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Tissue-specific expression of ALA synthase-1 and heme oxygenase-1 and their expression in livers of rats chronically exposed to ethanol

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Abstract 5-Aminolevulinic acid synthase-1 (ALAS1) and heme oxygenase-1 (HO-1) are the rate-controlling enzymes for heme biosynthesis and degradation, respectively. Expression of these two genes showed tissue-specific expression pattern at both mRNA and protein levels in selected non-treated rat tissues. In the livers of rats receiving oral ethanol for 10 weeks, ALAS1 mRNA levels were increased by 65%, and the precursor and mature ALAS1 protein levels were increased by 1.8- and 2.3-fold, respectively, while no changes were observed in HO-1 mRNA and protein levels, compared with pair-fed controls. These results provide novel insights into the effects of chronic ethanol consumption on hepatic heme biosynthesis and porphyrias.

Keywords: 5-Aminolevulinic acid synthase-1 (ALAS1); Heme oxygenase-1 (HO-1); Ethanol; Gene expression; Porphyria; Rat liver

1. Introduction

5-Aminolevulinic acid synthase-1 (ALAS1) is the first and normally rate-controlling enzyme of hepatic heme synthesis and is the house keeping form of ALA synthase [1–4]. 5-Aminolevulinic acid synthase 2 (ALAS2) is another isoform of ALA synthase, which is expressed virtually only in developing red blood cells [3,5]. Both ALAS1 and ALAS2 use glycine and succinyl-CoA as substrates and are synthesized in the rough endoplasmic reticulum (ER) as precursor proteins. Both isoforms, however, are believed to function only in mitochondria, the site of succinyl-CoA synthesis. Thus, like other nuclear-encoded mitochondrial proteins, ALAS1 and ALAS2 must be transported into mitochondria, with cleavage of a leader sequence [6]. In contrast to ALAS2, which is not responsive to chemicals or drugs, ALAS1 is up-regulated by chemicals and drugs [7–9]. Heme oxygenase-1 (HO-1), the rate-controlling enzyme for heme degradation, is the inducible form of heme oxygenase. Induction of HO-1 serves as an adaptive response to protect cells from oxidative stress and other types of stress through breakdown of unassigned heme to biliverdin, bilirubin, and carbon monoxide [10,11].

Studies suggest that a single ALAS1 mRNA is expressed in all rat tissues; however, the expression levels in each tissue measured by different groups remain controversial [12,13]. Additionally, it is not clear whether there is a correlation or correspondence between levels of expression of ALAS1 mRNA and those of ALAS1 protein in particular tissues and organs. The same is true of HO-1 mRNA and protein.

Earlier work showed that ethanol administration increases hepatic ALAS1 enzyme activities in rats [14–16]. Some have proposed that this effect is secondary to depletion of a heme regulatory pool, which is depleted during increased formation of cytochrome(s) P-450 [15]. In rat hepatocyte cultures, the addition of 16 mM ethanol for 24 h, causes a three-fold increase in the activity of ALAS1 [17]. In humans, increased ALAS1 activity may lead to the accumulation of neuro- and dermato-toxic intermediates of the heme biosynthetic pathway, such as may occur in the porphyrias [1,18–20]. Thus, ingestion of alcoholic beverages may trigger or exacerbate acute porphyric attacks. It is critical to understand how ethanol exerts its effects on inducing hepatic ALAS1 enzyme activity.

In the present study, the questions of: (1) tissue-specific expression of ALAS1 and HO-1 mRNA and protein levels in selected non-treated rat tissues, and (2) effects of chronic ethanol administration on ALAS1 and HO-1 mRNA and protein levels in rat liver were investigated by quantitative real-time polymerase chain reaction (PCR) and Western blotting analysis. We show that the expression of both ALAS1 and HO-1 is tissue-specific, and there is a correspondence between levels of HO-1 mRNA and protein, but not between those of ALAS1 mRNA and protein. Chronic ethanol feeding induces ALAS1 mRNA, cytosolic ALAS1 and mature ALAS1 protein levels, but not those of HO-1 in rat livers. These provide further evidence for alcohol consumption as trigger of acute porphyric attacks in human.

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Abbreviations: ALAS1, 5-aminolevulinic acid synthase-1; ALAS2, 5-aminolevulinic acid synthase 2; ER, endoplasmic reticulum; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HO-1, heme oxygenase-1; LDC, Lieber-DeCarli; PCR, polymerase chain reaction; qRT-PCR, quantitative real-time PCR; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis

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2. Materials and methods

2.1. Materials

Lieber-DeCarli (LDC) liquid diet was purchased from Dyets Inc. (Bethlehem, PA). All the reagents for reverse transcription and quantitative real-time PCR (qRT-PCR) were purchased from Sigma (St. Louis, MO). Rabbit anti-human ALAS1 antibody was supplied by Abcam (Cambridge, MA). Rabbit anti-human HO-1 polyclonal antibody was from StressGen (Victoria, BC, Canada). Goat anti-GAPDH antibody was from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). ECL-Plus was from Amersham Biosciences Corp. (Piscataway, NJ). Ponceau S was from Sigma (St. Louis, MO).

2.2. Animal model of chronic ethanol feeding

All experimental procedures were approved by the Institutional Animal Care and Use Committee and performed in accordance with the National Institutes of Health criteria for care and use of laboratory animals. Untreated male Sprague–Dawley rats (250–300 g; Charles River Laboratories, Wilmington, MA) were anesthetized with xylazine (3 mg/kg) and ketamine (70 mg/kg), and livers, forebrains, kidneys, lungs, spleens, recturs abdominis and hearts were rapidly resected, snap-frozen in liquid nitrogen, and stored at −80 °C.

Chronic ethanol administration was described previously [21]. Male Sprague–Dawley rats as described above were randomly assigned to experimental groups, and fed either control or ethanol-containing LDC liquid diet for 10 weeks. In ethanol feeding groups, the amount of ethanol in the LDC was sequentially increased during the first week of feeding until the amount of ethanol in the diet comprised 36% of total dietary calories. In subsequent weeks, the amount of food provided was altered according to consumption and adjusted for changes in body weight. Control groups received an isocaloric liquid diet in which maltodextrins were substituted isocalorically for ethanol. During the course of the studies, food intakes were measured daily, body weights were measured weekly, and animals were allowed free access to drinking water. During euthanasia, livers were rapidly resected, snap-frozen in liquid nitrogen, and stored at −80 °C for RNA and protein analysis.

2.3. RNA preparation and quantitative real-time PCR

Total RNA isolation, reverse transcription and qRT-PCR were performed as previously described [22]. Briefly, total RNA was isolated from frozen liver tissue (approximately 50 μg) using TRIzol (Invitrogen). For reverse transcription, 1 μg of total RNA was used with the following specific primers for qRT-PCR. The ALAS1 sense primer was 5′-GGC AGC ACA GAT GAA TCA GAG AG-3′, and the ALAS1 antisense primer was 5′-TTC AGC AAC CTC TTT CCT CAC GG-3′. In order to confirm the specificity of qRT-PCR, samples without template or without reverse transcriptase were used as negative controls. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an invariant internal control for liver samples. The sense primer for GAPDH was 5′-TTG TTG CCA TCA ATG ACC C-3′ and the antisense primer for GAPDH was 5′-CTT CCC GTT CTC AGC CTT G-3′. Primers for HO-1 were as described previously [22].

2.4. Western blots

Western blotting analysis was as described previously [23]. Liver samples were homogenized in lysis buffer (150 mM sodium chloride, 50 mM Tris–HCL, pH 7.2 and 5% Nonidet P-40) with protease inhibitor cocktail from Pierce (Rockford, IL). Thirty-five to seventy-five micrograms of protein were separated on 7.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) from Bio-Rad (Hercules, CA). After electrophoretic transfer onto Immun-Blot polyvinylidene fluoride membranes (Bio-Rad), the membranes were blocked with 5% non-fat dry milk in phosphate-buffered saline with Tween-20 (0.1% Tween-20) for 1 h, and then incubated with primary antibody for 1 h at room temperature. The dilutions of the primary antibodies were 1:5000 for anti-ALAS1 antibody, 1:2000 for anti-HO-1 antibody, and 1:1000 for anti-GAPDH antibody. The membranes were then incubated for 1 h with horseradish peroxidase-conjugated secondary antibodies (dilution 1:10 000). Finally, the bound antibodies were visualized with the ECL-Plus chemiluminescence system, according to the manufacturer's protocol (Amersham, Piscataway, NJ). A Kodak 1DV3.6 computer-based imaging system (Eastman-Kodak, Rochester, NY) was used to measure the relative optical density of each specific band obtained after Western blotting. Data are expressed as percentage of the control.

2.5. Statistical analysis

All experiments included at least triplicate samples for each treatment group. Experiments were repeated at least three times with similar results. Representative results from a single experiment are presented. Statistical analyses were performed with JMP 4.0.4 software (SAS Institute, Cary, NC). Initial descriptive statistics showed that the results for continuous variables were distributed normally. Therefore, the differences in mean values were assessed by ANOVA, with the Tukey–Kramer correction for multiple pair-wise comparisons, or Dunnett's test versus a control. Values of $P < 0.05$ were considered significant.

3. Results and discussion

3.1. Determination of ALAS1 and HO-1 mRNA levels in selected rat tissues

In order to determine ALAS1 mRNA levels, seven tissues, including liver, forebrain, kidney, spleen, lung, abdominal skeletal muscle and heart were collected. Total RNA was isolated and ALAS1 mRNA levels were determined by qRT-PCR. As shown in Fig. 1A, ALAS1 mRNA levels (relative to 28S ribosomal RNA) were set as 1 in the liver, and were compared to this level in other tissues. The relative levels of ALAS1 mRNA were about 40% those of liver in the forebrain, kidney and heart, and were less than 20% in the lung, spleen and muscle. These results differ slightly from those published previously [13] in which the levels of ALAS1 in lung, heart and muscle were similar or higher than those in the liver. These differences may indicate age or measuring technique variations since tissues from rats of 250–300 g body mass were collected followed by qRT-PCR in this study, while those from rats of 100 g body mass were collected followed by Northern blot and densitometric scanning in the previous study. The low ALAS1 levels in the muscle observed in this study might be due to the specific abdominal skeletal muscle being utilized.

In comparison to ALAS1, mRNA levels of another key enzyme in the heme metabolic pathway, namely, heme oxygenase-1 (HO-1), the rate-controlling enzyme for heme breakdown, were also determined. As shown in Fig. 1B, by far, the highest expression of HO-1 mRNA levels were observed in the spleen: 6.6-fold of those in the liver. This agrees well with the known roles of the spleen to engulf and digest senescent erythrocytes. In forebrain, HO-1 mRNA levels were 66% of those in liver, whereas, they were nearly undetectable in other tissues tested.

When measuring mRNA levels in cell cultures, we and others usually "normalize" ALAS1 or HO-1 mRNA levels to those of "house keeping genes", such as GAPDH or 18S ribosomal RNA (rRNA). However, in rat tissues, these house keeping genes were expressed at different levels among tissues. For example, in forebrain, GAPDH mRNA levels were 3.3-fold of those in the liver, but were nearly undetectable in the lung and abdominal skeletal muscle (Supplementary Figure 1). For 18S rRNA, in the kidney, spleen and heart, the mRNA levels were only about 20% of those in the liver (data not shown). We therefore, determined the quality and quantity of total RNA (represented by 28S rRNA in Fig. 1C) from each tissue by electrophoretic analysis, and found that total RNA from all tissues studied was of good quality and similar quantity.

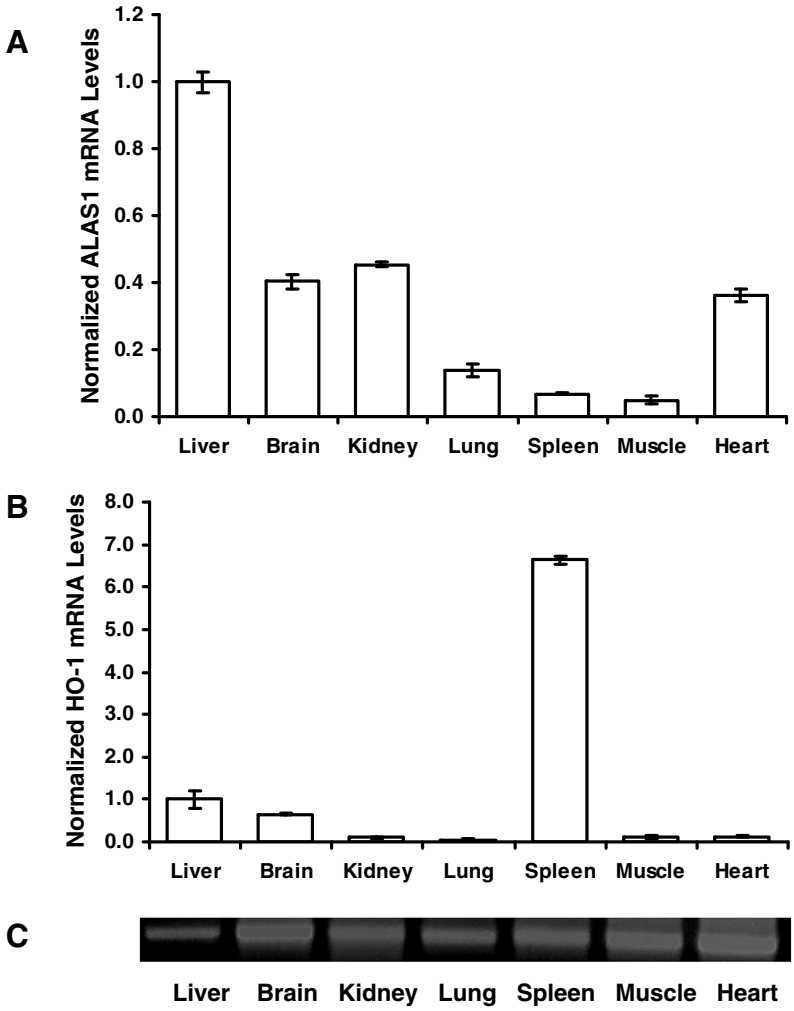

Fig. 1. Relative expression of ALAS1 (A) and HO-1 (B) mRNA in selected rat tissues. Seven tissues were collected from each of three control rats. Total RNA was isolated from each tissue by TRIzol. ALAS1 (A) and HO-1 (B) mRNA levels were determined by qRT-PCR. To confirm the quality and quantity of total RNA, 1 μg RNA from each tissue was loaded on 1% ethidium bromide agarose gel. The top band corresponding to the 28S ribosomal RNA is shown (C). Data in (A) and (B) represent means ± S.D., $n = 3$, normalized to the level of 28S ribosomal RNA of the respective tissue in (C). Liver ALAS1 (A) and HO-1 (B) mRNA levels are set to 1, and mRNA levels from other tissues are normalized to those in the liver. Brain represents the forebrain; muscle represents the abdominal skeletal muscle.

Both ALAS1 and HO-1 are key enzymes of heme metabolism. Expression of these genes at mRNA levels, however, is distinct among tissues. Interestingly, in the forebrain, ALAS1 and HO-1 mRNA expressions were about 40% and 60%, respectively, of those found in the liver (Fig. 1A and B), suggesting they are important for adult brain. Several recent lines of evidence suggest that heme deficiency might lead to neuronal dysfunction and aging [24,25]; it will be important to investigate further how ALAS1 and HO-1 are expressed and regulated in the brains of animals as they age.

3.2. Determination of ALAS1 and HO-1 protein levels in selected rat tissues

As shown in Fig. 2A, ALAS1 protein levels were greater in the liver, lung, spleen and heart than those in the forebrain, kidney and abdominal skeletal muscle. The relative protein levels are quantitated and shown as a bar graph in Fig. 2D. The same blot was reprobed with anti-HO-1 antibody, and is shown in Fig. 2B. HO-1 was the highest in the spleen, slightly detectable in the abdominal skeletal muscle, and almost undetectable in other tissues. Since GAPDH protein levels differed among the seven tissues tested (Supplementary Figure 2), to confirm the equal loading, the same membrane was stained with Ponceau (Fig. 2C). We found that although there was no single band that consistently appeared among all these tissues, it is reasonable to consider that the loadings are equal among different lanes (tissues), based on the total intensities of the bands on each lane.

Although ALAS1 mRNA levels in the forebrain, kidney and heart were about 40% of those in the liver (Fig. 1A), ALAS1 protein levels were less than 40% in the forebrain and kidney, but about 40% higher in the heart than those in the liver (Fig. 2D). On the other hand, ALAS1 mRNA levels were less than 20% in the spleen and lung, however, ALAS1 protein levels were similar or higher (two-fold) in spleen and lung than those in the liver. To further compare the expression level of protein versus mRNA in each tissue, the ratio(s) of ALAS1 protein/mRNA was calculated as shown in Fig. 2E. The ratio of ALAS1 protein/mRNA was 15 in the lung and spleen, and was about 40 in the heart, whereas, it was similar in the

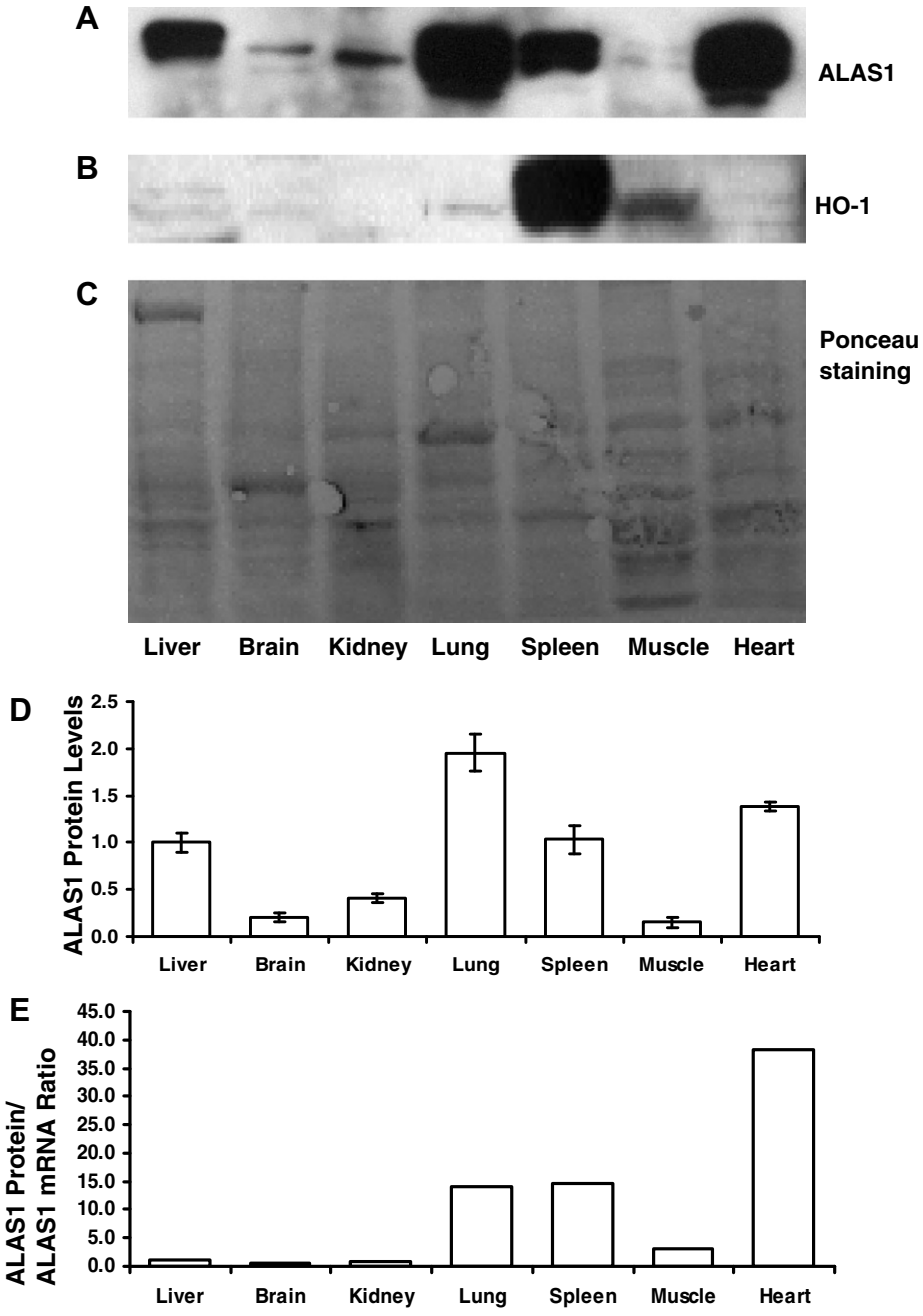

Fig. 2. Western blot analysis of ALAS1 and HO-1 protein levels in selected rat tissues. Seven tissues were collected from each of three control rats, homogenized, and 35 μg of proteins were separated on 7.5% SDS–PAGE, transferred to a polyvinylidene fluoride membrane, and probed with anti-ALAS1 antibody (1:5000 dilution) (A) and anti-HO-1 antibody (1:2000 dilution) (B). Equal loading of total protein from each tissue was confirmed by Ponceau S staining of the polyvinylidene fluoride membrane after gel transfer and before Western blot (C). Representative results from one of the three rats are shown in (A)–(C). ALAS1 protein levels were quantitated from each of the three rats and the average level is shown as a bar graph in (D). The ratio of ALAS1 protein and RNA levels calculated based on this figure (D) and Fig. 1A is shown as a bar graph in (E). Brain represents the forebrain; muscle represents the abdominal skeletal muscle.

forebrain, kidney and abdominal skeletal muscle compared to that in the liver. These results suggest a single ALAS1 mRNA molecule is subject to differential regulatory mechanism in translation into its protein in distinct tissues. Due to the low expression of HO-1 protein in many tissues, we did not perform similar calculations for HO-1. However, it is reasonable to speculate that although there is tissue-specific expression at both mRNA and protein levels, the ratio(s) of HO-1 protein/mRNA was similar among different tissues tested.

3.3. Effects of chronic ethanol administration on ALAS1 and HO-1 mRNA levels

As shown in Fig. 3A, hepatic ALAS1 mRNA levels were significantly increased (+65%) after ethanol administration for 10 weeks, compared with the pair-fed control ($P < 0.05$). On the other hand, no significant changes were observed in HO-1 mRNA levels between ethanol treated and pair-fed control (Fig. 3B). We also measured GAPDH mRNA levels in ethanol administrated livers and pair-fed controls. As shown in Fig.

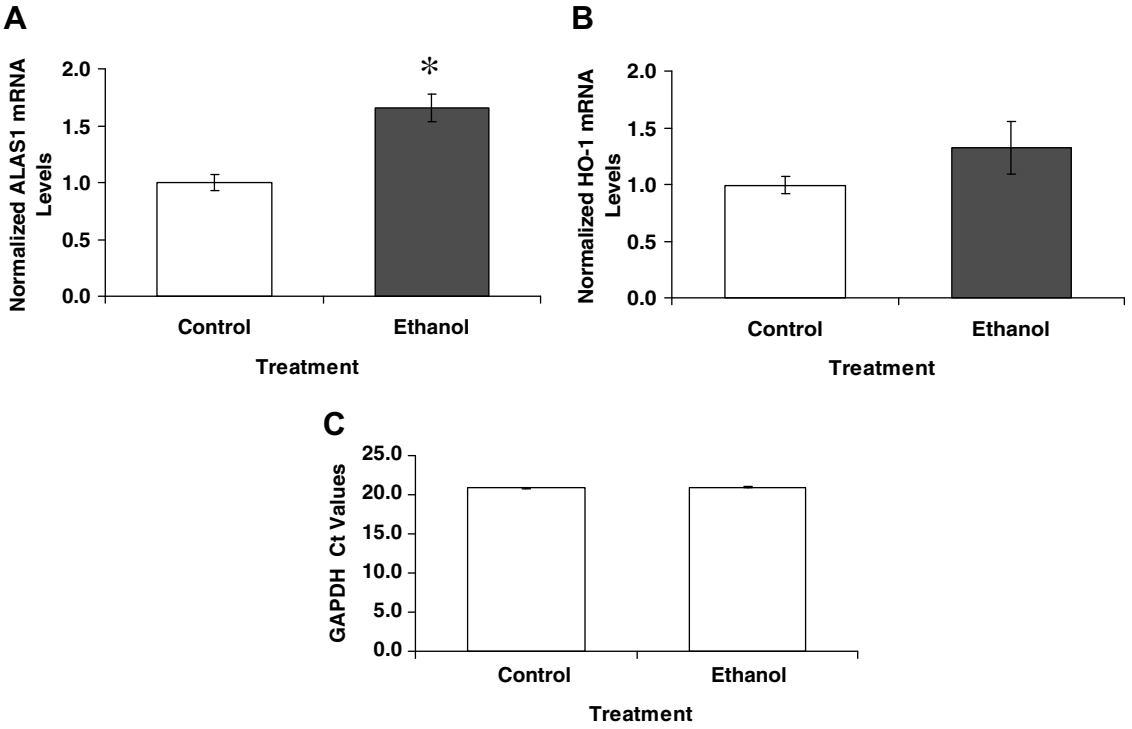

Fig. 3. Effects of chronic administration of ethanol on rat liver ALAS1 and HO-1 mRNA levels. Rats were administrated ethanol or isocalorically substituted maltodextrins for 10 weeks. Total RNA was isolated from livers of each of three rats. ALAS1 (A) and HO-1 (B) mRNA levels were determined by qRT-PCR, and normalized to an invariant GAPDH control (C). Data represent means ± S.D. for each group of three rats with triplicates of each rat. *Differs from control, $P < 0.05$.

3C, there were no changes in Ct values (expression level of a gene) of qRT-PCR, suggesting ethanol had no effects on the liver GAPDH mRNA levels. Therefore, in Fig. 3A and B, ALAS1 and HO-1 mRNA levels were normalized to GAPDH mRNA levels. These results suggest that ethanol specifically induces ALAS1 mRNA levels, although it is not clear whether this induction is through an enhanced transcription and/or an increase in mRNA stability. Further studies will be needed to address this issue.

3.4. Effects of chronic ethanol administration on ALAS1 protein levels

To further determine the effects of chronic ethanol administration on ALAS1 gene expression, ALAS1 protein levels were determined by Western blotting analysis. As shown in Fig. 4A, the anti-ALAS1 antibody detected two bands, which may correspond to the precursor form (higher MW, 71 kDa), and the mature form (lower MW, 65 kDa). Ethanol treatment increased the levels of both forms, especially the 65 kDa form. Since ethanol had no effect on liver GAPDH protein levels (Fig. 4A, bottom panel), ALAS1 protein levels were quantitated and normalized to GAPDH control (Fig. 4B). In Fig. 4B, without ethanol treatment, the lower molecular weight (MW) form was 44% of the higher form. After ethanol treatment, both forms increased significantly: the higher MW form increased by 1.8-fold ($P < 0.05$), while the lower form increased by 2.3-fold ($P < 0.05$). These results suggest that chronic ethanol administration induces both the precursor and mature forms of ALAS1 and has a relatively greater effect on the formation of mature form. Using these same sets of animals in another independent experiment, we found that cyto-

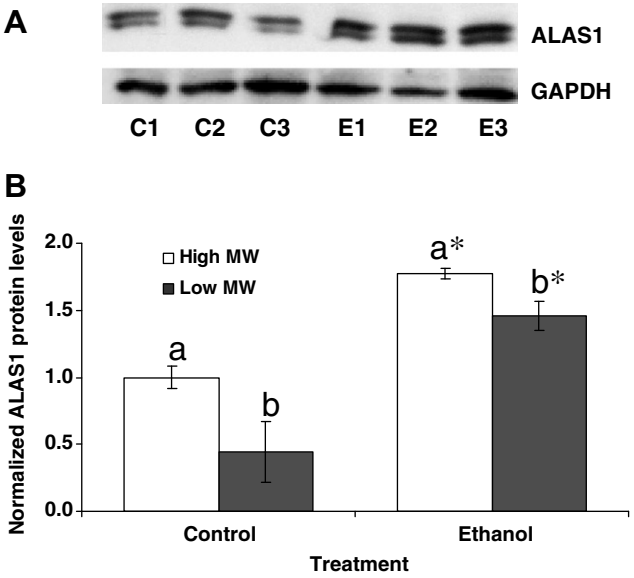

Fig. 4. Effects of chronic administration of ethanol on rat liver ALAS1 protein levels. Rats were administrated ethanol or isocalorically substituted maltodextrins for 10 weeks, as described in Section 2. Livers were harvested, frozen, homogenized, and subject to SDS–PAGE and Western blotting analysis, as described in Fig. 2. The ALAS1 antibody detected two bands: higher molecular weight (high MW) and lower molecular weight (low MW) bands of ~ 71, and 65 kDa, respectively (Panel A). Intensity of staining of each band was quantified and normalized to the GAPDH control as shown in the bar graph (Panel B). Data represent means ± S.D. for each group of three rats. *Differs from control, $P < 0.05$. In (A) "C" and "E" represent control and ethanol, respectively. In (B) "a" and "b" represent high MW and low MW band, respectively.

chrome P450 2E1, an inducible marker for ethanol treatment was significantly increased [21]. Our findings agree well with the previously published results in which chronic ethanol administration increased hepatic ALAS1 enzyme activities by two- to three-fold compared with those pair-fed controls [14–16]. Considering that the intra-mitochondrial mature ALAS1 is the functional form, and 2.3-fold increase in the levels of the mature protein after ethanol treatment provides a rationale for the ethanol induced increase (two- to three-fold) in ALAS1 enzyme activities observed by other investigators [14–16]. In this same experiment, HO-1 protein levels were nearly undetectable (data not shown), suggesting ethanol had no effect on HO-1 protein levels.

Abundant evidence from animal and cell culture models indicates that ethanol induces hepatic ALAS1 enzyme activities. Results from this study suggest that ethanol induces ALAS1 gene expression at the level of transcription, translation, and also exerts post-translational regulation (precursor processing) in the livers of rats. Our finding that chronic ethanol treatment induces ALAS1 but not HO-1 gene expression provides further understanding of ethanol as a trigger of acute porphyric attacks.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2008.04.047.

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