Heat-Shock Gene Expression in Alcoholic Liver Disease in the Rat Is Related to the Severity of Liver Injury and Lipid Peroxidation (43918)

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Abstract. **To evaluate the relationship between heat-shock gene expression and the severity of pathologic liver injury and lipid peroxidation in experimental alcoholic liver disease, we used the intragastric-feeding rat model. Six groups of male Wistar rats weighing between 225 and 250 g were fed liquid diets containing different dietary fats (saturated fat, corn oil, and fish oil) and ethanol or dextrose for 1 month. Pathologic injury, mRNA and protein levels of HSP70, microsomal conjugated dienes, and hydrogen peroxide were evaluated at sacrifice. Fish oil-ethanol fed rats developed the most severe injury, while the saturated fat-ethanol group showed no liver injury. The highest levels of HSP70 mRNA were seen in the fish oil-ethanol group. There was no difference in HSP70 protein levels between the groups. The levels of HSP70 mRNA correlated significantly with microsomal conjugated dienes ($r = 0.87$, $P < 0.01$) and hydrogen peroxide ($r = 0.90$, $P < 0.01$). Increased centrilobular HSP70 expression was seen in rats showing liver injury. The close relationship seen between oxidant stress and HSP70 mRNA but not protein suggest that the binding of HSP70 protein to other damaged proteins reduces free HSP70 protein leading to increased HSP70 expression. HSP70 expression may also be a cellular adaptive response to ethanol-induced oxidative stress.** [P.S.E.B.M. 1995, Vol 210]

The heat-shock proteins (HSPs) are a diverse family of proteins containing both constitutively expressed and stress-induced members (1–3). These stress proteins represent a large multigene family of intracellular proteins ranging in molecular size from 10 to 150 kDa. The most widely studied and abundant are the 70-kDa stress proteins (HSP70) family. HSC70 is constitutively expressed in unstressed cells, whereas HSP70 is induced in conditions of stress (1–3).

The induction of HSPs constitutes an important cellular defense mechanism against a variety of toxic and environmental insults (4). Results from several laboratories suggest that HSPs participate in the import and assembly of proteins and allowing these molecules to traverse biomembranes and promote proper folding (5–7). Most stress conditions, including alcohol administration, compromise the structural integrity of proteins (8). The binding of HSP70s to these damaged proteins stabilizes these proteins and provides the opportunity for either refolding or degradation of these proteins (5–7).

Several recognized stimuli of HSP expression are likely to be present in the alcoholic liver injury. These include increased cytokine expression (particularly tumor necrosis factor [TNF]) (9–11) and production of oxygen free radicals (12–14). HSP expression has been

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shown to be increased in rats (15) and in murine hepatocytes treated with ethanol (16). However, the degree of HSP expression was not related to liver pathology. In patients with alcoholic hepatitis, Koskinas *et al.* showed a close correlation between hepatic expression of HSP60 and the presence of an IgA-antiHSP60 in serum (17). However, we are not aware of any studies relating HSP70 expression to pathologic changes in alcohol-fed rats.

The present experiments were performed to analyze the response of HSP70 to ethanol feeding in different dietary models of alcoholic liver injury. Based on our previous epidemiologic studies, where we showed a relationship between the type of dietary fat and development of alcoholic liver disease (18, 19), we carried out studies in the intragastric feeding rat model for alcoholic liver disease (20). We showed that a diet rich in saturated fat was protective against alcohol-induced liver injury (21), whereas rats that were fed a diet rich in polyunsaturated fatty acids developed fatty liver, necrosis, inflammation, and fibrosis (22). Recently, we have shown that rats fed fish oil and ethanol develop severe liver injury (23). We have also demonstrated that the level of lipid peroxidation in the liver correlates with the severity of pathologic change in these different dietary models (ethanol with either saturated fat, corn oil, or fish oil) of alcoholic liver injury (24). In an attempt to relate HSP70 expression to lipid peroxidation and severity of pathologic changes, we studied the expression of HSP70 mRNA and protein in each of these different dietary ethanol-fed groups and their respective controls.

Materials and Methods

Animals. The experimental animals were male Wistar rats weighing between 225 and 250 g. Six groups of rats (three rats/group) were studied: saturated fat + dextrose (SD), saturated fat + ethanol (SE), corn oil + dextrose (CD), corn oil + ethanol (CE), fish oil + dextrose (FD), and fish oil + ethanol (FE). All animals sacrificed at 1 month and their livers examined for pathological changes, microsomal conjugated dienes and H_2O_2, and mRNA and protein levels for heat-shock protein (HSP) 70. All animals were pair-fed by continuous infusion of liquid diets through permanently implanted gastric tubes as described previously (20). The diet contained either saturated fat, corn oil, or fish oil as the source of fat. The percentage of calories derived from fat was 25% of total calories. The fatty acid composition of the diets is shown in Table I. Proteins, carbohydrates, minerals and vitamins were administered as described previously (25). The percentage composition of diets was such that the animals in all dietary groups consumed the same amount of calories, proteins, and vitamins. All control animals were pair-fed the same diet as ethanol-fed rats except that ethanol was isocalorically replaced by dextrose. All diets were prepared fresh daily. In particular, the fish oil diet was stored in air-tight containers, filled with nitrogen, in a cold room at 4°C. Malondialdehyde content of the diets was <5 nmol/g of sample, suggesting that very little autoxidation was taking place. The amount of ethanol was initially started at 8 g/kg/day and gradually increased as tolerance developed. Blood alcohol levels were maintained between 150 and 350 mg/dl. All animals were sacrificed at 1 month after the start of feeding. All animals received humane care in compliance with the National Institutes of Health criteria for care of laboratory animals.

Table I. Fatty Acid Composition (Percentage by Weight) of Saturated Fat, Corn Oil, and Fish (Menhaden) Oil Diets

Fatty acid	Saturated fat	Corn oil	Fish (menhaden) oil
Octanoic (C8:0)	52.5	0	0
Decanoic (C10:0)	33.2	0	0
Lauric (C12:0)	0.5	0	0
Myristic (C14:0)	0	0.1	11.6
Palmitic (C16:0)	1.4	11.3	13.0
Palmitoleic (C16:1 w7)	0	0	13.3
Stearic (C18:0)	1.0	1.9	2.1
Oleic (C18:1 w9)	1.0	25.5	6.7
C18:1 w7	0	0	3.3
Linoleic (C18:2 w6)	1.5	59.0	1.1
Arachidonic acid (C20:4 w6)	0	0	0.7
(C20:4 w3)	0	0	1.9
Eicosapentaenoic acid (C20:5 w3)	0	0	17.3
(C22:5 w6)	0	0	0.4
(C22:5 w3)	0	0	2.0
Docosahexenoic acid (C22:6 w3)	0	0	8.2

Note. Saturated fat: ICN Biochemicals Inc., Costa Mesa, CA. Corn oil: Mazola, Best Foods, CPC International, Engelwood Cliffs, NJ. Fish (menhaden oil): Zapata Haynie Corporation, Reedville, VA.

Histologic Analysis. A small sample of liver was obtained at sacrifice and formalin-fixed. Hematoxylin and eosin stain was used for light microscopy. The examination was carried out by a pathologist who had no prior knowledge of the treatment groups. The liver pathology was scored as follows (25): steatosis (the percentage of liver cells containing fat)—1+ = <25% of cells containing fat, 2+ = 26%–50%, 3+ = 51%–75%, 4+ = >75%; inflammation and necrosis—one focus/lobule = 1+, two or more foci/lobule = 2+.

RNA Isolation and Northern Blot Analysis. RNA was isolated from liver according to the procedure described by Chomczynski and Sacchi (26), and then fractionated on 1% agarose gel containing 3% formaldehyde, 0.02 *M* MOPS pH 7.4 (3-[N-mor-

pholino] propanesulfonic acid) and 0.001 *M* disodium EDTA (ethylenediaminetetraacetic acid). HSP 70 mRNA was detected using a 2.3-kb insert from the human HSP 70 genomic clone pH 2.3, containing 58 bp of 5′ leader sequence, 1986 bp of continuous open reading frame, the termination codon and 274 bp of 3′ untranslated sequence containing the poly (A) signal sequence (27). The RNA was visualized by ethidium bromide staining and then transferred to nitrocellulose overnight with 10 × SSC (standard saline citrate, pH 7.0). Hybridization with labeled probes (^{32}Pd α ATP) was done for 24 hr at 42°C in 50% formamide, 2 × SSC (1 × SSC—150 m*M* NaCl, 15 m*M* sodium citrate, pH 7.0) 5× Denhardts, 0.1% sodium dodecyl sulfate (SDS); 10% dextran sulfate (Sigma Chemical Co., St. Louis, MO). The HSP mRNA was semiquantitated by autoradiography. The autoradiograms were scanned using laser densitometry (GELSCAN DU 600; Beckman Instruments, Brea, CA). Rat α-tubulin oligonucleotide ON-50 (28) was employed as a control mRNA probe to verify RNA load consistency and RNA integrity. The tubulin mRNA is generally unresponsive to the treatments used in this study (29). The results were expressed as a multiple of the mean value seen in the saturated fat plus dextrose (SD) group.

Protein Extraction and Western Blotting. Frozen liver specimens were homogenized in TS solution (10 m*M* Tris, pH 7.5, 250 m*M* sucrose and 0.1% phenolmethysulfanil fluoride). Two hundred micrograms of protein were subjected to electrophoresis on 10% SDS-PAGE and blotted to a nitrocellulose filter. Filters were incubated at 37°C for 1 hr in blocking buffer (3% BSA in PBS) and then incubated with a monoclonal antibody (StressGen Biotechnologies Corp., Sidney, BC, Canada) that recognizes HSP 72/73. After three 15-min washes in Tween buffer, the filters were incubated with a 1:1000 dilution of peroxidase conjugated rabbit IgG against mouse IgG (Dakapats, Carpinteria, CA). Streptavidin biotinylated horseradish peroxidase complex and DAB were used for color development according to the manufacturer's instructions. In addition to the gel where proteins were transferred to nitrocellulose for Western blotting, another identical gel was stained with Coomassie Brilliant blue (R250, BDH Chemicals) to visualize protein. Although protein concentrations were equalized between samples on the basis of protein measurements, the intervening boiling and centrifugation steps necessitated rechecking by direct Coomassie visualization. The blots were scanned using laser densitometry.

Detection of HSP-70 in Liver Tissue. Five-micrometer sections of formalin-fixed, paraffin embedded tissue were used for immunohistochemical staining. They were dewaxed in xylene, rehydrated in graded alcohol, and covered with 10% normal serum. The anti-HSP70 antibody was used at a 1:50 dilution with overnight incubation at 4°C. All staining steps were conducted according to the manufacturer's instructions (Vector Labs, Burlingame, CA). Addition of the secondary antibody (anti-mouse IgG) was followed by addition of the avidin-peroxidase complex, chromogenic substrate (DAB), counterstaining with methyl green and mounting. Negative controls, incubated with nonimmune mouse serum instead of primary antibody, were included.

Measurement of Microsomal Conjugated Dienes and Hydrogen Peroxide. Microsomes were prepared from the liver as previously described (29). Lipid was extracted according to the method of Bligh and Dyer (30), and conjugated dienes measured by the method of Recknagel and Glende (31). Hydrogen peroxide was determined by measuring the formation of formaldehyde from methanol (32). Reactions were carried out in 200 m*M* KCl, 50 mm TRIS-HCl (pH 7.4), 100 m*M* methanol, 300 units of catalase, and 0.5 mg of microsomal protein in a final volume of 100 μl. Reactions were initiated by addition of NADPH (1.2 m*M*) and terminated by the addition of 30 μl of 20% trichloracetic acid. The generation of formaldehyde was assayed by the Nash reaction (33).

Statistics. All results are expressed as mean ± SD except where indicated. Comparison between groups was done using analysis of variance. Pearson's correlation coefficient (*r*) was used to evaluated associations.

Results

In each of the groups studied, the rats increased their body weight at a constant rate. Although the average weight gain was lower in the ethanol-fed rats than in the respective dextrose-fed controls, the differences between the various groups were not significant. The blood alcohol levels (mg/dl, mean ± SD) in the ethanol-fed group were: SE 226 ± 29, CE 236 ± 38, FE 223 ± 40. The differences between the groups were not significant. The pathologic changes in the different groups are shown in Table II. As previously observed, rats fed saturated fat and ethanol did not develop pathologic changes; rats fed fish oil and ethanol showed the most severe pathologic changes. Rats fed corn oil and ethanol had changes intermediate between those seen in the other two groups (SE and FE). The changes in HSP70 mRNA in the various groups are shown in Figure 1. The highest level of HSP70 mRNA was seen in the FE group (Fig. 2), which was also the group showing the most severe liver injury. The lowest level of HSP70 mRNA among the ethanol-fed groups was seen in the SE group (no liver injury). Also of interest is that the induction of HSP70 mRNA was also dependent on the degree of saturation of dietary fatty acids. In the control dextrose-fed groups, the highest

Table II. Pathological Changes in the Different Treatment Groups

Treatment group	Animal no.	Pathological changes		
		Fatty liver	Necrosis	Inflammation
Corn oil + ethanol	1	3	1	0
	2	2	1	0
	3	2	1	1
Fish oil + ethanol	1	4	1	2
	2	4	2	2
	3	3	2	2

Note. None of the dextrose-fed controls or animals fed saturated fat and ethanol developed pathological changes. See Materials and Methods for details on method of scoring pathologic changes.

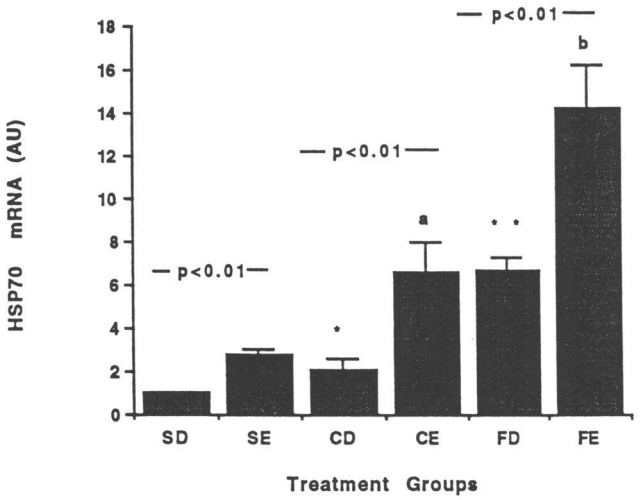

Figure 1. HSP70 mRNA levels in (mean ± SD) in the various experimental groups (n = 3/group). The level in each dietary group is related to an assigned value of one in the saturated fat plus dextrose (SD) group. Ethanol significantly increased the HSP mRNA in the saturated fat group (2.8 ± 0.28, $P < 0.01$). Ethanol also increased HSP70 mRNA in the corn oil (CE = 6.6 ± 1.3, CD = 2.1 ± 0.5, $P < 0.01$) and fish oil group (FE = 14.3 ± 2.0, FD = 6.7 ± 0.6, $P < 0.01$). *$P < 0.01$ compared with SD; **$P < 0.01$ compared with CD and SD; $^{a}P < 0.01$ compared with SE; $^{b}P < 0.01$ compared with CE; AU, arbitrary units.

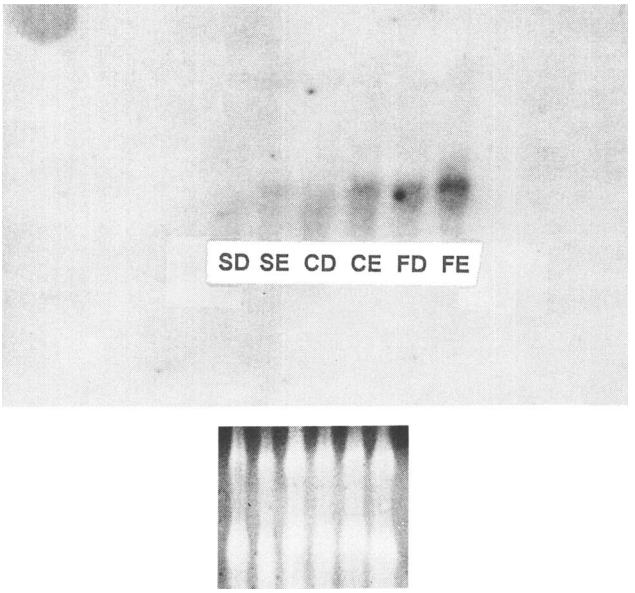

Figure 2. Northern blot analysis of hepatic HSP70 mRNA. Total RNA was extracted from liver samples in rats in the individual groups. Northern blots were probed using a 2.3-kb insert from the human HSP70 genomic clone pH 2.3, containing 58 bp of 5′ leader sequence, 1986 bp of continuous open reading frame, the termination codon and 274 bp of 3′ untranslated sequence containing the poly (A) signal sequence (see Materials and Methods). Uniformity of lane-to-lane loading was confirmed by inspection of the ethidium bromide stain gel (Fig. 2b) and normalized to the tubulin signal. SD, saturated fat and dextrose; SE, saturated fat and ethanol; CD, corn oil and dextrose; CE, corn oil and ethanol; FD, fish oil and dextrose; FE, fish oil and ethanol.

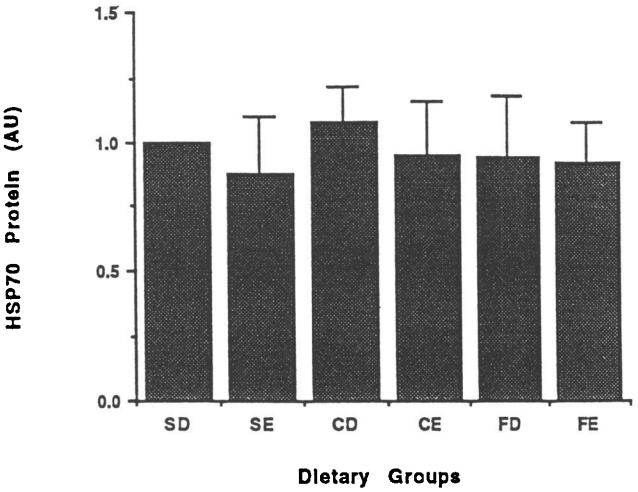

Figure 3. Comparison of HSP70 protein levels (arbitrary densitometric units) in the various experimental groups. Clarified liver homogenates were subjected to SDS-polyacrylamide gel electrophoresis and probed with a monoclonal antibody specific for HSP70. The HSP70 protein levels were normalized to a value of 1.0 for the SD group. Mean ± SD for HSP protein levels in the groups are: SE, 0.88 ± 0.22; CD, 1.08 ± 0.14; CE, 0.95 ± 0.21; FD, 0.94 ± 0.24; FE, 0.92 ± 0.16. There was no significant difference between the various groups.

level of HSP70 mRNA was seen in the fish oil-dextrose (FD) group. Although the presence of more than one HSP70 transcript has been observed in other organs, in the liver only the 2.3-kb transcript is seen (34).

There was no difference in inducible HSP70 protein levels between the various groups (Fig. 3). Liver sections from both dextrose and ethanol-fed rats showed diffuse cytoplasmic staining with HSP70 antibody. Occasional hepatocytes showed dense paranuclear staining. There was some nuclear staining in the ethanol-fed rats showing pathologic liver injury (CE and FE) with clusters of strongly positive hepatocytes seen in the perivenular zone (Fig. 4). Hepatocytes with fatty degeneration demonstrated a submembranous pattern of staining (Fig. 5).

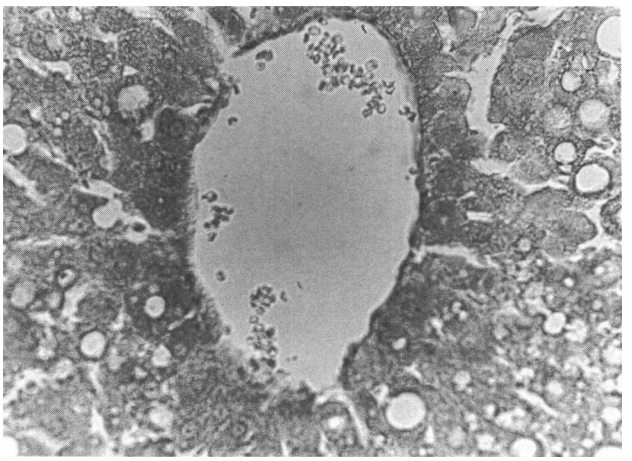

Figure 4. Section from liver of rat fed fish oil and ethanol for 1 month immunostained with anti-HSP70 antibody. Centrilobular hepatocytes that rim the central vein show positive staining for HSP70. Note also the positive staining of nuclei of cells lining the central vein as well as in the hepatocytes. No centrilobular immunoreactivity was seen in the centrilobular areas in the dextrose fed rats. Also no staining was seen in liver tissue where the primary antibody to HSP70 was left out (photographs not shown).

Since HSP expression is believed to be related to oxidant stress, we correlated the levels of HSP70 mRNA in the various groups with the degree of lipid peroxidation (A_{232}) and microsomal hydrogen peroxide. Significant correlations were obtained between HSP70 mRNA levels and A_{232} ($r = 0.87$, $P < 0.01$) (Fig. 6) and H_2O_2 ($r = 0.90$, $P < 0.01$) (Fig. 7).

Discussion

The results of our study indicate that increased hepatic HSP70 mRNA accumulation in the intragastric feeding rat model was associated with pathologic liver injury and that the increase in mRNA levels was not

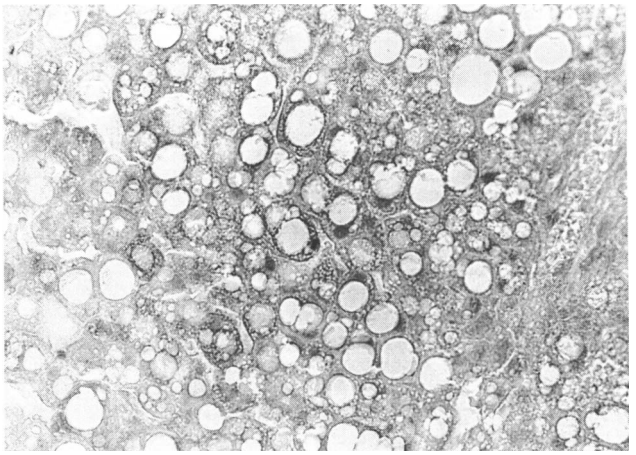

Figure 5. Section from liver of rat fed fish oil and ethanol for 1 month immunostained with anti-HSP70. Note the submembranous pattern of staining in cells showing fatty degeneration. Some hepatic nuclei also show positive staining. The staining of hepatic nuclei likely reflects the fact that with stress, HSP translocates to the nucleus where it associates with nucleoli (59, 60).

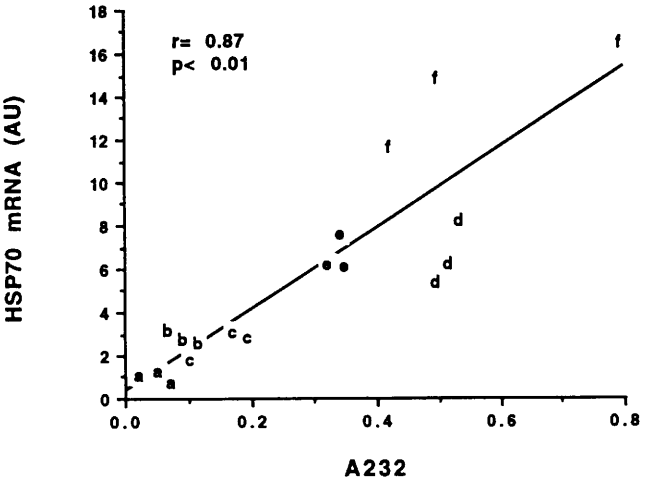

Figure 6. Correlation ($r = 0.87$, $P < 0.01$) between microsomal conjugated diene concentrations (A_{232}) and HSP70 mRNA levels (arbitrary units). A total of 18 samples had both measurements performed. a, saturated fat and dextrose; b, saturated fat and ethanol; c, corn oil and dextrose; d, corn oil and ethanol; e, fish oil and dextrose; f, fish oil and ethanol.

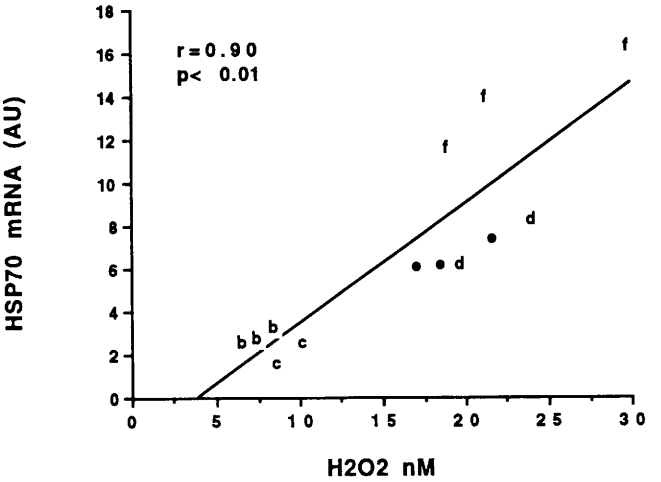

Figure 7. Correlation ($r = 0.90$, $P < 0.01$) between microsomal hydrogen peroxide (H_2O_2, nmol/mg protein) and HSP70 mRNA levels (arbitrary units). A total of 13 samples had both measurements performed. The letter symbols are same as for Figure 6.

coordinated with an increase in HSP70 protein. An increase in HSP70 mRNA was also seen in the fish oil-dextrose group in which no liver injury was obvious. However, it is recognized that feeding fish oil and dextrose for 2–3 months causes hepatic fibrosis (35). In addition, significant correlations between HSP mRNA and liver microsomal conjugated dienes and hydrogen peroxide suggest an inter-relationship between HSP induction and lipid peroxidation. We, however, caution that the correlation between HSP70 expression and markers of lipid peroxidation does not, of course, define HSP induction as a consequence of lipid peroxidation. However, some studies have shown that the superoxide radical is a possible mediator of HSP70 expression (36).

In eukaryotic cells, the heat-shock response in-

volves transcriptional activation mediated by a transcriptional activation factor called heat-shock factor (HSF). In response to physiologic and metabolic stress, activation and binding of HSF to DNA recognition sequences (heat-shock elements) result in the heat-shock transcriptional response (36, 37). Increased concentrations of misfolded and aggregated proteins, produced by a variety of stresses and toxins, sequester HSP70 and result in a reduction of "free" HSP70. This reduction in free HSPs consequently results in an increase in expression of new HSP70 mRNA and protein. It has been shown that the severity of physiologic stress determines the extent of new HSP70 synthesis (38). Our results, which showed a correlation between HSP70 mRNA levels and conjugated dienes and hydrogen peroxide, are consistent with these observations.

Ethanol causes damage to hepatocyte proteins by a variety of mechanisms. Acetaldehyde generated from ethanol in the liver forms covalent links with proteins (39). Israel *et al.* have proposed that these acetaldehyde adducts may serve as neoantigens and provoke an immune response (40). Binding of acetaldehyde to proteins (such as tubulin) also impairs their polymerization (41). Reactive oxygen metabolites may cause formation of malondialdehyde and 4-hydroxynonenal protein adducts in ethanol-fed rats (42). These protein adducts may participate in the pathogenesis of alcoholic liver injury. Ethanol also disorders protein trafficking in the liver; both protein secretory and plasma membrane assembly pathways have been shown to be impaired in the livers of ethanol-fed animals (43).

The cytoprotective effect of heat shock proteins appears to be mediated through several activities including the dissociation of protein aggregates, folding of newly synthesized polypeptides and translocation of proteins across membranes (36, 37). Binding of HSP70s to damaged proteins in livers of ethanol-fed rats provides the opportunity for re-folding or degradation of these proteins. The absence of any differences in HSP70 protein between the various groups in our study suggests that once HSP70 is bound to the damaged protein, these proteins are probably degraded in the liver cells. The depleted pool of "free" HSP70 allows for new HSP70 synthesis, which then binds to additional proteins (44). In this setting, the increase in HSP mRNA would be expected to correlate with the amount of protein damage, which in turn is probably a result of enhanced oxidative stress. The strong correlation between HSP mRNA and markers of oxidative stress (conjugated dienes and H_2O_2) supports the above hypothesis.

An additional factor important in enhancing the release of HSP70 from newly synthesized protein is hydrolysis of ATP. All HSP70 proteins examined bind ATP with high affinity. The affinity of HSP70 for denatured proteins is increased by ADP and decreased by ATP (45). A low ATP:ADP ratio increases the HSP70-protein binding affinity and blocks the release of HSP70 from newly-synthesized protein. Low ATP concentrations (and a high ADP:ATP ratio) have been described in ethanol-fed rats (46), including the intragastric feeding model (47). Thus the low ATP concentration would, in addition to generation of misfolded proteins, also lead to decreased "free" HSP and further stimulation of HSP70 mRNA synthesis.

Other factors likely to be present in ethanol-fed rats that are also stimuli for enhanced HSP expression include: cytokines (especially TNF), altered mitochondrial electron transport, impaired oxidative phosphorylation, and hypoxia (48–50). The selective localization of HSP to the centrilobular area in rats developing liver injury may be related to the occurrence of hypoxia secondary to alcohol administration (50). This is supported by the *in vivo* observation that liver slices rendered hypoxic show increased HSP expression (51). The centrilobular expression of HSP70 may also occur as a result of formation of acetaldehyde adducts which are also localized in the perivenular areas in ethanol-fed rats (52, 53).

The heat-shock response is also believed to be a cellular adaptive response to stress (54). The mechanism(s) by which HSPs protect cells from damage is unclear, but the fact that numerous stresses induce the HSP response suggests that a common signal pathway exists. Induction of HSP70 renders the cells resistance to the cytotoxic effects of TNF (55). Jaattela showed that HSP70 inhibits TNF-induced activation of phospholipase A_2 without interfering with receptor binding of signal transduction (55). Our finding that the induction of heat-shock mRNA is related to the severity of oxidant stress and pathologic injury is consistent with the observations of other investigators who show that the extent of new HSP70 mRNA synthesis is determined by the relative severity of the physiologic stress (37). Activation of HSF but not an increase in HSP70 protein has been described with the HSP response to reactive oxygen intermediates (56). This observation may also partly explain the failure to see increased HSP70 protein in the present study. An *in vitro* study using high ethanol concentrations did not detect increased HSP72 expression in rat hepatocytes (57).

Another observation of note in our study is the relationship between HSP mRNA levels and dietary unsaturated fatty acids in the dextrose-fed controls. Saturation of fatty acids is an important regulator of many genes. Jurivich *et al.* (58) have recently shown that arachidonate is an inducer of the heat-shock response and these investigators suggested that effectors of inflammation such as arachidonic acid metabolites may activate the heat-shock response. The association

between the highest HSP70 mRNA levels and severe pathologic changes in the fish oil-ethanol fed rats is consistent with this hypothesis.

In conclusion, we have demonstrated a close correlation between the degree of oxidant stress and HSP70 mRNA levels in ethanol-fed rats. We propose that cell injury results in protein damage which causes HSP70 to bind to these proteins. In our study, it appears that the endogenous pool of HSP70 available for accomplishing these cytoprotective functions was inadequate, leading to an increase in HSP70 expression. This increased HSP70 expression satisfies the demand for more HSP70 chaperones. In addition, ATP depletion, which is commonly seen with alcohol administration, may further exacerbate the decline in "free" HSP by increasing the affinity of HSPs for denatured protein.

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