of the experimental groups, they were housed in group cages (10 per cage) during ethanol treatment. The animals were segregated into two groups of 24 mice which were submitted to an alcoholization of 6 months or 10 months, respectively. When mice were 8 weeks old, each group of 24 was segregated into three groups (alcoholic, pair-fed and controls).

Mice of the ethanol-treated group were given, as their only source of fluid, an increasing progression of ethanol as follows: 4% (v/v) solution per week during 3 weeks until 12%. Dry food (pellets) was freely available throughout the experiment. Mice from the first control group were pair-fed to the ethanol-treated group. They received an isocaloric solution of dextri-maltose and dry food that was equivalent to the quantities consumed by animals from the ethanol-treated group [11]. Mice assigned to the second control group had access to dry food and tap water ad libitum.

2.2. *Properties of nuclear receptors*

Receptors preparation. The nuclei were obtained as described by De Groot et al. [12]. TR were obtained as described by Torresani et al. [13]. To obtain RAR, the nuclei were washed three times with binding buffer (HMK: 10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, pH 7.9) and then submitted to a DNase I (Sigma No. D 4527) digestion during 30 min at room temperature, followed by a high salt extraction (0.5

M NaCl). The nuclear extract was then obtained by centrifugation [14]. Protein contents of the nuclear extracts were determined using the Bradford assay [15]

Binding studies The TR binding was performed according to Torresani et al [13] The RAR binding was performed according to Daly et al. [14] modified by Audouin-Chevallier et al [16]

2.3. *Enzyme assays*

Alcohol dehydrogenase and aldehyde dehydrogenase activities were measured in liver according to the method of Poupon et al. [17]. Results were expressed in nanomol of NADH formed per milligram of protein per minute. The rate of NADH appearance was recorded spectrophotometrically at 340 nm and 37°C using a Monarch (International Laboratories) analyser.

2.4. *Quantification of mRNAs*

The absolute levels of RAR mRNAs cannot be determined directly but the proportion can be deduced by comparing with a β-actin internal standard simultaneously reverse-transcribed and amplified in the same test tube Extraction of RNA was performed according to Chomczynski and Sacchi [18]. Oligonucleotides primers used for PCR were these used by Higueret et al. [19] Preparation of cDNA was carried out as described by Pailler-Rodde et al. [20]. Synthesized cDNA (15 μl) was amplified by the polymerase chain reaction (PCR) using *Taq* polymerase [22]. The amplification was performed according to Higueret et al. [19]. The reaction was carried out for a total of 34 cycles.

For quantitative analysis of PCR products, 10 μl of the PCR reaction were sampled after each amplification cycle (from cycles 12 to 29) and then at the last one [19] and the co-amplified fragments were separated by electrophoresis on a 10% acrylamide gel. The incorporated radioactivity was visualized by autoradiography, the bands were excised from the gels and quantified by scintillation counting.

3. RESULTS

3.1. *Effect of chronic alcoholization on the properties of TR (results summarized in Table I)*

3.1.1. Hormone binding

After 6 months of chronic alcoholization, the capacity (C_{max}) of TR was strongly modified. It was increased by 138% relative to the control and pair-fed values. This difference was increased after 10 months of alcoholization until 156% (Fig. 1). Moreover, if we compared the two groups of mice, we could observe that the C_{max} was significantly increased between 6 and 10 months.

3.1.2. mRNA abundance

As early as 6 months of alcoholization the mRNA expression of c-*erb*-A was significantly increased by around 100% relative to the control and pair-fed mice (Fig. 1). This increase was maintained after 10 months. Like for the hormone binding we could observe that the mRNA amount was strongly increased between 6 and 10 months.

3.2. *Effect of chronic alcoholization on the properties of RAR (results summarized in Table I)*

3.2.1. Hormone binding

In contrast to the TR, 6 months of alcoholization had no effect on retinoic acid nuclear receptor properties. The difference observed between the C_{max} of alcoholized mice (159 ± 17 fmol/mg protein), control (136 ± 10 fmol/mg protein) and pair-fed (135 ± 13 fmol/mg protein) was not significant. In contrast, when mice were submitted to 10 months of alcoholization, their RAR binding capacity was modified, it was increased by 50% relative to the control and pair-fed values.

3.2.2. mRNA abundance

In our experiment it appeared that the RAR mRNA was not significantly different between the three groups of mice (control, pair-fed and alcohol). This result is consistent with those concerning the binding capacity of RAR under the same conditions. So it is clear that up until 6 months, alcohol had no effect on the expression of the retinoic acid nuclear receptors.

After 10 months of chronic alcoholization we observed a significant increase of RAR mRNA relative to the control and pair-fed values (+90%). This result re-

Table I

Effect of ethanol consumption on retinoic acid and triiodothyronine nuclear receptors in liver

	RAR		*c-erb-A*	
	Capacity (1) (fmol/mg protein)	mRNA (2) ($A_{RAR}/A_{\beta actin}$)	Capacity (1) (fmol/mg protein)	mRNA (2) ($A_{erb\text{-}A}/A_{\beta actin}$)
6 months of treatment				
Control	136 ± 10	0.100 ± 0 025	91 ± 37	0.092 ± 0.015
Pair-fed	135 ± 13	0.064 ± 0.011	101 ± 41	0.075 ± 0.013
Ethanol	159 ± 17	0.068 ± 0.010	218 ± 33*	0.140 ± 0.019*
10 months of treatment				
Control	193 ± 27	0.106 ± 0.020	104 ± 5	0.143 ± 0.018
Pair-fed	200 ± 40	0.142 ± 0.025	78 ± 10	0.115 ± 0.021
Ethanol	290 ± 12*	0.232 ± 0.018*	267 ± 53*	0.220 ± 0.020*

Capacity is the C_{max} value obtained by Scatchard analysis. (1) Each value represents the mean ± S E.M. of four to nine determinations performed on pools of 2–6 animals (2) A_{RAR}, $A_{erb\text{-}A}$, $A_{\beta actin}$ are absolute values of RAR, c-*erb-A* and β-actin mRNA, respectively. Control: mice receiving drinking water only; pair-fed: mice receiving drinking water containing 18% dextrimaltose (w/v); ethanol: mice receiving drinking water containing 12% ethanol (v/v) and 3% sucrose (w/v); *significantly different (Student's *t*-test) $P < 0.05$ from corresponding control and pair-fed values.

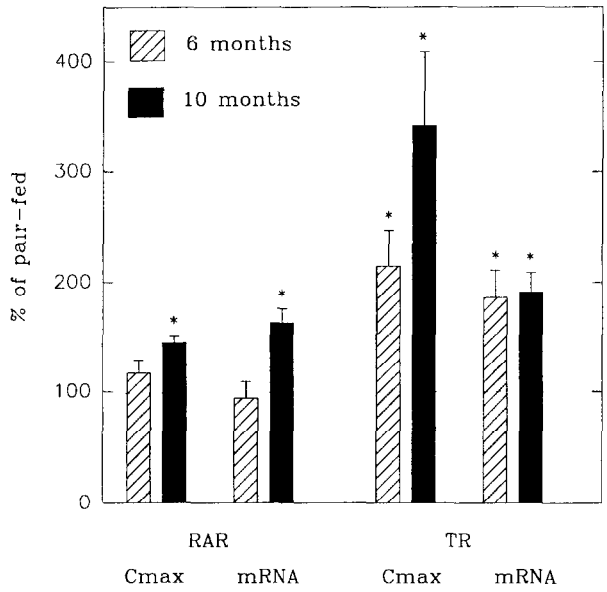

Fig. 1. Changes of binding capacity and mRNA level of triiodothyronine receptors (TR) and retinoic acid (RA) receptors (RAR) in the liver of mice submitted to a chronic alcoholization of 6 or 10 months (Ethanol: mice receiving drinking water containing 12% ethanol (v/v) and 3% sucrose (w/v); Pair-fed: mice receiving drinking water containing 18% dextrimaltose (w/v) isocaloric to the ethanol mice). C_{max} and mRNA levels were determined as described in section 2, and is expressed as the average ± S.E.M. from pools of 2 to 6 animals. *Significantly different (Student's t-test) $P < 0.05$ from pair-fed values.

veals a specific action of alcohol on the expression of RAR mRNA. The increase of RAR mRNA at 10 months of alcoholization could be compared to that of the RAR C_{max} under the same conditions. Indeed it was generally admitted that the C_{max} is correlated to the mRNA abundance.

3.3. *Effect of chronic alcoholization on alcohol and aldehyde dehydrogenase activities*

After 6 months of chronic alcoholization the ADH activities stayed the same as controls and pair-fed. In contrast, the ALDH activity was modified when mice were submitted to ethanol consumption: it was significantly enhanced (Table II). Moreover, at the tenth month of chronic ethanol ADH and ALDH were the same as the control and pair-fed values. We can observe that ALDH activity was increased between 6 months and 10 months for control and pair-fed and this is not the case for ADH activity (Table II).

4. DISCUSSION

The increased expression of TR observed in our study as soon as the sixth month of chronic alcoholization are in agreement with previous data obtained in humans. Indeed in alcoholic cirrhosis there is an increase of the amount of TR mRNA in liver [22]. Also an increased binding capacity of TR was shown in mononuclear blood cells from patients with liver cirrhosis [23]. These data can be related to the liver hypermetabolism state reported during chronic ethanol consumption [8] and in the etiology of which hyperthyroidism was suspected [9].

The increased expression of RAR, which constitutes an original result, can be related to the increased metabolization of retinol observed during chronic alcoholization [24]. It is not evident that the ethanol-induced microsomal oxidizing systems are involved in biosynthesis of retinoic acid from retinol [1] but alcohol also stimulates other metabolic pathway(s) involved in RA synthesis. Our results showing an increased activity in 6 months alcoholized mice were in agreement with a previous study which reports that aldehyde dehydrogenase (ALDH) has an increased activity during the first stages of alcoholic liver diseases [25]. Such a stimulated enzymatic activity could be responsible for increased biosynthesis of RA and subsequently of RAR expression since RA up-regulates its own receptors [26,27]. After 10 months of considerable ethanol intake, we observed an increase TR and RAR expression while ADH activity was not different relative to pair-fed animals (Table II). Thus it could be hypothesized that the increased RAR expression was the consequence of the increased TR expression which appeared earlier. So it was reported for a long time that administration of thyroid hormones induces an increased vitamin A metabolism [28] and recently it was shown in our laboratory that in hyperthyroid rats there was an increased amount of RAR mRNA [19].

Increased TR and RAR expression could be involved in some liver diseases occurring after long-term chronic ethanol consumption. Thus increased TR expression could be related to liver necrosis and fibrosis predominantly in the centrilobular area occurring in a large percentage of humans who died of thyrotoxicosis [8]. Moreover relations are more obvious concerning increased RAR expression and ethanol liver diseases. Thus high doses of vitamin A induced liver alterations similar to those induced by alcoholism [29]. Also, the

Table II

Effect of alcohol consumption on hepatic alcohol and aldehyde dehydrogenase activities

	ADH activity (mIU/mg protein)		ALDH activity (mIU/mg protein)	
	6 months	10 months	6 months	10 months
Control	81 ± 3	80 ± 3	75 ± 3	96 ± 5
Pair fed	80 ± 9	92 ± 5	77 ± 7	89 ± 4
Alcohol	78 ± 5	81 ± 3	100 ± 8*	96 ± 4

ADH: alcohol dehydrogenase; ALDH: aldehyde dehydrogenase Each value represents the mean ± S.E.M. of three to six determinations performed on pools of 2 animals. *Significantly different (Student's t-test) $P < 0.05$ from corresponding control and pair-fed values.

mechanism responsible for producing many of the malformations in infants exposed to RA are the same as those as a consequence of alcoholism [30]. Moreover, retinoic acid can be related to the severe liver lesions occurring in later stages of alcoholism and involving cell death. So it is known that retinoic acid is involved in apoptosis. This cell death is catalyzed by 'tissue' transglutaminases (tTG) which form covalent bonds between polypeptide chains. It was shown that RA induces a tTG transglutaminase in rat hepatocytes [31] and then that the level of this enzyme activity was determined by the level of expression of RAR [32].

So deregulated expression of nuclear receptors such as TR and RAR probably plays a major role in the development of liver lesions occurring in alcoholism. Indeed it is well known that the RAR expression is involved in the pathology of hepatocellular carcinoma [33].

Acknowledgements We are indebted to the Laboratoire de Neurosciences Comportementales et Cognitives du Pr. R. Jaffard University Bordeaux I for providing experimental mice used in this study.

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