

## PHARMACOLOGY AND CELL METABOLISM

### Reduction of Ethanol Consumption in Alcohol-Preferring Rats by Dual Expression Gene Transfer

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**Abstract** — **Aims:** To mimic, in an animal model of alcoholism, the protective phenotype against alcohol consumption observed in humans carrying a fast alcohol dehydrogenase (ADH1B\*2) and an inactive aldehyde dehydrogenase (ALDH2\*2). **Methods:** We developed a multiple expression cassette adenoviral vector (AdV-ADH/asALDH2) encoding both a fast rat ADH and an antisense RNA against rat ALDH2. A control adenoviral vector (AdV-C) containing intronic non-coding DNA was also developed. These adenoviral vectors were administered intravenously to rats bred as high alcohol-drinkers (University of Chile bibulous) that were previously rendered alcohol dependent by a 75-day period of voluntary 10% ethanol intake. **Results:** Animals administered AdV-ADH/asALDH2 showed a 176% increase in liver ADH activity, whereas liver ALDH2 activity was reduced by 24%, and upon the administration of a dose of ethanol (1 g/kg, i.p.), these showed arterial acetaldehyde levels that were 400% higher than those of animals administered AdV-C. Rats that received the AdV-ADH/asALDH2 vector reduced by 60% their voluntary ethanol intake versus controls. **Conclusion:** This study provides evidence that the simultaneous increase of liver ADH and a reduction of ALDH activity by gene transfer could constitute a potential therapeutic strategy for the treatment of alcoholism.

## INTRODUCTION

Alcoholism is one of the most important public health problems in the Western world (Rehm *et al.*, 2006; WHO, 2009). Currently, the pharmacotherapy of alcoholism is based mainly on the use of three Food and Drug Administration-approved drugs: disulfiram, acamprosate and naltrexone (Johnson, 2008). However, drawbacks in their long-term effectiveness and compliance have limited their use, prompting the search for new therapeutic agents (Kranzler and Van Kirk, 2001; Fuller, 2004; Anton *et al.*, 2006).

Despite the relevance of socio-cultural factors in the drinking patterns of a population, there are a number of studies showing that genetic factors account for 50–60% of the susceptibility to developing alcoholism (Heath *et al.*, 1991; Prescott and Kendler, 1999). These genetic factors may protect or predispose against the development of this condition (see Ducci and Goldman, 2008). The protective genetic factors against alcoholism are related to polymorphisms in the genes coding the enzymes that metabolize ethanol (Chen *et al.*, 1999; Zintzaras *et al.*, 2006). In humans, ethanol is degraded mainly by hepatic alcohol dehydrogenase (ADH) to acetaldehyde, which is further oxidized to acetate by mitochondrial aldehyde dehydrogenase (ALDH2).

In some East Asians, a point mutation in the ALDH2 gene (ALDH2\*2) abolishes the activity of this enzyme. Upon ethanol consumption, these individuals display marked elevations of blood acetaldehyde, which generates a dysphoric reaction (e.g. facial flushing, hypotension, headaches and nausea) that deters individuals from drinking (Mizoi *et al.*, 1994; Peng *et al.*, 2007). This aversive reaction to ethanol is responsible for the 60–90% of protection against alcoholism shown in humans who carry the ALDH2\*2 allele (Thomasson *et al.*, 1991; Higuchi, 1994; Tu and Israel, 1995; Chen *et al.*, 1999). A study in rats showed that the hepatic levels of ALDH2 can be reduced by the systemic administration of antisense oligonucleotides against the mRNA of this enzyme (Garver *et al.*, 2001), which reduced ethanol intake

by 60%. However, in these proof-of-principle studies, the oligonucleotides were delivered by an infusion pump implanted subcutaneously. In a subsequent work, Ocaranza *et al.* (2008) showed, in high alcohol-drinking rats, that the single intravenous administration of an anti-ALDH2 antisense-coding gene carried by an adenoviral vector reduced liver ALDH2 activity by 85% and reduced voluntary ethanol intake by 50% for 35 days.

Another important polymorphism existent mainly in East Asian and Polynesian populations is a variant of ADH1B (ADH1B\*2; Arg47His), which is 100 times more active than the normal enzyme ADH1B\*1 (Hurley *et al.*, 1990, 1991). Several studies have reported that carriers of ADH1B\*2 showed a marked protection (~50%) against the development of alcoholism (Chambers *et al.*, 2002; Kim *et al.*, 2008; Chen *et al.*, 2009a). But in spite of the high activity of ADH1B\*2, there are no reports of elevated levels of acetaldehyde on venous blood upon ethanol consumption in carriers of the ADH1B\*2 allele (Mizoi *et al.*, 1994; Peng *et al.*, 2007). However, it was recently reported (Rivera-Meza *et al.*, 2010) that ‘naïve’ University of Chile bibulous (UChB) alcohol-preferring rats injected with an adenoviral vector coding for a rat ADH analog (rADH<sup>47His</sup>) of the fast human enzyme ADH1B\*2, showed 90% increase in hepatic ADH activity and 5-fold higher arterial blood acetaldehyde levels soon after ethanol administration. The treated animals also markedly (50%) reduced their voluntary ethanol intake, showing that the mechanism of protection against developing alcoholism in carriers of the ADH1B\*2 allele is likely a brief increase in arterial blood acetaldehyde.

Since individuals carrying both protective genes are virtual abstainers (Chen *et al.*, 2009a), it would be interesting to develop a new therapeutic strategy to treat alcoholism based on mimicking this maximally protective Asian phenotype. We propose that the simultaneous increase in the liver of ADH activity along with a reduction of ALDH2 activity would produce higher blood levels of acetaldehyde during ethanol metabolism and a marked protection against ethanol consumption.

In this work, we report studies aimed at mimicking by gene transfer, this fully protective phenotype in a rat model of alcoholism. For this purpose, we developed a multiple expression cassette adenoviral vector (AdV-ADH/asALDH2) encoding both (a) a fast rat ADH and (b) an antisense RNA against rat ALDH2. A control adenoviral vector (AdV-C) containing intronic non-coding DNA was also developed. These adenoviral vectors were first delivered to rat hepatoma cells to verify its ability to be expressed in rat liver cells. Thereafter, the adenoviral vectors were administered to UChB alcohol-preferring rats (Mardones and Segovia-Riquelme, 1983; Quintanilla *et al.*, 2006) that were previously rendered alcohol dependent. In these rats we determined: (a) liver ADH and ALDH2 activities, (b) arterial blood acetaldehyde levels following ethanol administration and (c) voluntary ethanol consumption.

The obtained results show that simultaneous increase of ADH and decrease of ALDH2 activities in the liver markedly reduces the voluntary ethanol intake of alcohol-dependent animals and may provide the basis to develop a gene therapy for alcoholism.

## MATERIALS AND METHODS

### *Gene construct encoding the fast rat ADH enzyme (rADH<sup>47His</sup>) and the rat ALDH2 antisense RNA*

The rat ALDH2 antisense RNA (asALDH2) expression cassette (3288 bp) was excised from pACCMV-pALDH2-I (Karahanian *et al.*, 2005) with NotI, blunt ends were generated with Klenow and subsequently cloned in pAdTrack (ATCC, Manassas, VA, USA), which was previously digested with BglIII/KspAI and blunted with Klenow, to generate pAd-asALDH2. The rADH<sup>47His</sup> expression cassette (2996 bp) was excised from pShuttle-rADH<sup>47His</sup> (Rivera-Meza *et al.*, 2010) with NotI and cloned, in inverted orientation respect of asALDH2 cassette, in the plasmid pAd-asALDH2 previously digested with NotI. The resulting plasmid was named pAd-ADH/asALDH2.

### *Production of the adenoviral vector coding for rADH<sup>47His</sup> and asALDH2*

#### Cell culture conditions

Human embryonic kidney (HEK)-293 cells were used to generate and propagate adenoviral vectors. HEK-293 cells were obtained from ATCC and grown in Dulbecco's modified Eagle's medium containing 4.5 mg/ml glucose, 1.5 mg/ml NaHCO<sub>3</sub>, 100 U/ml penicillin, 0.1 mg/ml streptomycin and 0.25 µg/ml amphotericin-B. The culture medium was supplemented with 10% fetal bovine serum.

#### Production of adenoviral vectors

The first-generation adenoviral vectors AdV-ADH/asALDH2 and AdV-C were generated by the AdEasy System (He *et al.*, 1998). To obtain the AdV-ADH/asALDH2 vector, the pAd-ADH/asALDH2 plasmid was linearized with PmeI and recombined with pAdEasy-1 in the *Escherichia coli* BJ5183 strain to generate a plasmid containing the recombinant adenoviral genome. The viruses were propagated in HEK-293 cells, purified in two consecutive CsCl gradients, and dialyzed for 24 h against 10 mM Tris-HCl, 2 mM

MgCl<sub>2</sub> and 5% sucrose (storage buffer). Total viral particles were estimated by absorbance at 260 nm (Mittereder *et al.*, 1996). Adenoviral vectors were kept at -80°C in storage buffer. The control adenoviral vector (AdV-C), which only carries the human β-globin intron, was generated as previously described (Rivera-Meza *et al.*, 2010).

### *Expression of rADH<sup>47His</sup> and asALDH2 in rat hepatoma cells*

#### Cell culture conditions

Rat hepatoma cells (H4-II-E-C3) were used to study the *in vitro* expression of rADH<sup>47His</sup> and asALDH2 in hepatic cells. H4-II-E-C3 cells were obtained from ATCC and grown as indicated for HEK-293 cells. The culture medium was supplemented with 10% equine serum and 5% fetal bovine serum.

#### Transduction of H4-II-E-C3 cells with adenoviral vectors

The rat hepatoma cells were plated on 6-well plates at 2 × 10<sup>6</sup> cells/well and transduced with different volumes (0–1000 µl) of a crude HEK-293 cell lysate containing AdV-ADH/asALDH2 vector. Seventy-two hours after the transduction, the cells were harvested, lysed in 1% Triton X-100 with 0.33 mM dithiothreitol (DTT), centrifuged at 20,800g for 20 min at 4°C and samples of the supernatant were collected. Total protein concentrations in the samples were determined using the Bio-Rad Protein Assay kit.

#### Assay of ADH and ALDH2 activities

The activity of ADH and ALDH2 were determined spectrophotometrically by the measurement of absorbance (340 nm) of nicotinamide adenine dinucleotide reduced (NADH) generated from NAD<sup>+</sup>. The ADH activity was measured as reported previously and was expressed as nanomoles of NADH per minute per milligram of protein (Rivera-Meza *et al.*, 2010). The activity of ALDH was determined in duplicate in a final volume of 0.8 ml at 37°C in 34 mM Na<sub>2</sub>HPO<sub>4</sub> (pH 8.5) containing 10 mM pyrazole, 5 mM MgCl<sub>2</sub>, 4 mM DTT, 0.8 mM NAD<sup>+</sup> and 10 µM NADH. After 10 min of stabilization, the reaction was initiated by the addition of 21 µM propionaldehyde for the low K<sub>m</sub> ALDH2 and 1 mM propionaldehyde for total ALDH activity (Ocaranza *et al.*, 2008). The ALDH activity was expressed as nanomoles of NADH per minute per milligram of protein.

### *Expression of rADH<sup>47His</sup> and asALDH2 in UChB rats*

#### Animals

Wistar-derived rats of the UChB lineage were used; this line has been bred selectively for high alcohol preference over several decades (Mardones and Segovia-Riquelme, 1983; Quintanilla *et al.*, 2006). Twelve alcohol-naïve female UChB rats weighing between 150 and 200 g (~16-week-old) were housed in individual cages in a temperature and humidity controlled room for 60 days, and offered a 24-h free choice between 10% (v/v) and water. After this period, the animals were assigned to two groups and voluntary ethanol intake was next followed for 15 days, but on a limited access paradigm in which 10% ethanol was available for only 1 h each day with food and water freely available. Animal

experimentation procedures were approved by the Institutional Animal Experimentation Ethics Board (FCQF-240805).

#### Systemic administration of adenoviral vectors and voluntary ethanol intake

Following 15 days of limited access to ethanol, the ethanol solution was removed and 24 h later a single dose of the adenoviral vectors AdV-ADH/asALDH2 or AdV-C ( $3 \times 10^{12}$  pv/kg; six animals/group) was administered via the tail vein (500  $\mu$ l in saline). Seventy-two hours after AdV administration, rats were allowed access to 10% ethanol solution for only 1 h each Day (1–2 p.m. in the normal light cycle). The voluntary ethanol intake was recorded for 23 days and expressed as g of ethanol per kg body weight per hour. Water intake was recorded for the total 24 h. Seven days after the voluntary ethanol consumption determinations, the abstinent rats were given a standard dose of ethanol, and arterial acetaldehyde levels were determined (see below). Thereafter, animals were decapitated and liver was removed immediately, weighed, and stored at  $-80^{\circ}\text{C}$  for analysis of ADH and ALDH activities.

#### Arterial acetaldehyde determination

To determine arterial acetaldehyde levels, ethanol was administered i.p. (as a 20% solution in saline) at a dose of 1 g/kg. Blood samples for acetaldehyde determination were drawn from the carotid artery of anesthetized rats (60 mg/kg ketamine hydrochloride plus 2 mg/kg acepromazine) at 2.5, 5, 10, 15 and 30 min after ethanol administration. The blood samples (0.1 ml) were diluted 10-fold in distilled water, and acetaldehyde was measured by head-space gas chromatography (Quintanilla *et al.*, 2007).

#### Determination of liver ADH and ALDH2 activity

Small samples (0.5 g) of the liver were weighed, immediately cut in small pieces, washed twice with ice-cold phosphate-

buffered saline, and homogenized in five volumes of 1% Triton X-100 and 0.33 mM DTT. Cell debris was removed by centrifugation at 20,800g for 20 min at  $4^{\circ}\text{C}$ , and the supernatant was collected. The ADH and ALDH activity in the samples were measured by duplicate in 10 and 5  $\mu$ l of supernatant respectively, as described previously. ADH and ALDH activities were expressed as micromoles of NADH per minute per gram of tissue.

#### Statistical analyses

Data were expressed as means  $\pm$  SE. Statistical differences were analyzed by Student's *t*-test or analysis of variance (ANOVA) for repeated measures for the time factor. A level of  $P < 0.05$  was considered statistically significant.

## RESULTS

#### Transduction of rat hepatoma cells with an adenoviral vector encoding both the fast rADH<sup>47His</sup> and the asALDH2 (AdV-ADH/asALDH2)

The cDNAs coding for the fast rADH<sup>47His</sup> and asALDH2, each one in an expression cassette under the control of the CMV promoter, were incorporated into an adenoviral vector (AdV-ADH/asALDH2) and propagated in HEK-293 cells (Fig. 1). The correct expression of the viral construction was tested by transduction of H4-II-E-C3 rat hepatoma cells and its capacity to increase ADH and to decrease ALDH activity on these cells was also assessed. Figure 2 shows the effect on the ADH and ALDH activity of H4-II-E-C3 cells upon its transduction with a crude lysate of AdV-ADH/asALDH2. The results showed that the transduction of H4-II-E-C3 cells with 800  $\mu$ l of crude viral reaches a maximal effect on the activity of the enzymes, resulting in 7-fold increase of ADH activity and a 95% reduction of ALDH activity. These results indicate that the viral construct is functional in rat liver cells and its encoding cDNAs were correctly expressed.

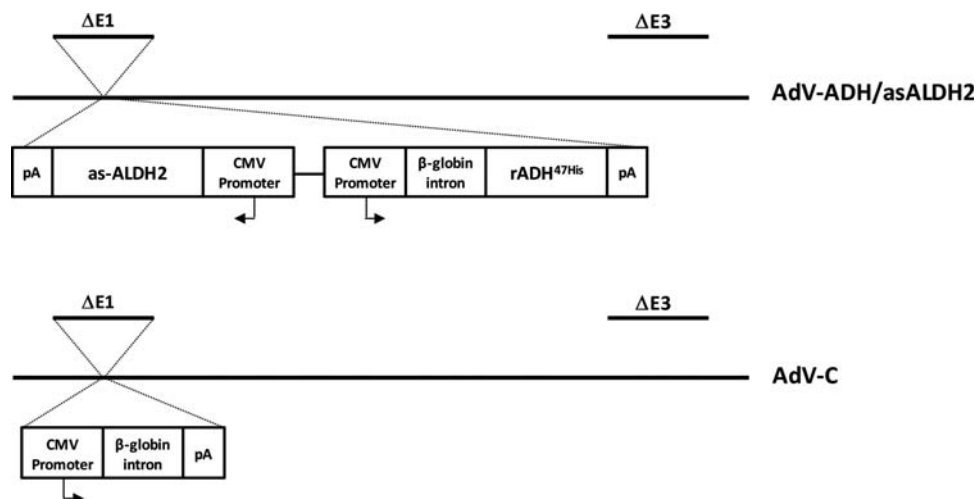


Fig. 1. Diagrammatic representation of the first-generation adenoviral vectors used in this study. In the AdV-ADH/asALDH2 vector, the antisense rat ALDH2 (asALDH2) and fast rat ADH (rADH<sup>47His</sup>) expression cassettes were inserted in inverted orientation in place of the E1 region. In the control vector (AdV-C), an expression cassette encoding the human  $\beta$ -globin intron was inserted. CMV promoter: immediate-early human cytomegalovirus promoter; pA, polyadenylation signal.



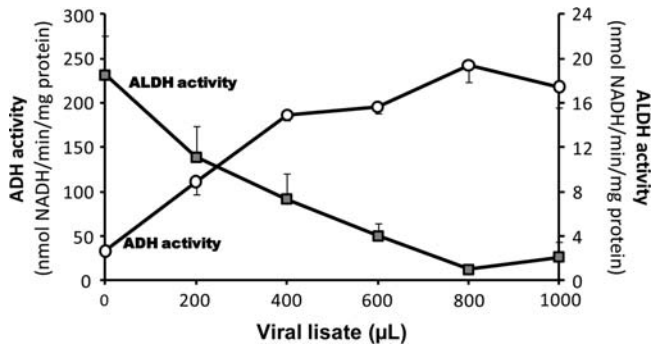


Fig. 2. ADH and ALDH2 activities in rat hepatoma cells transduced with a cell lisate containing the adenoviral vector coding for rADH<sup>47His</sup> and asALDH2. H4-II-E-C3 cells were transduced with different volumes of a HEK-293 cell lisate (AdV packaging cells) containing the AdV-ADH/asALDH2 vector. After 72 h of transduction, cells were lysed and the ADH and ALDH activities were measured. Left y-axis represents ADH activity and right y-axis represents ALDH activity. Points are the mean  $\pm$  SE of three experiments.

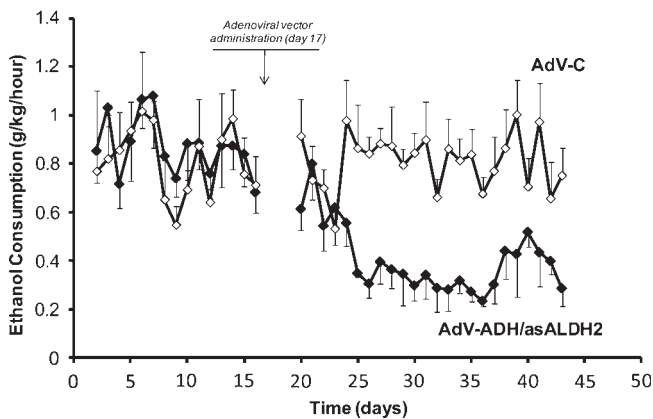


Fig. 3. Voluntary ethanol intake of UChB rats treated with the adenoviral vector coding for rADH<sup>47His</sup> and asALDH2. Twelve rats from the high alcohol-drinking UChB line previously rendered alcohol-dependent were assigned into two groups and subjected to a 15-day (Day 2–16) period of limited access to 10% ethanol for only 1 h-day. During this period, their average voluntary ethanol intake was 0.8–0.9 g ethanol/kg/h. At Day 17, the access to ethanol was removed and the animals were injected with  $3 \times 10^{12}$  pv/kg of one of the adenoviral vectors AdV-ADH/asALDH2 or AdV-C. Seventy-two hours after the administration of the adenoviral vectors (Day 20), limited access to 10% ethanol was reinstated for the next 23 days. Points represent means  $\pm$  SE of daily ethanol intake during the 1 h access to ethanol; six animals/group. Rats treated with AdV-ADH/asALDH2 vector showed, between Days 24 and 43, a 60% reduction in voluntary ethanol intake compared with control animals [ANOVA;  $F(1, 39) = 400$ ,  $P < 0.001$ ].

#### *In vivo administration of the AdV-ADH/asALDH2 vector*

Effect on the voluntary ethanol intake of alcohol-dependent animals

Rats of the high alcohol-drinking UChB line were rendered alcohol dependent by an initial 60-day period of voluntary ethanol intake (24 h/day) in which animals reached a voluntary ethanol intake of 6–7 g ethanol/kg/day (see also Ocaranza *et al.*, 2008). After such a period of alcohol self-administration, animals were assigned to two groups and

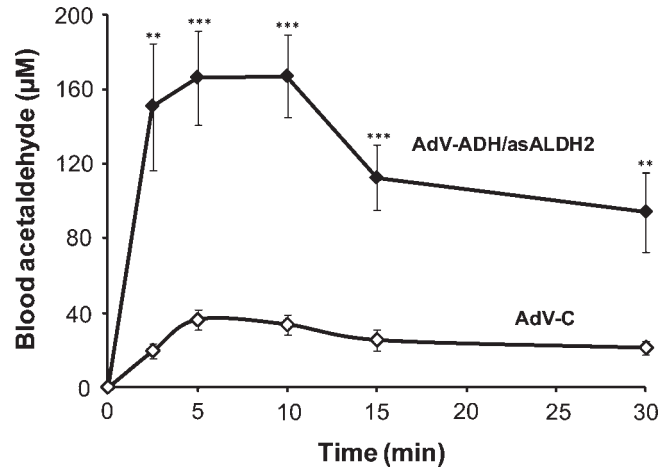


Fig. 4. Arterial blood acetaldehyde levels after a dose of ethanol to UChB rats treated with the adenoviral vector coding for rADH<sup>47His</sup> and asALDH2. Seven days after finishing voluntary ethanol determination, the animals were administered with a dose of ethanol (1 g/kg, i.p.) and acetaldehyde levels in arterial blood were measured by gas chromatography. Points represent the mean  $\pm$  SE blood acetaldehyde concentration; six animals/group. At 5 min after ethanol administration, maximal levels of acetaldehyde were measured in animals that received AdV-ADH/asALDH2 vector which were 5-fold higher than those measured in control animals ( $t = 5.02$ ,  $df = 10$ ). \*\*\* $P < 0.001$ ; \*\* $P < 0.01$ .

access to the 10% ethanol solution was restricted to only 1 h per day for a period of 15 days. Figure 3 shows that during this limited access to ethanol (Days 2–16) both groups of animals showed a similar baseline voluntary ethanol intake of 0.8–0.9 g ethanol/kg/h. At Day 17, ethanol was removed and 24 h later rats were injected with either the AdV-ADH/asALDH2 or the AdV-C vector. Ethanol access was reinstated at Day 20 under the same 1-h per day limited access paradigm.

Figure 3 shows that animals that received a single administration of the AdV-ADH/asALDH2 vector significantly reduce their voluntary ethanol intake for the 3 weeks tested (60% reduction, ANOVA  $P < 0.001$ ) compared with rats that received the AdV-C vector. The results also showed that the control group (AdV-C) maintained its baseline ethanol consumption indicating that the adenoviral vector administration *per se* did not affect ethanol intake. The daily water consumption in animals receiving the AdV-ADH/asALDH2 vector was not different from that of animals that received the control vector (data not shown).

#### Determination of blood acetaldehyde levels

After completing the voluntary ethanol consumption period, ethanol was removed and the animals were allowed a 7-day period of abstinence. Thereafter, arterial acetaldehyde levels were measured at different times following the administration of a dose of ethanol (1 g/kg, i.p.). Figure 4 indicates that upon ethanol administration, rats treated with the AdV-ADH/asALDH2 vector displayed elevated levels of blood acetaldehyde with a peak at 5–10 min after ethanol administration which was 400% ( $P < 0.001$ ) higher than those in animals that received the AdV-C.

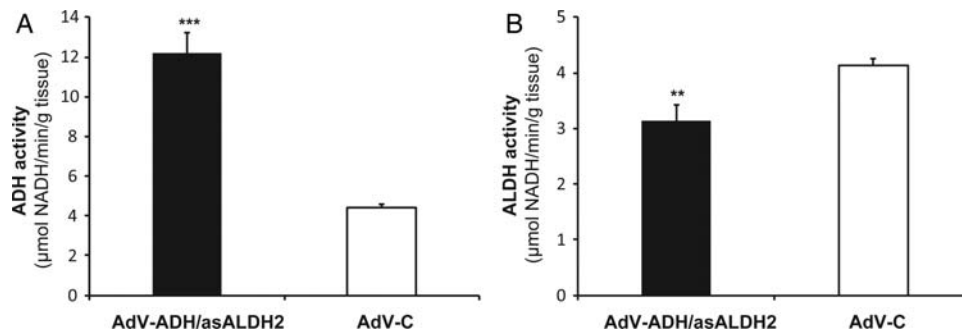


Fig. 5. Liver ADH and ALDH2 activity of UChB rats treated with the adenoviral vector coding for rADH<sup>47His</sup> and asALDH2. Thirty-three days after the administration of the adenoviral vectors, the animals were killed, and the ADH and ALDH2 activities were determined in the liver. Bars represent mean  $\pm$  SE of enzyme activity in liver ( $\mu\text{mol NADH}/\text{min}/\text{g}$  tissue); six animals/group. (A) Liver ADH activity: Rats treated with AdV-ADH/asALDH2 vector showed a 176% increase in liver ADH activity compared with animals that received AdV-C vector ( $t=7.4$ ,  $df=10$ ,  $P<0.001$ ). (B) Liver ALDH2 activity: Rats treated with AdV-ADH/asALDH2 vector showed a 24% reduction in liver ALDH2 activity versus control animals ( $t=-3.3$ ,  $df=10$ ,  $P<0.01$ ).

#### Determination of liver activities of ADH and ALDH2

The *in vivo* effects elicited by the administration of the AdV-ADH/asALDH2 vector on ethanol consumption and blood acetaldehyde levels were found to be consistent with the changes measured in the liver activities of ADH and ALDH2 enzymes. Figure 5A shows that liver ADH activity in animals transduced with the AdV-ADH/asALDH2 vector was 176% ( $P<0.001$ ) higher than that of the animals that received the AdV-C vector. Figure 5B data shows that liver ALDH2 in animals transduced with the AdV-ADH/asALDH2 vector was 24% ( $P<0.01$ ) lower than that of control animals. Total liver ALDH was not significantly different between both groups (data not shown).

#### DISCUSSION

As indicated above, it is well established that elevations in blood acetaldehyde following ethanol consumption in humans carrying an inactive form of ALDH (ALDH2\*2) lead to a marked protection against alcoholism (Thomasson *et al.*, 1991; Higuchi, 1994; Tu and Israel, 1995; Peng *et al.*, 2007). Several studies have shown also that a point mutation in the ADH gene that codes for a fast enzyme (ADH1B\*2) lead to a marked protection against alcoholism (Chambers *et al.*, 2002; Kim *et al.*, 2008; Chen *et al.*, 2009a). Furthermore, carriers of both protective genes showed a practically complete protection against alcoholism (Chen *et al.*, 2009a). In this study, we aimed at investigating in an animal model of alcoholism (UChB rats) whether the replication of this fully protected phenotype would result in a marked reduction of the voluntary ethanol intake. To mimic the human condition, we administered to high alcohol-drinking UChB rats a single dose of an adenoviral vector carrying the genes for both (a) a fast rat ADH (rADH<sup>47His</sup>) analogous to the fast ADH1B\*2 human enzyme and (b) an antisense RNA against the mRNA of rat ALDH2 which blocks its translation and therefore inhibits the synthesis of this enzyme. The strategy chosen of incorporating both expression cassettes in a single construction allowed the expression of both protective genes from a single adenoviral vector. Such a type of multiple expression cassette vector permits the use of lower adenoviral doses compared with the use of one independent vector for each gene, which also results in a lesser immunological

response to the adenoviral administration since these reactions are dependent on the viral dose (Lozier *et al.*, 2002).

The animals transduced with a single dose of the AdV-ADH/asALDH2 vector showed a 3-fold increase of in their liver ADH activity compared with the control animals, whereas liver ALDH2 activity showed a 24% reduction (see Fig. 5). Thus, upon the administration of the active adenoviral vector (AdV-ADH/asALDH2), the relative activity ratio of liver ADH/ALDH2 increased from  $\sim 1.0$  to 4.0 (see Fig. 5). The *in vivo* effect of the antisense RNA against *Aldh2* expression was substantially lower compared with the effect of rADH<sup>47His</sup>.

Upon the administration of a moderate dose of ethanol (1 g/kg, i.p.), the animals treated with AdV-ADH/asALDH2 showed a marked elevation in the arterial acetaldehyde levels reaching a peak at 5–10 min post-injection, which was 5-fold higher than that detected in the control animals. Since blood acetaldehyde levels reflect the balance between its hepatic generation by ADH and its degradation by ALDH, the kinetics of blood acetaldehyde levels in the animals treated with AdV-ADH/asALDH2 are consistent with the changes detected in the relative ADH and ALDH liver activities. Therefore, the initial burst of blood acetaldehyde (2.5–10 min) after ethanol administration would likely be due to the high activity of the fast rADH<sup>47His</sup>, while the prolong-lived elevated levels of blood acetaldehyde is likely associated with the decrease in the ALDH2 activity by the antisense mRNA. It should be noted that the levels of acetaldehyde ( $\sim 160 \mu\text{M}$ ) reached by the animals treated with AdV-ADH/asALDH2 are in the range of those obtained in rats treated with disulfiram, an ALDH inhibitor (Tampier *et al.*, 2008) and in humans carrying the inactive ALDH2\*2 (Mizoi *et al.*, 1994; Peng *et al.*, 2007).

The systemic administration of a single dose of the AdV-ADH/asALDH2 vector to alcohol-dependent UChB resulted in a marked reduction in their voluntary ethanol intake ( $\sim 60\%$ ) which correlates with the increased levels of blood acetaldehyde displayed for these animals upon ethanol administration. It should be noted that rats that received the AdV-C vector did not show changes in their baseline ethanol consumption, indicating that the adenoviral vector *per se* does not have effects on ethanol intake. Also, the adenoviral dose administered to the animals ( $3 \times 10^{12}$  pv/kg) did not elicit alterations in their behavior, water consumption or

body weight (data not shown). However, it has been reported in rats that the intravenous administration of first-generation adenoviral vectors at a dose of  $1 \times 10^{12}$  pv/kg can elicit alterations in platelet counts, increases in the activity of alanine transaminase and aspartate transaminase enzymes and hepatotoxicity (Kim *et al.*, 2001; Morrisey *et al.*, 2002). Taking in account both (a) the intrinsic hepatotoxic effects of ethanol and (b) the prolonged exposure to ethanol of the animals used in this study (75 days), it is likely an enhancement of the liver toxicity elicited by the systemic administration of adenoviral vectors. These toxicological aspects were not covered in this work and additional studies are needed to rule out a possible increase of the acute and chronic hepatotoxicity of adenoviral vector administration to animals previously treated with ethanol.

It should be noted that the adenoviral dose used in this animal study ( $3 \times 10^{12}$  pv/kg) would not be clinically acceptable, since fatal toxicity has been observed in one human clinical trial in which second generation (E1, E4-deleted) adenoviral vectors ( $6 \times 10^{11}$  pv/kg) were injected through the hepatic artery (Raper *et al.*, 2003). Considering the limited capacity of animal studies to predict the response in humans, more studies are needed to determine the potential clinical relevance of this Ad-based therapeutic approach to alcoholism.

A question that arises is why the combination of the fast ADH and a reduction in ALDH2 activity did not fully inhibit ethanol intake. Three possible explanations can be offered: (a) the engineered rat ADH<sup>47His</sup> has an activity that is considerably lower than the human ADH<sup>47His</sup> (Hurley *et al.*, 1990; Rivera-Meza *et al.*, 2010), (b) the reduction of ALDH2 activity was below 30%; while in heterozygous humans carrying the ALDH2\*2 allele the enzyme activity is reduced by 80% and (c) the rat may be less sensitive to the aversive effects of peripheral acetaldehyde than humans. Moreover, the potential reinforcing effects of acetaldehyde in the brain (Karahanian *et al.*, 2011) and the development of tolerance to its peripherally aversive effects (Chen *et al.*, 2009b) would affect the efficacy of the proposed gene-based therapy of alcoholism. An additional matter that should be considered is the rat model used in this study. After 60 days of voluntary ethanol intake, the drinking behavior of the animals is mediated mainly by (a) the reinforcing properties of ethanol and (b) the conditioning to contextual cues (alcohol taste or smell) which have been paired during the acquisition of the habit (Quintanilla *et al.*, 2011). Thus, in future studies this conditioning to the contextual cues should be extinguished prior the administration of the combined vector to maximize its effect on reducing ethanol drinking.

Although the importance of first-generation adenoviral vectors as a powerful research tool, its therapeutic application is hindered by the limited duration of its gene expression *in vivo*. The host immunological response against the viral proteins coded in the adenoviral vector genome limits the duration of *in vivo* expression of the therapeutic genes to a maximum of 6–8 weeks (Quantin *et al.*, 1992; Yang *et al.*, 1994). In this work, the therapeutic genes (i.e. rADH<sup>47His</sup> and asALDH2) encoded by the AdV-ADH/asALDH2 was seen to be actively expressed in the liver of the animals after 4 weeks of the systemic administration of the vector (see Fig. 5). However, the development of helper-dependent adenoviral vectors that was devoid of all viral sequences in

their genome has allowed the expression of therapeutic genes in the liver of rats and primates for years (Toietta *et al.*, 2005; Brunetti-Pierri *et al.*, 2009). Their non-integrative nature, large cloning capacity (~37 kb), their ability to accommodate multiple transgenes and lower chronic toxicity have made of the helper-dependent adenovirus an excellent vector for liver-directed gene therapy (see Brunetti-Pierri and Ng, 2011). However, it has been reported in animals that the presence of pre-existing liver diseases reduce the efficiency of adenoviral vectors to transduce the liver and could exacerbate their toxic effects (Smith *et al.*, 2004). In this regard, the use of helper-dependent adenoviral vectors would not avoid this enhanced hepatotoxicity in livers with pre-existing diseases, particularly at early times upon their systemic administration (Reddy *et al.*, 2002). These effects should be carefully considered in a possible clinical application of the proposed therapy in severe alcoholics, which are prone to develop liver cirrhosis.

The specific liver tropism of adenoviral vectors administered intravenously is also highly desirable (see Rivera-Meza *et al.*, 2010), since tissues such as those in the upper GI and laryngopharyngeal areas, which are prone to develop neoplasias in alcoholics carrying the ubiquitous inactive ALDH2\*2, would not have their ALDH2 activity decreased. It is in fact noteworthy that the ADH1B\*2 gene (ADH<sup>47His</sup>) although expressed mainly in the liver has been shown to have a marked protective effect against alcohol related cancers in these tissues (Chen *et al.*, 2006; see also Israel *et al.*, 2011).

Overall, the present study shows that the concomitant increase of ADH activity and a reduction of ALDH2 activity in the liver of alcohol-dependent rats is an effective method to reduce the voluntary ethanol intake in rodents. These results combined to improved methods for prolonged liver-directed gene transfer, added to an extinction of contextual cue conditioning, would provide elements for new therapeutic strategies for the treatment of alcoholism.

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