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EFFECT OF ALCOHOL ON EXPRESSION OF HEAT SHOCK PROTEIN 73 IN MONOLAYERS OF HEPATOCYTES FROM ALCOHOL-EXPOSED RATS

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Alcohol dramatically reduced loss of heat shock proteins (HSP73) and prevented morphological damage in monolayers of hepatocytes prepared from alcohol-fed rats. The monolayers were treated with 0, 5, 25, or 100 mM alcohol and triplicate samples were assayed at 24, 48, 72, and 96 hr after exposure. The content of HSP73 was measured by PAGE electrophoresis and Western blotting with a mouse monoclonal anti-HSP70 IgG antibody. HSP72 is not expressed under these conditions. Damage to the hepatocytes, quantified by leakage of lactate dehydrogenase (LDH), was also decreased by 100 mM alcohol. Although the initial 100 mM alcohol concentration decreased logarithmically to 1.7 mM over the first 24 hr, the effect of alcohol on HSP73 loss, LDH leakage, and morphological damage was most pronounced at 96 hr.

In the present study the ability of alcohol to alter the expression or degradation of heat shock proteins (HSP73) in monolayers of rat hepatocytes was assayed. Monolayers of hepatocytes isolated from alcohol-fed rats were treated with 0, 5, 25, or 100 mM alcohol and triplicate samples were assayed at 24, 48, 72, and 96 hr after exposure. Damage to the hepatocytes was quantified by leakage of lactate dehydrogenase (LDH) and was visualized by phase contrast photomicroscopy. The content of heat shock proteins was measured by PAGE electrophoresis and Western blotting. A mouse monoclonal anti-HSP70 IgG antibody, which recognizes both the constitutive (HSP73) and inducible (HSP72) forms, showed that alcohol decreases the rate of HSP73 loss.

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MATERIALS AND METHODS

Mouse monoclonal anti-HSP70 IgG1 (clone BRM-22) antibodies that bind both HSP72 and HSP73 and other chemicals were from Sigma (St. Louis MO). Horseradish peroxidase-conjugated goat-anti-rabbit IgG (H + L) was from BioRad (Richmond, CA)

The experimental protocol was approved by the Stanford panel for animal studies. Male Sprague-Dawley rats were maintained on a Purina rat chow diet for three days or until they weighed at least 200 gm. Nutritionally adequate diets formulated according to the method of Lieber and DeCarli (1,2) were purchased from Dyets Inc. (Bethlehem, PA). The alcohol diet consisted of 18% of total calories as protein, 35% as fat, 11% as carbohydrate, and 36% as alcohol. The rats were acclimated to a control liquid diet for 1 day and then the alcohol content was increased to 5% over a period of 4 days (3,4). The 5% alcohol diet was continued for 2 weeks.

The rats were anesthetized with 65 mg/kg sodium pentobarbital and hepatocytes were isolated by *in situ* perfusion of the livers with collagenase, as previously described (5-7). For the preparation of hepatocyte monolayers, 0.5 ml aliquots containing approximately 1.5 x 10^6 cells were added to collagen-coated 35 mm Lux Permanox Contur (Miles, Naperville, NY) culture dishes pre-incubated for 3 hr with 20% bovine calf serum. The hepatocyte monolayers were then washed twice after the 90 min attachment period. Cells at this point were approximately 95% viable.

These monolayers were separated into groups of three dishes each for exposure to 0, 5, 25, or 100 mM alcohol for up to 96 hr. Each monolayer was covered with 1 ml of alpha-MEM growth medium (alpha-MEM containing ascorbic acid, albumin, transferrin, sodium pyruvate, amino-levulinic acid, insulin, folic acid, and 10% bovine calf serum) containing the appropriate concentrations of alcohol. At 24, 48, 72 and 96 hr, triplicate plates from each alcohol concentration were sampled as follows: The supernatant of each plate was removed and assayed for leakage of lactate dehydrogenase (LDH). The kinetics of oxidation of NADH by pyruvate was measured at 340 nm on a 96 well plate reader (Molecular Devices, Menlo Park, CA). The values were measured in triplicate. The assays were duplicated with nearly identical results.

One of the hepatocyte monolayers from each set was viewed and photographed with a phase contrast microscope (Nikon Diaphot, Japan). The monolayers were then viewed under epifluorescence after adding 2 ml PBS containing 10 µg Hoechst 33342 and 10 µg propidium iodide. The Hoechst dye caused nuclei of viable cells to fluoresce blue whereas the propidium iodide caused nuclei of nonviable cells to fluoresce orange.

The remaining two monolayers from each set of triplicates were lysed by addition of 1 ml of 1% sodium dodecyl sulfate. The protein concentration of each lysate fraction was determined with BCA reagent (Pierce Chemical Co.) and an aliquot was mixed with a 4 fold concentrated solution of Laemmli's sampling buffer (8). The mixture was heated in boiling water bath for 3 min. To prepare the high resolution separations shown in Fig. 1, 50 µg of protein was loaded into each lane of a 14 X 14 cm 10% Tricine-SDS-PAGE gel prepared by a modification of the procedure of Schagger and Jagow (9). In this modified procedure, only a 4% acrylamide mixture was used for the stacking gel and a 10% acrylamide mixture was used for the separating gel. In addition, the sampling buffer described by Laemmli was used instead of that described by Schagger and Jagow. To prepare the lower resolution separations, either 30 µg (Fig. 2) or 10 µg (Fig. 5) of protein was loaded into each well of an 8 X 6 cm minigel prepared as described above. In each case, a set of prestained protein standards (BioRad, Richmond, CA) was run in an adjacent lane on each gel. After electrophoresis the 14 X 14 cm PAGE gels were cut to form two 7 X 1.5 cm pieces that divided the horizontal width and included the 50-90 kDa vertical range. The cutting of the gels allowed the proteins to be transferred to

nitrocellulose membranes by Western blotting in a Mini-BioRad apparatus (Richmond, CA) as described by the manufacturer. In the case of the 8 X 6 minigels, they were also cut into 7 X 1.5 cm pieces such that four gel sections could be transferred onto the same piece of nitrocellulose. The latter technique resulted in optimum reproducibility when comparing lane densities. After transfer, membranes were blocked with 5% BSA and 0.1% Tween 20 in PBS for 1 hr. Membranes were then incubated 1 hr with a 1:3500 dilution of mouse monoclonal anti-HSP-70 IgG_1 antibodies in PBS containing 0.1% Tween 20, 1% BSA and 0.1% sodium azide. The membranes were washed in PBS containing 0.1% Tween 20 and then incubated for 1 hr with a 1:5,000 dilution of peroxidase-conjugated goat-anti-mouse IgG antibodies in PBS containing 0.1% Tween 20 and 1% BSA. These membranes were then washed and incubated with an enhanced chemiluminescence substrate (ECL, Amersham) to provide an X-ray film record. The film was scanned with a PDI scanner (PDI, Huntington Station, NY) to produce digital images from which background was subtracted. These digital images were corrected for contrast and the intensity of each band was quantified digitally. The digital images were then transferred to Adobe Photoshop (Adobe, Sunnyvale, CA) where labels were applied and the images were formatted to be printed at 203 pixels/in in the Stanford Medical Illustrations facility.

Loss of alcohol from the culture dishes during 24 hr was determined. One ml of 100 mM alcohol in PBS, pH 7.2, was transferred to each of 25 tissue culture dishes (35 X 10 mm). These dishes were incubated at 37° C and assayed for alcohol (n = 5) at 2, 4, 6, 12, and 24 hr with an alcohol assay kit (332-A, Sigma, St. Louis, MO).

RESULTS

The positive control (PC) in Fig. 1 is from a monolayer of hepatocytes that was heat shocked at 42.5° C for 30 min, incubated at 37° C for 6 hr, lysed with 1% sodium dodecyl sulfate, and then probed for heat shock proteins with Western blotting technique. It exhibits both HSP72 and HSP73. It is seen that the use of a 14 X 14 cm 10% Tricine-SDS-PAGE gels prepared by a modification of the procedure of Schagger and Jagow (9) allowed sharp resolution of HSP72 from HSP73. Because of the good resolution, it was possible to use mouse monoclonal anti-HSP70 IgG1 antibodies that recognize both HSP72 and HSP73 (10). In contrast to the positive control, none of the control or alcohol-exposed hepatocytes (from 2 - 96 hr after exposure) expressed HSP72. Fig. 1 shows a representative control monolayer at 24 hr (C) and a monolayer exposed to 100 mM alcohol for 24 hr (E). Since the inducible HSP72 was not observed, all the later samples were run on 8 X 6 cm PAGE minigels under conditions where the HSP72 and HSP73 co-migrate.

The effect of 100 mM alcohol on the time course of loss of HSP73 in monolayer cultures is clearly seen in Fig. 2. Each of the bands in Fig. 2 was quantified digitally and the values are plotted in Fig. 3. The supernatants of each treatment group in Fig. 2 were analyzed for leakage of lactate dehydrogenase (LDH) (Fig.4). The values were analyzed by two-tailed Student's t-test. Differences were statistically significant at 72 and 96 hr.

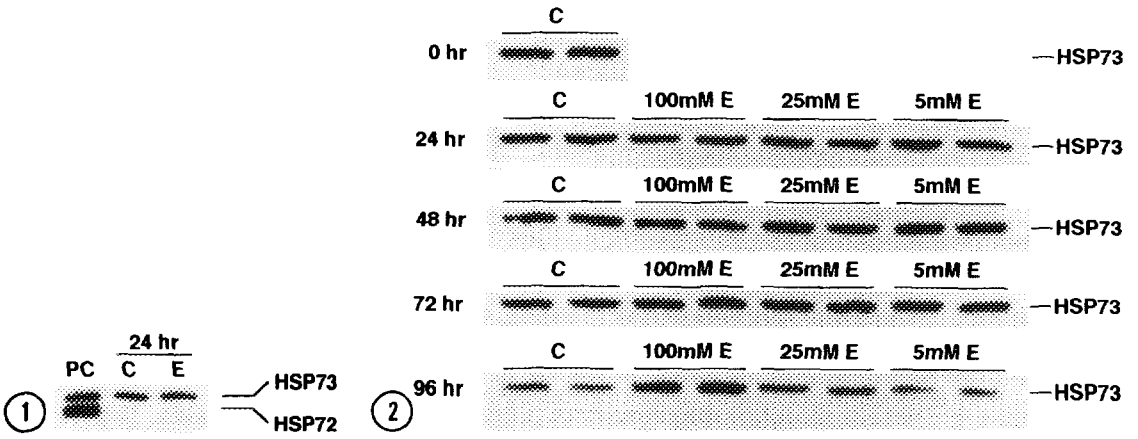


Figure 1. Western blot analysis of HSP72 induction. A positive control (PC) was prepared from a monolayer of hepatocytes that was heat shocked at 42.5° C for 30 min and incubated at 37° C for 6 hr. A control (C) was prepared from a monolayer incubated at 37° C for 24 hr. A treated sample (E) was prepared from a monolayer exposed to 100 mM alcohol for 24 hr. The monolayers were lysed with 1% sodium dodecyl sulfate. The lysates were separated on 14 X 14-cm 10% Tricine-SDS-PAGE gels and transferred to nitrocellulose membranes by Western blotting. The membranes were incubated with mouse monoclonal anti-HSP70 IgG1 antibodies and then with peroxidase-conjugated goat-anti-mouse IgG antibodies. Binding of the primary antibodies was visualized with enhanced chemiluminescence detection and converted into a digital image with a PDI scanner.

Figure 2. Western blot analysis of protection by alcohol from loss of HSP73. Rats were fed alcohol *in vivo*. Monolayers of hepatocytes were prepared and treated with either 0, 5, 25, or 100 mM alcohol for the indicated times. Lysates of the monolayers were separated on 8 X 6-cm 10% Tricine-SDS-PAGE minigels and transferred to nitrocellulose membranes by Western blotting. The membranes were incubated with mouse monoclonal anti-HSP70 IgG1 antibodies and then with peroxidase-conjugated goat-anti-mouse IgG antibodies. Binding of the primary antibodies was visualized with enhanced chemiluminescence detection and converted into a digital image with a PDI scanner.

In order to differentiate the protection of HSP73 by alcohol from a nonspecific blocking of protein degradation or increase in expression, two experiments were performed: (1) Duplicates of each PAGE gel in Fig. 2 were prepared and stained with Commassie Blue to visualize the protein bands (Fig. 5). It can be seen that the alcohol-induced changes in HSP73 in Fig. 2 are much more dramatic than the changes in the general spectrum of proteins in Fig. 5. The overall pattern of stained proteins changes somewhat with time and there is a small effect of alcohol at 96 hr. (2) Total protein in the lysates of the monolayers was measured with the BCA protein assay. There was a clear difference between the control monolayers and those treated with 100 mM alcohol (Fig. 6). The loss of protein in the monolayers with time is due to alterations in protein degradation

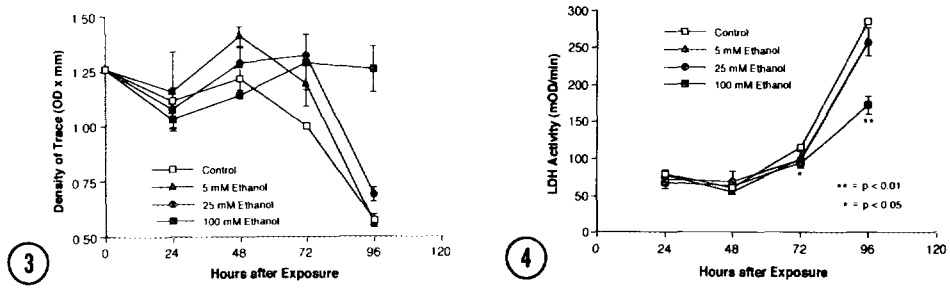


Figure 3. Integrated intensities of HSP73 bands. Each band in the enhanced chemiluminescence image in Fig. 2 was quantified digitally with a PDI scanner. The integrated band intensities are plotted versus time (n = 2, +/- range).

Figure 4. Leakage of lactate dehydrogenase (LDH) from damaged hepatocytes. The supernatant of each treatment group in Fig. 2 was analyzed for leakage of LDH at the indicated times (n = 3, +/- s.e.m.). The assays were duplicated with nearly identical results. Statistical significance was determined by two-tailed Student's t-test.

and synthesis within hepatocytes, as well as hepatocytes dying and floating off the monolayer. It is seen that 100 mM alcohol offers protection against the sum of these effects.

It was necessary to determine the rate of reduction of the original alcohol concentration in the culture dishes during the experiment. Alcohol would be lost by both

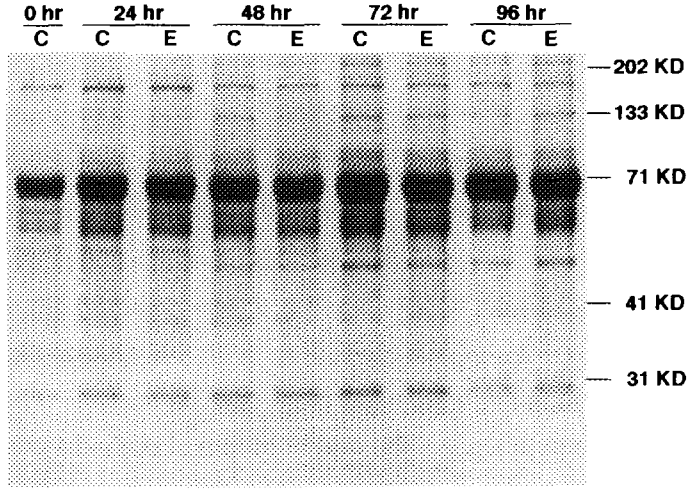
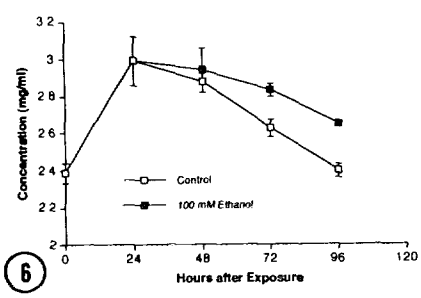


Figure 5. Changes in the spectrum of total proteins as a function of time and alcohol. Lysates of hepatocytes treated as control (C) or with 100 mM alcohol (E) were separated on an 8 X 6-cm 10% Tricine-SDS-PAGE minigel and stained with Coomassie Blue to visualize proteins. Pre-stained molecular weight markers run in an adjacent lane provided a calibration of molecular weights.



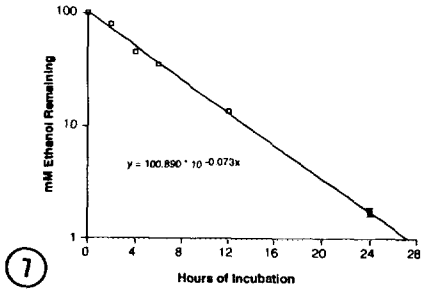


Figure 6. Total protein in the lysates of the monolayers. The supernatants and unattached cells were decanted from the monolayers. The monolayers were lysed with 1% sodium dodecyl sulfate and total protein was measured with the BCA protein assay. The value at 24 hr is the mean +/- s.e.m. of 8 samples. The other values are the mean and range of duplicate samples.

Figure 7. Loss of alcohol from the culture dishes during 24 hr. One ml of 100 mM alcohol in PBS, pH 7.2, was transferred to culture dishes that were incubated at 37° C and assayed for alcohol at 2, 4, 6, 12, and 24 hr. The evaporative loss of alcohol is plotted as a function of time and fitted with a logarithmic function. Each point is the mean +/- s.e.m. of 5 samples. Error bars are smaller than the symbols.

evaporation and metabolism by the hepatocytes. However, the metabolic rate for alcohol by monolayers of hepatocytes has been measured (11) and it amounts to a reduction of only 1.3 µmol (1.3 mM in 1 ml of buffer) per hr. Therefore, only evaporative loss was measured to avoid the complications of cells and serum on the assay. In Fig. 7 it can be seen that the concentration in the dishes containing 100 mM alcohol is reduced considerably by evaporation during 24 hr. Therefore, the dramatic effect of alcohol at 96 hr (Figs. 2-6) was triggered by changes produced in the hepatocytes during the first 24 hr.

The effects of alcohol on HSP73 (Figs. 2 and 3), LDH (Fig. 4), and total protein (Fig. 6) are consistent with the phase contrast photomicrographs (Fig. 8) that show that alcohol prevented morphological damage of monolayers after 96 hr of incubation. These photomicrographs are representative of monolayers observed in three repetitions of this entire experiment. The phase contrast results in Fig. 8 are also consistent with observations using epifluorescence of the same monolayers after they were stained with Hoechst dye and propidium iodide.

DISCUSSION

It is noteworthy that HSP72 is not expressed in hepatocyte monolayers under any of the conditions used in this study (Fig. 1). Alcohol has been shown to induce heat shock

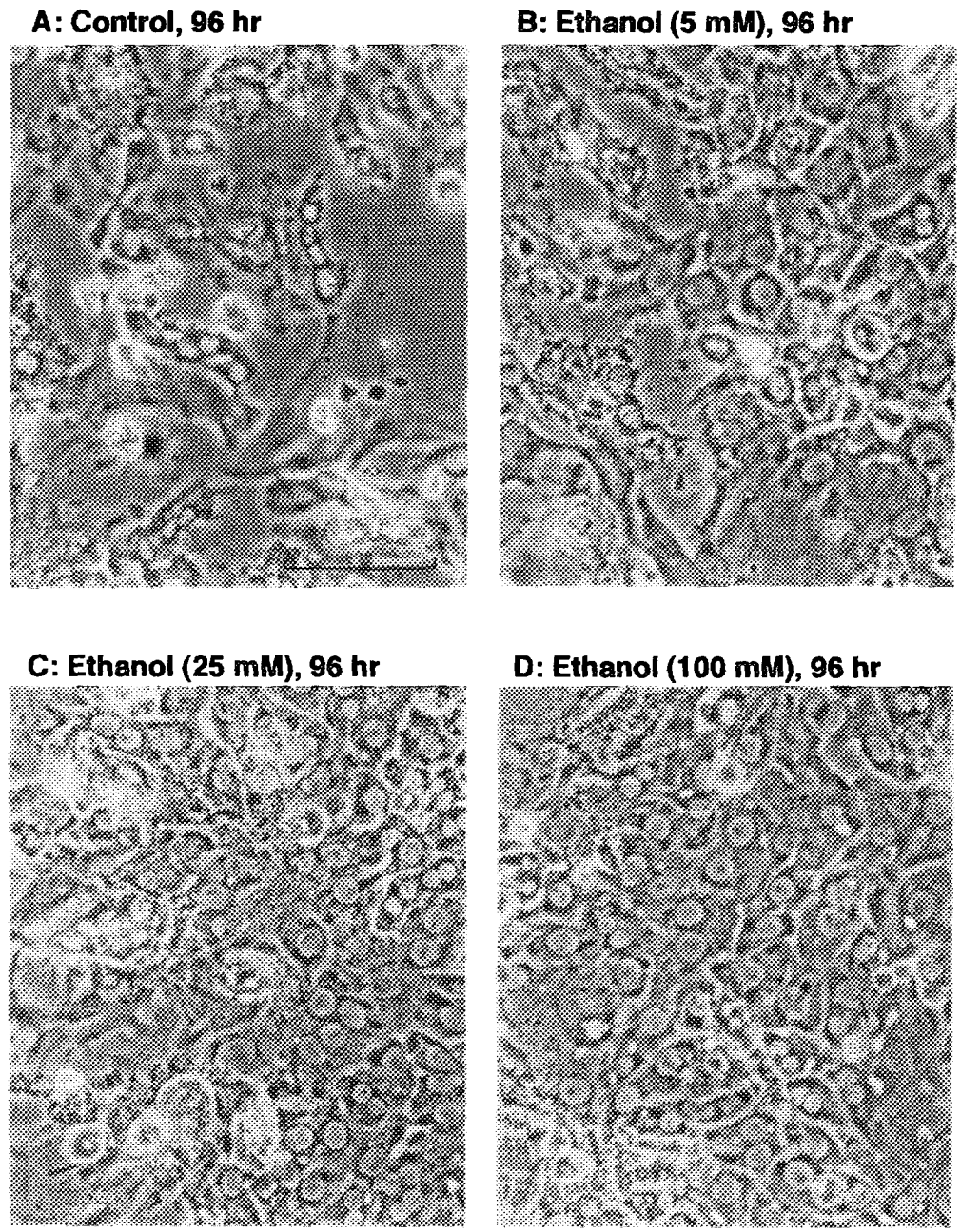


Figure 8. Phase contrast photomicrographs of monolayers at 96 hr. One monolayer of hepatocytes from each experimental group was photographed with phase contrast (X 20, the scale bar is 100 μm). These photomicrographs are representative of three separate replications of the entire experiment.

proteins in C6 rat glioma cells (12) and NG108-15 neuroblastoma cells (13). The combination of alcohol and nicotine can coinduce heat shock proteins in HA-1 Chinese hamster ovary cells (14). Heat shock proteins are also expressed *in vivo* during alcoholic

liver disease (15,16). Although we could find no references to alcohol-induced expression of HSP72 in monolayers of hepatocytes, these cells are capable of expressing HSP72 because we (unpublished observations) and others (17,18) have shown that heat shock (42° to 44° C) results in expression of HSP72 in hepatocytes within 2-72 hr.

The time-dependent protection from loss of HSP73 by alcohol at 24, 48, 72, and 96 hr is shown in Fig. 2. The integrated densities of each band in Fig. 2 are plotted as a function of time in Fig. 3. Fig. 5 shows the time-dependent change in the spectrum of total proteins in the lysate fractions in a PAGE gel identical to that in Fig. 2. Although there are discernable changes with time and alcohol in Fig. 5, the changes are not as dramatic as those in Figs. 2 and 3. In addition, Fig. 6 shows that at 96 hr after exposure, 20% of total protein was lost from the control monolayers compared with 11% in the presence of 100 mM alcohol. In contrast, Fig. 3 shows that in control hepatocytes 49% of HSP73 was lost at 96 hr after exposure whereas 100 mM alcohol completely prevented loss over the same time period. Therefore, reduced loss of total protein after alcohol exposure is only part of the reason for retention of HSP73 in these hepatocytes. It is especially interesting that, although the initial 100 mM alcohol concentration decreased logarithmically to 1.7 mM over the first 24 hr (Fig. 7), the effect of alcohol on HSP73 loss (Fig. 3) and LDH leakage (Fig. 4) was most pronounced at 96 hr. Both of the latter changes at 96 hr are consistent with the dose-dependent improvement in morphology of the hepatocytes seen in the photomicrographs in Fig. 8.

We have previously shown that *in vivo* exposure of rats to halothane in a hypoxic gas mixture causes nuclear translocation of HSP72 (19). In addition, the time course of the halothane-induced expression of HSP72 coincides with the progression of hepatocellular damage but not with the degree of formation of trifluoroacetylated adducts (20). Similarities in the metabolism of halothane and alcohol with respect to formation of free radicals and utilization of glutathione motivated the present study of the possible effect of alcohol on heat shock protein expression. In the *in vivo* studies HSP73 remained constant and expression of HSP72 was increased by halothane (19,20). In contrast, in this *in vitro* study loss of HSP73 was prevented by alcohol and HSP72 was not expressed. Whereas halothane is toxic, both *in vivo* (19) and *in vitro* (5), alcohol was clearly protective in the present study.

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REFERENCES

1. Lieber, C.S., DeCarli, L.M., and Sorrell, M.F. (1989) Hepatology 10, 501-510.
2. Lieber, C.S. and DeCarli, L.M. (1989) Alcohol and Alcoholism 24, 197-211.
3. Casey, C.A., Kragskow, S.L., Sorrell, M.F., and Tuma, D.J. (1991) Hepatology 13, 260-266.
4. Lin, R.C. and Lumeng, L. (1990) Adv. Exp. Med. Biol. 2283, 139-149.
5. Schieble, T.M., Costa, A.K., Heffel, D.F., and Trudell, J.R. (1988) Anesthesiology 68, 485-494.
6. Heffel, D.F., Costa, A.K., Schieble, T.M., and Trudell, J.R. (1989) Biochem. Arch. 5, 229-235.
7. Trudell, J.R., Ardies, C.M., Green, C.E., and Allen, K. (1991) Alcohol. Clin. Exp. Res. 15, 295-299.
8. Laemmli, U.K. (1970) Nature 227, 680-685.
9. Schagger, H. and von Jagow, G. (1987) Anal. Biochem. 166, 368-379.
10. Margulis, B.A., Nacharov, P.V., Tsvetkova, O.I., Welsh, M., and Kinev, A.V. (1991) Electrophoresis 12, 670-673.
11. Berry, M.N., Fanning, D.C., and Wallace, P.G. (1980) Adv. Exp. Med. Biol. 132, 403-411.
12. Neuhaus-Steinmetz, U., Xu, C., Fracella, F., Oberheitmann, B., Richter-Landsberg, C., and Rensing, L. (1994) Mol. Pharmacol. 45, 36-41.
13. Miles, M.F., Diaz, J.E., and DeGuzman, V. (1992) Biochim. Biophys. Acta 1138, 268-274.
14. Hahn, G.M., Shiu, E.C., and Auger, E.A. (1991) Mol. Cell Biol. 11, 6034-6040.
15. Omar, R., Pappolla, M., and Saran, B. (1990) Arch. Pathol. Lab. Med. 114, 589-592.
16. Koskinas, J., Winrow, V.R., Bird, G.L.A., Iau, J.Y.N., Portmann, B.C., Blake, D.R., Alexander, J.M., and Williams, R. (1993) Hepatology 17, 1047-1051.
17. Heydari, A.R., Wu, B., Takahashi, R., Strong, R., and Richardson, A. (1993) Mol. Cell Biol. 13, 2909-2918.
18. Wu, B., Gu, M.J., Heydari, A.R., and Richardson, A. (1993) J. Gerontol. 48, B50-B56.
19. Lin, W.Q., Van Dyke, R.A., Marsh, H.M., and Trudell, J.R. (1994) Biochem. Biophys. Res. Commun. 199, 647-652.
20. Lin, W.Q., Van Dyke, R.A., Marsh, H.M., and Trudell, J.R. (In Press) Mol. Pharmacol.