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The Effect of Ethanol-Induced Cytochrome p4502E1 on the Inhibition of Proteasome Activity by Alcohol

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The present investigation was undertaken to determine the effect of CYP2E1 induction by ethanol on the inhibition of proteasomal activity in wild-type and CYP2E1 knockout C57 black mice. The proteasomal chymotrypsin-like activity decreased significantly in ethanol-fed wild-type mice liver, but was not reduced in ethanol-fed knockout mice liver. The 26S proteasomal activity was decreased more by ethanol feeding than was the 20S proteasomal fraction. Individual hepatocytes lost immunostaining of the proteasomes in the centrilobular zone in the livers of ethanol-fed wild-type mice and the knockout mouse liver. There was increased product of protein oxidation in the liver in the wild type but not in the knockout mice given ethanol. Taken together, these results suggest that CYP2E1 induction was responsible for the decrease in proteasome activity seen in the wild-type mice which head to the accumulation of oxidized proteins which were increased as the result of free radicals generated by CYP2E1 metabolism of ethanol.

In the chronic administration of alcohol, P450 CYP2E1 is significantly induced. The induction of CYP2E1 is associated with a production of free radicals (1), and with an increase in lipid peroxidation (2, 3) which causes liver injury. When CYP2E1 inhibitors were fed with ethanol, the CYP2E1 inhibition protected the liver from ethanol-induced injury (4–6), suggesting that the CYP2E1 induction by ethanol is strongly associated with increased oxidative damage in the ethanol-treated liver (7).

The ethanol-dependent induction of CYP2E1 is also associated with decreased proteolysis in the liver (8, 9). The ATP-ubiquitin-proteasome pathway was significantly decreased in intragastric ethanol-fed animals (10). The mechanism responsible for this decline was not shown, but the ubiquitin level and expression were decreased (6). The inhibition of CYP2E1 reduced the inhibition of the ethanol-related proteasome activities (10, 11).

In the present work, the effects of ethanol treatment on 20S and 26S proteasome components and the role of CYP2E1 induction on these components were investigated. CYP2E1 knockout mice (gift of Dr. Gonzalez at NIH) were used for this study. The proteasomal components were analyzed in wild-type and CYP2E1 knockout mice receiving ethanol intragastrically for 20 days. The question was: What is the role of CYP2E1 induction in the reduction of proteasomal activity by ethanol?

MATERIALS AND METHODS

Male mice C57 black weighing 25 g were purchased from Charles River Laboratory (Hollister, CA). The CYP2E1 knockout C57 black mice were obtained from Dr. F. Gonzalez Laboratory and bred in our animal care unit (12). The polyclonal antibodies raised in rabbits against CYP2E1 and against ubiquitin were obtained respectively from StressGen (Victoria, BC, Canada) and from DAKO (Carpinteria, CA). The mouse monoclonal antibodies anti p25 and p31 (respectively, anti proteasome α-subunit C3 and C8) were obtained from ICN (Aurora, Ohio).

The mice were pair-fed ethanol or isocaloric dextrose. They were continuously infused via a permanent intragastric cannula for 20 days as previously reported, except that AIN93 vitamin and mineral mixes replaced AIN76 (5).

The mice were maintained according to the Guidelines of Animal Care, as described by the National Academy of Sciences and published by the National Institute of Health. After 20 days of alcohol treatment, the mice were sacrificed and the livers were weighed. Portions of liver were fast frozen in liquid nitrogen for biochemical analysis. The rest was fixed in zinc formalin for light and immunofluorescent microscopy.

Homogenization and subcellular fractionation. Frozen livers were homogenized with ultra-turrax T25 and Potter-Elvehjem glass homogenizer in 50 mM Tris–HCl pH 7.5, 10% glycerol, 5 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride (PMSF), 50 nM E64, and 2.5 μM pepstatin-A in 2 volumes for the determination of peptidase activities. For Western blot analysis, the liver was homogenized in 20 mM Tris–HCl pH 8.8, 100 mM NaCl, 1 mM EDTA, 0.5% nonidet P-40 (v/v), 12 mM Na-deoxycholate.

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The total liver homogenate was centrifuged at 100,000*g* for 1 h and the supernatant (cytosolic fraction) was used for protein and enzyme assays, and for Western blot analysis of proteasome subunits.

The protein concentration was determined using Bio-Rad assay reagent (13). Bovine serum albumin was the protein standard.

Enzyme assay. The assay was performed according to Bardag-Gorce *et al.* (14). Chymotrypsin-like activity of the proteasome, was measured in the cytosolic fraction. Reaction mixtures (final volume = 200 μL) contained 5–50 μg of protein from the cytosolic fraction, 50 mM Tris–HCl pH 8, 1 mM DTT, and 40 μM Succinyl-leucine-leucine-valine-tyrosine-7-amino 4-methylcoumarine (Suc-LLVY-AMC). The mixture was incubated for 30 min at 37°C. The reaction was stopped by adding 0.8 ml 100 μM monochloroacetate and 30 mM sodium acetate pH 4.3. Fluorescence was determined by measuring the release of AMC (λ excitation: 370 nm, λ emission: 430 nm) using a Perkin Elmer LS 30 spectrofluorometer. A standard curve for AMC was made to calculate the concentration of liberated products in the assay.

Western blots. Protein (5–10 μg) from the cytosolic fraction was used in SDS–PAGE electrophoresis (15) performed on a 12% separating gel. Proteins were transferred to PVDF membrane (Bio-Rad) for 1 h in 25 mM Tris–HCl (pH 8.3), glycine 192 mM, and 20% methanol. The immunoblot stain was done using various antibodies against proteasome subunits. The immunodetection was performed using the enhanced chemiluminescence (ECL) kit from Amersham for the proteasome subunit analysis. An alkaline phosphatase kit from Bio-Rad was used for CYP2E1 analysis. Densitometric measurements of the bands were made using the GS-710 imaging densitometer (Bio-Rad, CA).

Morphological analysis. Liver tissue sections fixed in zinc formalin and embedded in paraffin were double stained with p25 or p31 mAB (green: 2nd antibody, anti mouse conjugated with fluorescein isothiocyanate (FITC) (DAKO Co., Carpinteria, CA) and ubiquitin antibody (red: 2nd antibody, anti rabbit conjugated to Texas-Red dye (Jackson Immuno Research Lab., West Grove, PA). Stained slides were mounted with 0.1% ρ-phenylendiamine in 70% glycerol phosphate-buffered saline and photographed using a Nikon epifluorescent microscopic system. This was done to show the proteasome and ubiquitin in the same hepatocytes using immunofluorescence microscopy.

Protein carbonyl measurement. As a consequence of protein oxidation, carbonyl groups are introduced into protein side chains. These carbonyl groups were detected by derivatization to 2, 4-dinotrophenylhydrazone (DNP-hydrazone) by reaction with 2, 4-dinotrophenylhydrasine (DNPH) (15, 16) using an oxyblot kit (ONCOR, Gaithersburg, MD). The DNP-derivatized protein samples were separated by polyacrylamide gel electrophoresis followed by Western blotting. The DNP-moiety of protein was detected by a rabbit anti-DNP antibody supplied in the kit, and the second antibody was goat anti-rabbit HRP-conjugated. The immunodetection was performed using the ECL kit.

Statistic analysis. For the wild-type animals, data are from four dextrose-fed mice and three ethanol-fed mice. The CYP2E1 knockout data, are from three dextrose-fed and four ethanol-fed mice. Bars represent mean values ± SE. P values were determined by ANOVA and the PLSD Fisher post-hoc test (Sigma-Stat software, San Francisco, CA).

RESULTS

The body weight for both wild-type and mice lacking CYP2E1 expression was not affected by 20 days of ethanol treatment. However the liver weight increased significantly in the ethanol-fed mice (Fig. 1). The alcohol fed mice showed fatty liver (Fig. 2).

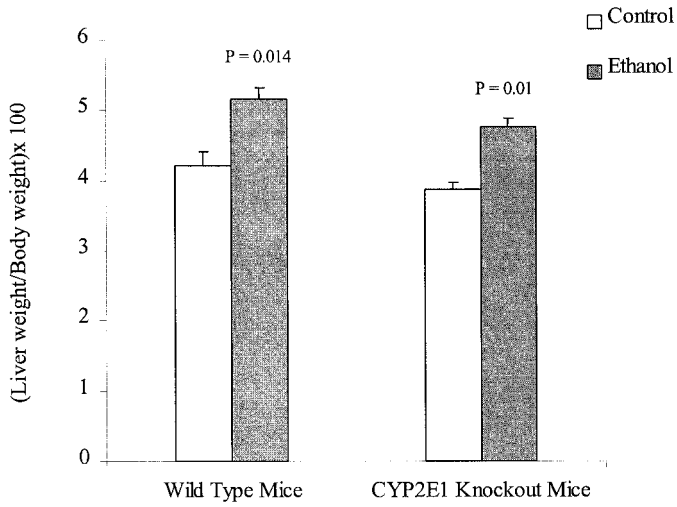

FIG. 1. Effect of ethanol on the liver weight: relative weight of liver between control and ethanol-treated wild-type mice (A) and CYP2E1 knockout mice (B) were compared. Liver weight increases with ethanol treatment.

Using the rabbit polyclonal anti CYP2E1 in the wild-type mice, it was shown that ethanol feeding increased the CYP2E1 level 5.75-fold over the control levels (Fig. 3). No band was detect in samples from control and ethanol-fed knockout mice.

Chymotrypsin-like activity was measured in the liver of mice using a fluorogenic substrate SucLLVY. Chymotrypsin-like activity was significantly decreased in the liver of wild-type ethanol-fed mice (Fig. 4). These results indicated that ethanol treatment decreased the basal activity of the proteasome. However, the chymotrypsin-like activity measured in the liver of CYP2E1 knockout mice did not show any decrease in the chymotrypsin-like activity in the liver of ethanol-fed mice.

The activities measured above are the basal activity. To further investigate this activity, the chymotrypsin-like activity of the SDS activated 20S proteasome and ATP-activated 26S proteasome activities were determined. It was observed that ethanol treatment decreased the chymotrypsin-like activity as seen in the basal activity for both 26S and 20S proteasomes. The 26S proteasome activity was decreased more by ethanol administration than was the 20S proteasome. The 26S proteasome decreased by 40% while the 20S proteasome was decreased by 20% in the ethanol-fed wild-type mice (Fig. 5).

In the CYP2E1 knockout mice, the 20S and 26S proteasome chymotrypsin-like activity was not significantly different between the controls and ethanol treated mice (Fig. 6).

In the Western blot analysis and the immunohistochemical study, using anti-alpha subunit C8 (p31), ethanol decreased the staining of the proteasome (Fig. 7).

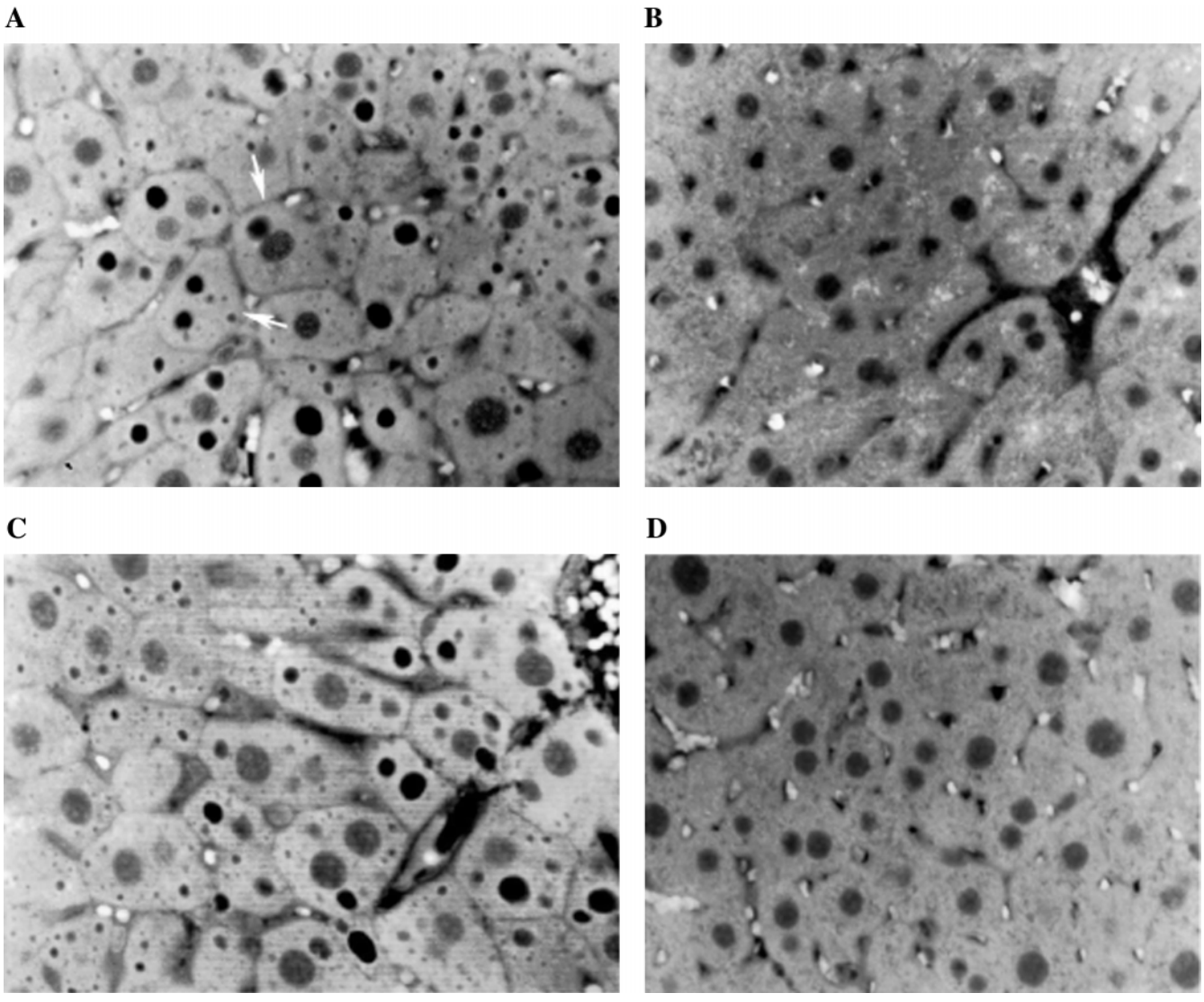

FIG. 2. p25 anti proteasome alpha-subunit C3 immunofluorescent staining. (A) Liver cells from wild-type mouse fed ethanol. The black holes indicate fat storage. The pale holes are nuclei. (B) Liver cells from wild-type mice fed dextrose. (C) Liver cells from CYP2E1 knockout mice fed ethanol. The black holes indicate increased fat storage. (D) Liver cells from CYP2E1 knockout mice fed dextrose. (B) and (D) show no fatty change and the pale holes are nuclei. (Original magnification × 624).

The morphological analysis correlated with the Western blot results (Fig. 8). The proteasomes appeared to aggregate in clusters within the cytoplasm of the hepatocytes. A marked decrease in staining for the subunit C8 of the proteasome was noted in individual hepatocytes around the central vein in the livers of mice fed ethanol. Adjacent hepatocytes showed normal staining of the proteasomal subunits. In the CYP2E1 knockout mice treated with ethanol (Fig. 8C) there was also a significant loss of proteasome staining. The focal nature of the histological change was not reflected in the enzyme assay because the magnitude of the focal changes were not extreme enough to be detected in the homogenate assay.

To determine whether ethanol caused the accumulation of oxidized proteins, the amount of protein carbonyls in the cytosolic fraction of the four different groups of mice were measured. Oxidized proteins accumulated in the ethanol-fed wild-type mice livers. But in the ethanol-fed CYP2E1 knockout mice, there was no accumulation of oxidized proteins. The results indicate that ethanol-induction of CYP2E1 and ethanol reduced proteasomal activity are both necessary before oxidized protein accumulation was observed (Fig. 9).

DISCUSSION

The intragastric ethanol feeding of the wild-type and knockout mice caused a significant increase in liver

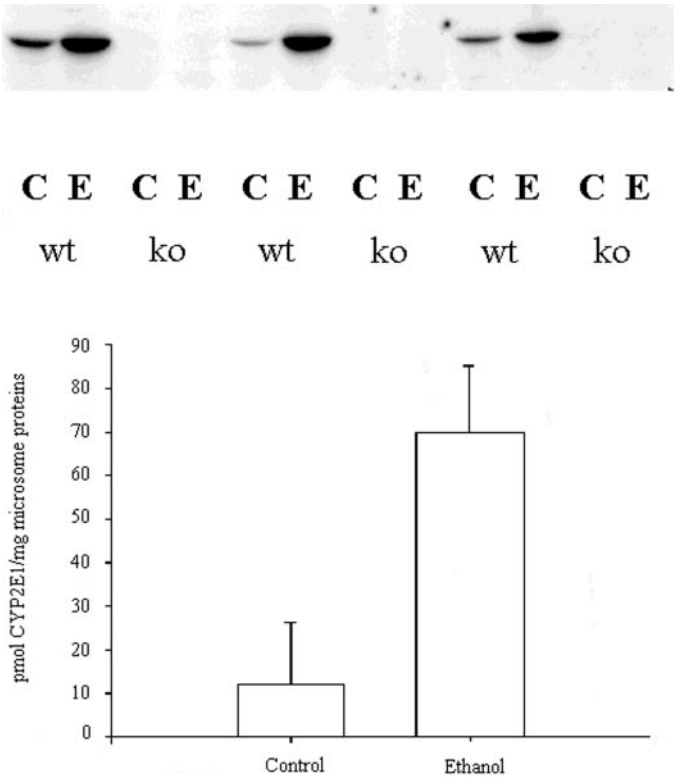

FIG. 3. Mice liver quantitation of CYP2E1: Western blot analysis was done using liver 9000*g* supernatants from fed dextrose (C) or ethanol (E) intragastrically wild-type (wt) or CYP2E1 knockout (KO) mice. Representative immunoreactive bands of CYP2E1 from three sets of different samples are shown on the upper part of the Fig. 3. A densitometric analysis was then performed. CYP2E1 was significantly increased 5.75-fold (P = 0.0015) in wild-type ethanol-fed mice.

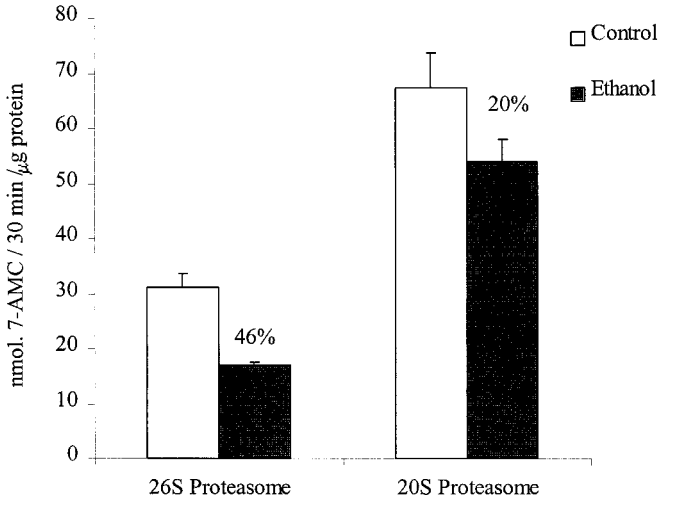

FIG. 5. ATP-stimulated (26S proteasome) and SDS–stimulated (20S proteasome) chymotrypsin-like activity in the liver cytosolic fraction of wild-type mice. The ethanol-related inhibition of proteasomes was significantly greater (P = 0.016) when ATP was added.

weight. Previous studies showed this phenomenon in rat liver after ethanol treatment for 2 months (1, 7). It was found that fat accumulated in the liver of wild-type and CYP2E1 knockout ethanol fed mice. Kono *et al.* (17) observed the same results. This indicates that the accumulation of fat was not causally related to ethanol inhibition of proteasome activity or vice versa.

The CYP2E1 increased fivefold in the wild-type mouse but not in the knockout mouse, after ethanol administration. CYP2E1 degradation by the ubiquitin-

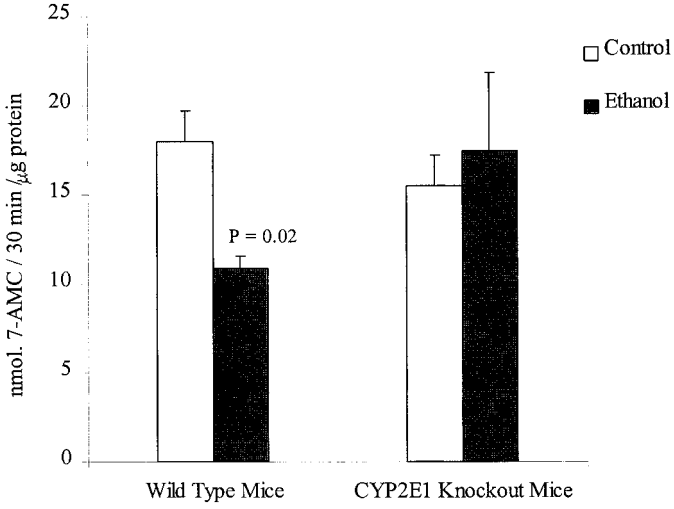

FIG. 4. Chymotrypsin-like activity in the liver cytosolic fraction of wild-type and CYP2E1 mice. Each determination was performed in duplicate. The chymotrypsin-like activity decreased significantly (P = 0.02) in the wild-type ethanol-treated mice.

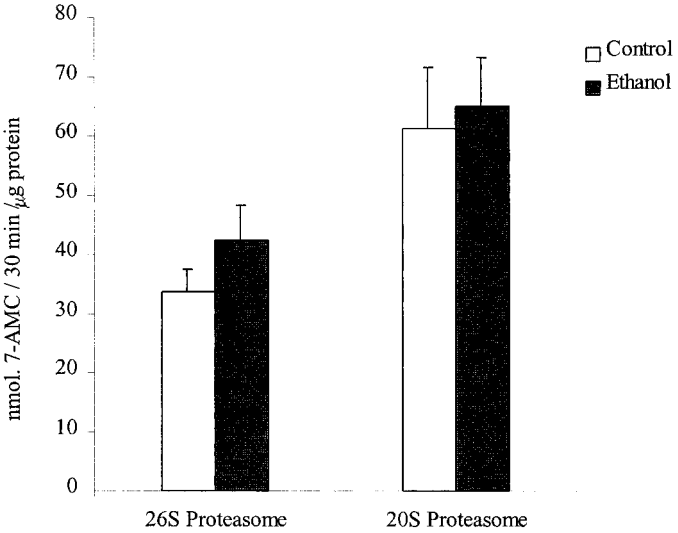

FIG. 6. ATP-stimulated (26S proteasome) and SDS–stimulated (20S proteasome) chymotrypsin-like activity in the liver cytosolic fraction of CYP2E1 knockout mice. There was no significant difference between the control and the ethanol fed mice.

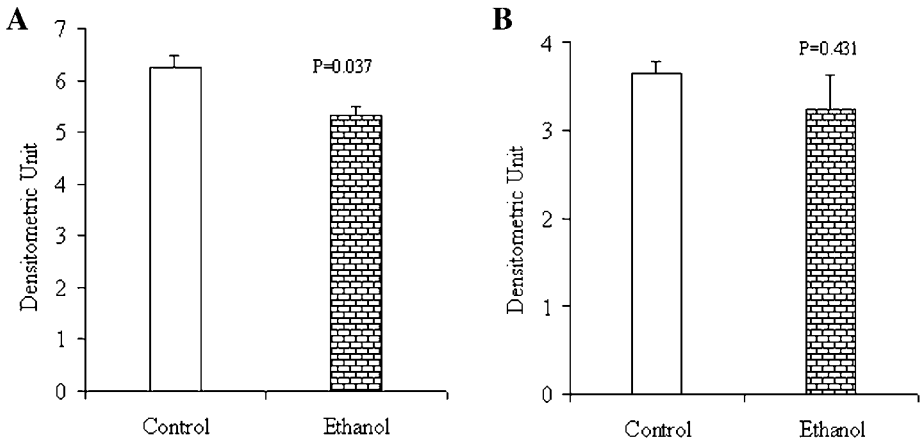

FIG. 7. Proteasome α-subunit C8 quantitation in the liver cytosolic fraction by p31 mAB. Western blot and densitometric analysis were performed in wild-type (A) and CYP2E1 knockout mice (B). The ethanol treatment reduced the proteasome subunit C8 staining in the wild-type mouse liver but the decrease was not significant in CYP2E1 knockout mouse liver.

proteasome pathway was inhibited by ethanol (18). This was due to the stabilization of CYP2E1 protein. This stabilization was associated with an increase in lipid peroxidation (19). The products of lipid peroxidation correlated with the degree of liver injury (8, 20, 21).

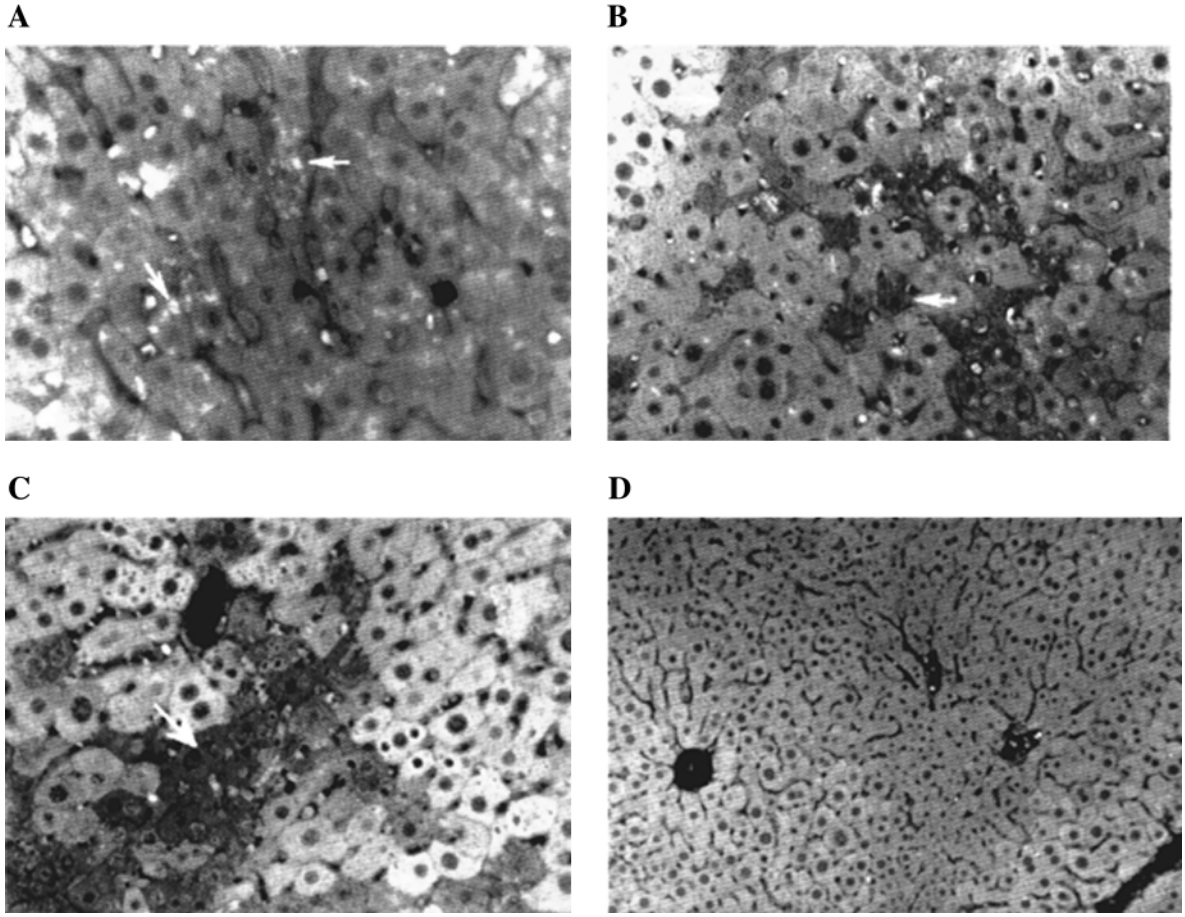

FIG. 8. Liver cells of wild-type and knockout mice fed ethanol, immunostained with p25 (A) and p31 (B–D) antibodies for the α-subunits of proteasome. (A) Aggregation of proteasomes (arrows). (B and C) Loss of proteasome staining in individual liver cells indicating the absence of proteasomes (arrows). (D) Control knockout. (Original magnification × 624 (A), × 312 (B), × 624 (C), and × 312 (D)).

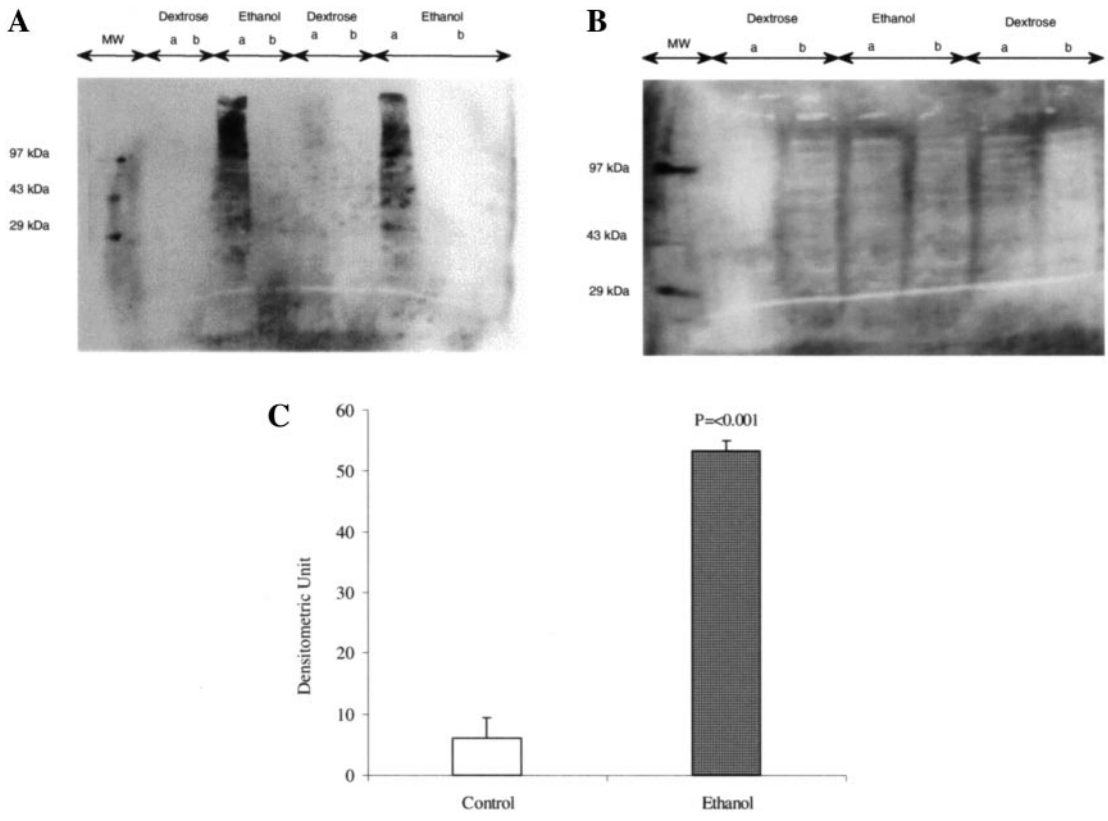

FIG. 9. Oxidized protein analysis in the liver cytosolic fraction. Immunodetection of carbonyl groups using Western blot analysis of sample from wild-type mice liver (A and C), and CYP2E1 knockout mice liver (B) was performed (DNP-derivatized sample (a), derivatization control (b)). The ethanol treatment increased the amount of oxidized proteins in the wild-type mouse liver but not in the CYP2E1 knockout mice liver.

This leads to the question: Does the oxidative damage caused by the ethanol-induced CYP2E1 affect the ATP-ubiquitin-proteasome pathway of protein removal in the liver cytosol?

To answer this question, CYP2E1 knockout mice were used to determine the role of CYP2E1 induction on the proteasomal peptidase activity. The results showed that the chymotrypsin-like activity decreased significantly in wild-type ethanol-treated mice. Thus, the mouse model was similar to rats fed ethanol (1, 10). On the other hand, CYP2E1 knockout mice did not show a decrease in the peptidase activities. Thus, the hypothesis that the ethanol-related stabilization of CYP2E1 is linked to the ethanol-induced inhibition of the chymotrypsin-like activity was supported. The proteasome peptidase activity which is responsible for the removal of oxidized protein (22) may itself undergo the oxidative damage (23). Therefore, it is possible that the ethanol-induced CYP2E1 caused oxidative damage to the proteasome which resulted in the loss of proteasomal peptidase activity and consequently the accumulation of the oxidized proteins. To further investigate this possibility, direct oxidative damage to individual subunits should be demonstrable.

The basal peptidase activity measured included both 20S and 26S proteasomes. To separate the activity derived from the two proteasomes, SDS was used (25) to measure the 20S proteasome activity and ATP was used to stimulate the 26S proteasome chymotrypsin-like activity (26). The results showed that both proteasomes had decreased chymotrypsin-like activity in wild-type ethanol-treated mice but the 26S proteasome was significantly more affected. The 26S proteasome is so labile and unstable that it is vulnerable to damage by oxidative stress (24, 27). The 20S proteasome which is responsible for the removal of most oxidized proteins was also decreased by ethanol but less so than 26S proteasome. However, in the knockout mice livers, there was no significant difference in the activity of both proteasomes when the control and the ethanol-treated mice data were compared. Therefore, it is possible that CYP2E1 was responsible for the oxidative damage to both the 20S and 26S proteasomes, which was the result of ethanol ingestion. However Kono *et al.* (18) have reported no difference in ethanol-related hepatotoxicity between the wild-type and CYP2E1 knockout mice. However, this observation may be due to

induction of other CYP families of isoenzymes by ethanol such as CYP3A and 4A (18).

The results suggest that the 20S proteasome is less vulnerable to damage due to CYP2E1 induced oxidative stress than is the 26S proteasome.

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