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Alcohol alters skeletal muscle heat shock protein gene expression in rats: these effects are moderated by sex, raised endogenous acetaldehyde, and starvation

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Abstract

Alcoholic myopathy is a common pathology characterized by wasting due to reduced protein synthesis, although the mechanisms involved remain unclear. Women are particularly sensitive and malnutrition exacerbates the myopathy. This study aimed to address (i) whether long-term alcohol feeding alters expression of heat shock proteins (HSPs) in male and female rats; (ii) the effect of immediate alcohol dosing with or without raised levels of endogenous acetaldehyde; and (iii) the effect of starvation. To address this, (i) male and female rats were fed alcohol in the long-term (6-7 weeks as 35% of energy in a liquid diet) and compared to controls fed the same diet with isoenergetic glucose; (ii) male rats given an immediate bolus (75 mmol ethanol per kilogram body weight intraperitoneally) 2.5 hours before sacrifice and compared to controls given a dose of saline (with or without pretreatment with cyanamide—an acetaldehyde dehydrogenase inhibitor which raises endogenous acetaldehyde); (iii) male rats starved for 1 or 2 days then immediately dosed with alcohol. Protein levels of HSP 27, HSP 60, and HSP 70 were measured in muscles of male rats fed alcohol and pair-fed control rats by SDS-PAGE and Western blotting in study I. Levels of HSP 27, HSP 60, HSP 70, and HSP 90 mRNA were analyzed in hind limb skeletal muscle by reverse transcription–polymerase chain reaction with an endogenous internal standard, glyceraldehyde-3-phosphate-dehydrogenase. (i) Long-term alcohol dosage reduced HSP 27 in male rats but not in females, whereas HSP 90 mRNA increased in long-term alcohol-fed female rats but not in male rats. These changes were reflected by a similar trend in HSP protein content, although statistical significance was not achieved. (ii) There was no effect on any of the HSP mRNAs in rats dosed immediately with alcohol or in combination with cyanamide. (iii) Starvation per se for 2 days was associated with an increase in HSP 27 mRNA. Alcohol administration after 2 days starvation caused a blunting of the increased HSP 27 mRNA in starvation alone. This suggests that long-term alcohol exposure affects HSP gene expression and that this effect is moderated by sex and starvation. This may contribute to, or reflect, the biochemical lesion in alcoholic myopathy.

1. Introduction

Alcoholic myopathy is a common pathology affecting 40% to 60% of all long-term alcohol misusers [1,2]. The chronic form of alcoholic myopathy is characterized by muscle weakness and loss of protein content, including contractile proteins such as myosin heavy chain (isoforms IIx and IIb) [3]. This is mediated by reduced protein synthesis and an induction of the proto-oncogene c-myc [1,4]. Histologically, the myocytes are unchanged in number but are atrophied [2]. The acute form is far more rare and presents with a picture of rhabdomyolysis [5]. It has been noted that women are more susceptible to alcoholic myopathy than men, with greater biochemical perturbations noted in animal models despite equal doses of alcohol per

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unit body weight [6]. The myopathic changes in muscle can occur independently of overt malnutrition, but nutritional deficiencies exacerbate the biochemical changes in muscles exposed to ethanol especially in situations of acute nutritional deprivation [7]. Although the causative agent is known, the precise sequence of steps between alcohol exposure and the manifestations of this disease are unknown. Nevertheless, suitable in vivo animal models, which display all the characteristics of its clinical counterpart, have facilitated studies into this area [8].

Recent investigation has concentrated on alcohol causing direct damage to tissues through oxidative stress. Damage to myocyte cell membranes causes increased cholesterol hydroperoxides and oxysterols, and aldehyde-derived protein adducts [9-11]. Heat shock proteins (HSPs) are involved in tissue response to a multitude of stimuli, from hormones and mitogens to cellular stresses such as heat, ultraviolet radiation, chemotherapy, and reactive oxygen species [12,13]. They act largely as molecular chaperones in post-translation processing of proteins in muscle as well as refolding and renaturing misfolded proteins [12,14,15]. They are also involved in various aspects of protein maturation and translocation to different organelles, as well as binding to nucleotides and aiding in RNA and DNA synthesis [12]. Heat shock proteins also have a key role in the control of apoptosis [13]. Pathogenic changes in these HSPs may also be involved in the etiology of skeletal muscle pathologies including aging and oxidative stress, and impaired expression may impair the ability of tissues to tolerate metabolic stresses [16,17]. However, the role of altered HSP mRNA in alcohol-exposed muscle is unknown and it is possible that down-regulation may be a contributory factor in the development of alcohol-induced muscle disease.

To resolve some of these issues and address the pathophysiological factors that seem to govern the etiology of alcohol-induced muscle disease, we measured HSP 27, 60, 70, and 90 mRNA levels in muscle in various experimental conditions in both long-term and immediate studies. We (i) investigated the response of male and female rats to long-term alcohol exposure. In immediate studies, we (ii) investigated the effects of alcohol with or without cyanamide (an inhibitor of acetaldehyde dehydrogenase) predosing to raise endogenous acetaldehyde and (iii) determined whether starvation exacerbated the immediate effects of immediate ethanol dosage on HSP mRNA levels. The latter study also allowed us an opportunity to investigate the extent to which starvation per se altered HSP mRNA expression in mammalian muscle.

2. Methods

Male or female Wistar rats were obtained from accredited commercial suppliers at about 60 g body weight. Three different groups of rats were obtained for the 3 separate studies. Rats were maintained and studied according to Home Office Guidelines and a specific Project License.

Rats were maintained in a temperature- and humidity-controlled animal house for approximately 1 week until they weighed approximately 0.1 to 0.15 kg. Each batch of rats was then ranked on the basis of weight and then divided into the appropriate number of groups of equal mean body weight in each experiment. There were 7 to 10 rats in each group. At the end of each study, the rats were killed by decapitation and representative skeletal muscle (hind limb musculature) dissected on ice. These were frozen immediately in liquid nitrogen and stored at −70°C until analysis. The studies were as follows.

2.1. Comparison of the effect of long-term alcohol exposure for 6 to 7 weeks in male and female rats

Rats were divided into the following groups: (1) control male (n = 9), (2) control female (n = 8), (3) ethanol male (n = 10), (4) ethanol female (n = 7). Rats were subjected to a Lieber-DeCarli alcohol-feeding regimen in which treated rats were fed a nutritionally complete liquid diet containing 35% of total energy as ethanol ad libitum [3,4,8]. Controls were pair-fed the same diet in which ethanol was replaced by isoenergetic glucose. There was no restriction on the amount of alcohol consumed by these rats. The liquid diets were prepared daily according to the recipe described in Table 1. The control and alcohol-containing diets were isolipidic, isonitrogenous, and isoenergetic (Table 1). The diets were freshly prepared each day and presented to the animals between 9:00 AM and 12:00 PM. After 6 to 7 weeks, animals were sacrificed.

2.2. Effects of immediate ethanol exposure and raising endogenous acetaldehyde with cyanamide

Rats were divided into the following groups: (1) saline + saline (n = 7), (2) cyanamide + saline (n = 7), (3) saline + ethanol (n = 7), (4) cyanamide + ethanol (n = 7). The dosage used was 75 mmol/kg body weight for alcohol, 0.5 mmol/kg body weight cyanamide, and controls were injected with identical volume of 0.15 mol/L NaCl. The experimental procedure involved intraperitoneal injection (0.5 mL/100 g body weight) “pretreatment” (30 minutes) with either saline or cyanamide, followed by intraperitoneal injection (1 mL/100 g body weight) “treatment” (150 minutes) with either saline or ethanol. Rats were therefore killed after a total time of 2.5 hours’ exposure to ethanol. Immediate ethanol dosage in this model reduces muscle protein synthesis between 1 and 24 hours postexposure [18]. The reduction is most pronounced at 2.5 hours, and hence this time point has been used since in numerous studies with a variety of biochemical perturbations noted [4,10,18].

2.3. Effects of starvation and immediate superimposition of alcohol

Rats were either treated as in the fed state or starved 1 or 2 days before use. Rats were thus divided into the following groups: (1) fed + saline (n = 8), (2) fed + ethanol (n = 8), (3)

Table 1
Composition of control and alcohol-containing liquid diets

(A) Control and ethanol-containing diets

	Content (g)	Total kilocalories	Total kilojoules
Alcohol diet			
Water	366	0.0	0.0
Fresubin (mL)	600	586	2448
Glucose	0.0	0.0	0.0
Casein	15.0	60	251
Orovite (sucrose)	2.46	9	39
Alcohol (61.8 mL)	49.08	348	1457
Total	1063	1003	4194
Control diet			
Water	366	0.0	0.0
Fresubin (mL)	600	586	2448
Glucose	93	349	1458
Casein	15.0	60	251
Orovite (sucrose)	2.46	9	39
Alcohol	0	0	0
Total	1106	1004	4196

(B) Composition of Fresubin and Orovite 7

	Fresubin/100 mL	Orovite 7/5 g
Protein	3.8 g	None
Fat	3.4 g	None
Carbohydrate	13.8 g	3.8 g
Energy	420 kJ	59.6 kJ
Vitamin A	60.0 μg	62.5 μg
Thiamine (vitamin B_1)	0.11 mg	1.4 mg
Riboflavin (vitamin B_2)	0.13 mg	1.7 mg
Vitamin B_6	0.12 mg	2.0 mg
Vitamin B_{12}	0.3 μg	None
Phylloquinone (vitamin K_1)	10 μg	None
Vitamin E	1.0 μg	None
Nicotinamide	0.9 mg	18.0 mg
Vitamin D	0.5 μg	300.0 μg
Folic acid	20 μg	None
Vitamin C	5 mg	60.0 mg
Biotin	12 mg	None
Pantothenic acid	0.69 mg	None
Calcium	75.0 mg	None
Iodine	7.5 μg	None
Iron	1.0 mg	None

Energy values were calculated as follows: ethanol, 7.1 kcal/g; carbohydrate, 3.8 kcal/g; protein, 4.0 kcal/g; fat, 9.0 kcal/g; and 4.18 kJ was assumed to be equal to 1.0 kcal. Compositions of Fresubin (Fresnius Kabi Ltd, Cheshire, UK) and Orovite 7 (Thornton & Ross Ltd, West Yorkshire, UK) were from manufacturers' specifications.

starved 1 day + saline (n = 8), (4) starved 1 day + ethanol (n = 9), (5) starved 2 days + saline (n = 7), (6) starved 2 days + ethanol (n = 8). The dosage used was 75 mmol/kg body weight for alcohol. Controls were injected with identical volume of 0.15 mol/L NaCl, both by intraperitoneal injection (1 mL/100 g body weight), and rats were killed after a total time of 2.5 hours exposure to alcohol.

2.4. RNA extractions and mRNA assays

Total RNA was prepared from the skeletal muscle as described by Chomczynski and Sacchi [19]. The levels of HSP mRNAs in the muscle were quantified by reverse transcription–polymerase chain reaction (RT-PCR) with an endogenous internal standard, glyceraldehyde-3-phosphate-dehydrogenase (GAPDH). Reverse transcription was performed on 1 μg total RNA for 90 minutes at 42°C in a 5-μL reaction mixture containing 25 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 5 mmol/L $MgCl_2$, 2 mmol/L dithiothreitol, 1 mmol/L each deoxynucleotide, 10 U avian myeloblastosis virus reverse transcriptase (Roche Molecular Biochemicals, Mannheim, Germany), 10 U ribonuclease inhibitor (Roche Molecular Biochemicals), and 0.8 μg oligo $(dT)_{15}$ primer (Roche Molecular Biochemicals). The reverse transcription was terminated by heating the sample at 95°C for 2 minutes.

The multiplexed PCR was carried out in a 20-μL reac-L reaction mixture containing 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 2% dimethyl sulfoxide, 0.2 mmol/L each deoxynucleotide, 0.1 μmol/L each of 5′ and 3′ GAPDH-specific primers, 1 μmol/L each of 5′ and 3′ HSP 27–, HSP 60–, HSP 70–, or HSP 90–specific primers, 25 ng of reverse-transcribed total RNA, and 0.5 U Taq DNA polymerase (Roche Molecular Biochemicals). The PCR amplification was performed for 24 (HSP 27 and HSP 90), 26 (HSP 60), or 30 (HSP 70) cycles, consisting of denaturation (94°C, 45 seconds), annealing (60°C, 45 seconds), and extension (72°C, 75 seconds). After 2 cycles (HSP 27 and HSP 90), 4 cycles (HSP 60), or 8 cycles (HSP 70), 0.1 μmol/L each of GAPDH primer pair was added to the reaction mixture and PCR cycles were further continued. The primer sequences used for amplification of the coding regions are summarized in Table 2. The PCR products were analyzed on a 10% (w/v) polyacrylamide gel electrophoresis. Gels were stained with ethidium bromide, visualized with ultraviolet trans-illumination, photographed, and submitted to image analysis. Fig. 1 shows representative gels which illustrate the clarity of band separation. Quantitative image analysis of the PCR fragments was performed using the NIH image program (National Institutes of Health, Bethesda, MD). The levels of mRNAs were calculated as the ratios of optical density of the PCR products to that of the GAPDH PCR product.

2.5. Proteins by Western blotting

Muscles were ground in liquid nitrogen and homogenized in a 1% (w/v) solution of SDS containing a range of protease inhibitors [20]. Homogenates were centrifuged at 14 000 × *g* and +4°C for 10 minutes, and the protein content of the supernatant was determined using a BCA assay kit (Sigma, Dorset, UK). One hundred micrograms of total protein was separated on a 12% (w/v) polyacrylamide gel by SDS-PAGE, followed by Western blotting [20]. The content of HSP 27, HSP 60, and HSP 70 was analyzed using monoclonal antibodies and secondary antibodies labeled with peroxidase (Bioquote Ltd, York, UK, and Sigma). Bands were visualized using SuperSignal Substrate (Pierce, Cheshire, UK) and quantified using a ChemiDoc chemiluminescence system (Biorad, Hemel Hempstead, UK). Exposure times were

Table 2
Oligonucleotide primers, product length, annealing temperatures, and number of PCR cycles

(A)

Gene		Sequence
HSP 27	Forward	5′- GTCTCAGAGATCCGACAGACG-3′
	Reverse	5′- GAATGGTGATCTCCGCTGAT-3′
HSP 60	Forward	5′- GATGCCATGCTTGGAGATTT-3′
	Reverse	5′- GGAACCTGCCTTGAGCTTC-3′
HSP 70	Forward	5′-GAGTCCTACGCCTTCAATATGAAG-3′
	Reverse	5′-CATCAAGAGTCTGTCTCTAGCCAA-3′
HSP 90	Forward	5′- AGGGCAGTTGGAATTCAGG-3′
	Reverse	5′- GCCAACTCAGAGAAGAGCTCA-3′
GAPDH	Forward	5′- CAGCAATGCATCCTGCAC -3′
	Reverse	5′- GAGTTGCTGTTGAAGTCACAGG -3′

(B)

Gene	PCR product (bp)	Annealing temperature (°C)	Cycles	Accession
HSP 27	292	60	24	M86389
HSP 60	274	60	28	X54793
HSP 70	347	60	30	L16764
HSP 90	288	60	24	S45392
GAPDH	429	60	21, 22	X02231

Details of (A) oligonucleotide primers, (B) product length, annealing temperatures, and number of PCR cycles. The top and lower primers of each amplification pair are forward and reverse primers, respectively.

varied to ensure that saturation had not occurred. Data are presented as arbitrary densitometric units [20].

2.6. Statistical analysis

All data are mean ± SEM. In studies II and III, the data were analyzed using a between-subjects 2 × 2 two-way analysis of variance (ANOVA), with treatment (ethanol, no ethanol) and sex (male, female) as independent factors (study I) or with pretreatment (cyanamide, no cyanamide) and treatment (ethanol, no ethanol) as independent factors (study II). Significance was indicated when $P < .05$ or $P = .05$. A Mauchly sphericity test was conducted on the data sets before the 2 × 2 ANOVA analysis to determine whether the data set centrality and spread characteristics were satisfactory for the conduct of a parametric statistical test. A Greenhouse-Geisser ϵ correction was applied to the data analysis to modify degrees of freedom and recalculate a corrected value of P. Posteriori statistical analysis was conducted to unpack any statistically significant main effects or higher order interactions observed using the least significant differences multiple comparison procedure. Posteriori comparisons were conducted after careful scrutiny of graphical and descriptive representation of the data to reduce the total number of multiple comparisons made to the absolute minimum and allowed α to remain at $P = .05$. In study III, the data were analyzed using a between-subjects 2 × 3 two-way ANOVA, with treatment (ethanol, no ethanol) and starvation status (fed, 1 day starved, 2 days starved) as independent factors. Otherwise, the data analysis procedure was identical to that of studies I and II.

3. Results

3.1. The effect of long-term alcohol exposure for 6 to 7 weeks in male and female rats

It has been demonstrated previously that this alcohol-feeding model results in reduced animal growth rates, muscle weights, muscle protein content, and atrophy of muscle fibers [8]. At the time of sacrifice, muscle weights were found to be reduced in the animals fed alcohol indicative of myopathy (data presented previously) [4,9]. For HSP 27 mRNA, there was no evidence of a main effect of sex (P = NS) or treatment (P = NS), but a statistically significant interaction was observed between sex and treatment ($P = .05$; Fig. 2). Posteriori analysis revealed male ethanol-treated rats to have significantly lower HSP 27 mRNA levels than male control rats ($P < .01$), although there was no significant differences in HSP 27 mRNA levels between female control and female ethanol-treated rats (P = NS; Fig. 2). For HSP 90 mRNA, there was no evidence of a main effect of sex (P = NS) or treatment (P = NS), but a statistically significant interaction was observed between sex and treatment ($P < .05$; Fig. 2). Although alcohol had no effect on HSP 90mRNA levels in

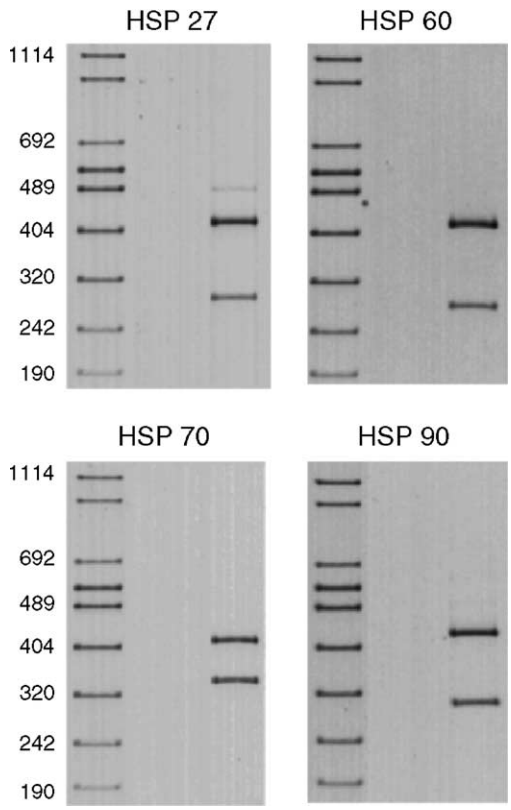

Fig. 1. Ethidium bromide–stained polyacrylamide gel showing PCR products amplified from rat muscle. Ethidium bromide–stained polyacrylamide gel showing PCR products amplified from rat muscle RNA. Total RNA extracted from the right-leg muscle was incubated in the absence (blank middle lanes) or in the presence (right lane) of reverse transcriptase. The reverse transcription products were coamplified with HSP 27, HSP 60, HSP 70, or HSP 90 primers, and GAPDH primers. A DNA standard lane is shown at the left of the gel with bands labeled in base pairs.

male rats, there was a significant increase in HSP 90 mRNA levels in female rats fed alcohol ($P < .05$; Fig. 2). There were no statistically significant changes for HSP 60 or 70 mRNAs in this study for any of the variables, treatments, or interactions (data not shown for brevity).

3.2. Effects of long-term alcohol exposure on HSP content of muscles of male rats in study I

The muscle content of HSP 27, HSP 60, and HSP 70 is shown in Table 3. Protein content was reduced by a mean of 25%, 5%, and 13%, respectively. Thus, data appear to generally reflect the pattern of changes observed in mRNA levels, although no significant differences were observed in any of the proteins studied.

Table 3
Mean HSP protein content for each HSP (27, 60, and 70)

	HSP protein (arbitrary densitometric units)		
	Control	Ethanol	*P*
HSP 27	218 ± 23	164 ± 19	NS
HSP 60	84 ± 16	80 ± 13	NS
HSP 70	193 ± 14	169 ± 15	NS

Male rats were fed nutritionally complete liquid diets containing ethanol as 35% of total energy intake (treated) or identical amounts of the same diet in which ethanol was replaced by isoenergetic glucose (controls) for approximately 6 to 7 weeks (from study II). At the end of the study, muscle was dissected for analysis of HSP by Western blotting. Data are presented as mean ± SEM.

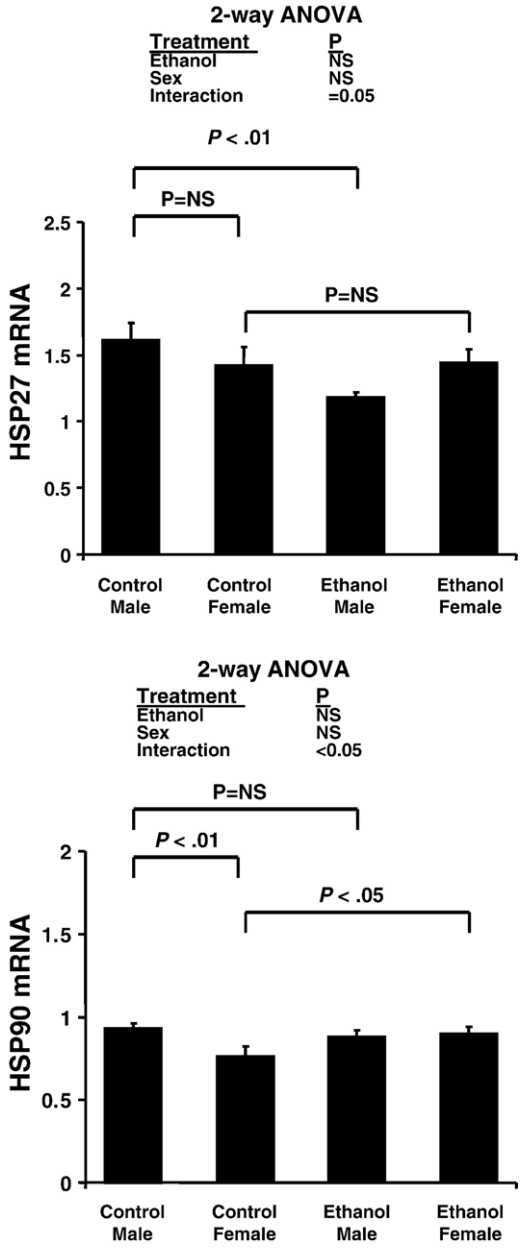

Fig. 2. Effect of alcohol exposure for 6 to 7 weeks on muscle HSP 27 and HSP 90 mRNA levels in male and female rats. Male and female rats were fed nutritionally complete liquid diets containing ethanol as 35% of total energy intake (treated) or identical amounts of the same diet in which ethanol was replaced by isocaloric glucose (controls) for approximately 6 to 7 weeks. At the end of the study, muscle was dissected for analysis of HSP mRNA relative to GAPDH mRNA. Data are presented as mean ± SEM.

3.3. Effects of immediate ethanol exposure and raising endogenous acetaldehyde with cyanamide

For HSP 60 mRNA, there was a statistically significant main effect of pretreatment with cyanamide associated with elevated HSP 60 mRNA levels compared to animals not pretreated ($P < .01$; Fig. 3). No main effect of treatment (P = NS) was observed. No statistically significant interaction was observed between pretreatment and treatment (P = NS). Posteriori analysis revealed the cyanamide and ethanol group to have significantly higher HSP 60 mRNA levels than the saline and ethanol group ($P <$

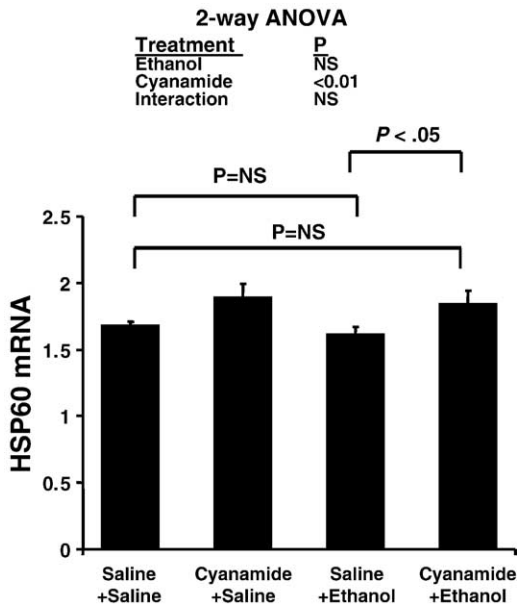

Fig. 3. Effects of immediate ethanol exposure and raising endogenous acetaldehyde with cyanamide on muscle HSP 60 mRNA levels in male rats. Male rats were administered immediately with the following (pretreatment of 30 minutes followed by treatment of 2.5 hours): saline + saline (control), cyanamide + saline, saline + ethanol, cyanamide + ethanol. At the end of the study, muscle was dissected for analysis of HSP mRNA relative to GAPDH mRNA. Data are presented as mean ± SEM.

.05; Fig. 3), although this was no different to the cyanamide and saline group. There was no significant effects of alcohol in animals pretreated with saline (P = NS). The cyanamide and ethanol group did not differ significantly in HSP 60 mRNA levels compared to the saline and saline group (P = NS). There were no statistically significant changes for HSP 27, HSP 70, or HSP 90 mRNAs in this study for any of the variables, treatments, or interactions (data not shown for brevity).

3.4. Effects of starvation and immediate superimposition of alcohol

An important component of this study was the examination of any interaction between alcohol and malnutrition in the pathogenesis of alcoholic myopathy, a novel approach to analyzing this pathology. Although there are various ways of examining this, the model used was that of starvation before administration of alcohol. It is recognized that there are alternative strategies such as long-term underfeeding and low protein diets, and that each of these may have different effects. For HSP 27 mRNA, no statistically significant main effects of treatment (P = NS), starvation (P = NS), or interaction were observed (Fig. 4). Posteriori analysis revealed that the group starved 2 days and given saline had an increased HSP 27 mRNA level compared to those fed and given saline (P = .05; Fig. 4). The group starved 2 days and given ethanol was observed to have significantly lower HSP 27 mRNA levels than the group starved 2 days and given saline (P < .05; Fig. 4). The group starved 2 days and given ethanol was unchanged compared to the fed group given saline. There were no significant changes for HSP 27, HSP 70, or HSP 90 mRNAs in this study for any of the variables, treatments, or interactions (data not shown for brevity).

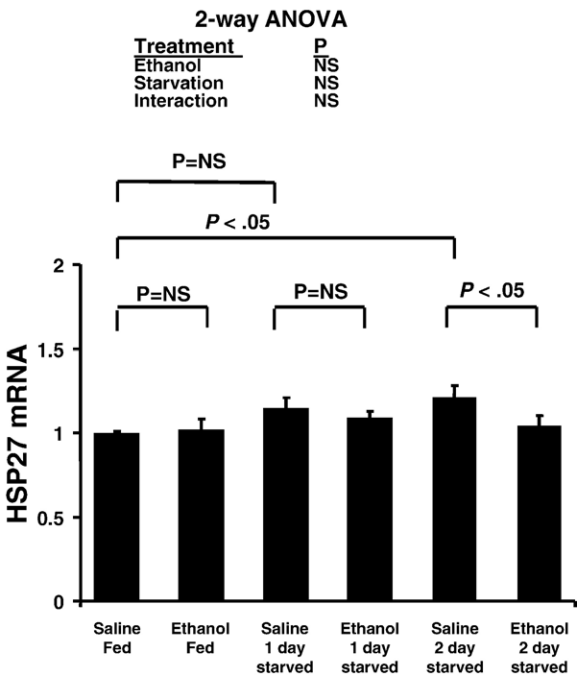

Fig. 4. Effects of starvation and immediate superimposition of alcohol on muscle HSP 27 mRNA levels in male rats. Rats were either treated as in the fed state or starved 1 or 2 days before use. Rats were then divided into the following groups (nutritional state + treatment with an exposure time of 2.5 hours): fed + saline; fed + ethanol; starved 1 day + saline; starved 1 day + ethanol; starved 2 days + saline; starved 2 days + ethanol. At the end of the study, muscle was dissected for analysis of HSP mRNA relative to GAPDH mRNA. Data are presented as mean ± SEM.

4. Discussion

Pathogenic changes in HSPs may be involved in the etiology of skeletal muscle pathologies including aging [16] and oxidative stress [17]. However, the effect of alcohol on muscle HSP expression is unknown and it is possible that impaired HSP expression may be a contributory factor in the development of alcohol-induced muscle disease. The 4 different HSPs measured have slightly different functions. HSP 27 and HSP 70 are highly inducible and are involved in various aspects of protein maturation and translocation to different organelles, as well as binding to nucleotides and aiding in RNA and DNA synthesis [12-15]. HSP 60 and HSP 90 are constitutively expressed [12]. HSP 60 seems primarily concerned with protein folding and assembly of protein complexes, whereas HSP 90 seems to serve a regulatory role by stimulating or inhibiting various proteins and may be important in muscle development [12,21]. All of these HSPs have a key role in tissue response to stress and the control of apoptosis [13].

Many of the earlier studies on HSPs were carried out on nonmammalian systems such as yeast and showed that alcohol increased HSP or mRNA expression [22,23]. Our experiments were focused on in vivo studies in the laboratory rats and in general showed opposite changes compared to the aforementioned citations. Due to these novel findings, we initially raise issues of methodological concern, then proceed to discuss cyanamide predosing, the effects of alcohol on HSP mRNA levels, and sex susceptibility.

4.1. Methodological considerations

A well-validated feeding regimen was used for the long-term alcohol feeding (study I), which ensured that control and alcohol-fed rats received identical amounts of the same diet in which ethanol or isoenergetic glucose comprised 35% of total dietary energy [3,4,8]. Otherwise, the micro- and macronutrient compositions of the 2 diets were identical, and any effect seen was due to the putative effects of ethanol or its ensuing metabolites rather than dietary limitations. This is an important point, as long-term ethanol exposure induces marked anorexia [8] and reductions in nutrient supply alter HSP mRNA or protein expression in a number of systems including increases seen in liver or yeast [24,25]. This latter aspect was also substantiated in our own experiments, which showed that starvation increased HSP 27 mRNA in muscle.

4.2. Effects of alcohol on HSP mRNA levels

The novel findings in these studies are that in male rats long-term alcohol feeding reduced HSP 27. There were also trends toward reduction in the protein content of the 3 HSPs in muscle of male rats also, but this did not reach significance. Although measurement of HSPs provides a static measure of tissue protein levels, measurement of their corresponding mRNA levels arguably provides a more dynamic reflection of production and turnover of these proteins. As the changes in mRNA levels are generally small (<60% change), and there is no significant change in the HSPs, it remains uncertain whether alcohol results in altered production of these proteins. There was no effect on any of the HSP mRNAs in male rats dosed immediately with alcohol alone. Starvation of male rats per se caused an increase in HSP 27 after 2 days. Immediate ethanol dosing after 2 days of starvation appears to blunt this rise in HSP 27 mRNA caused by starvation. This reduction in HSP expression can confer distinct biological disadvantages and may impair the ability of tissues to tolerate metabolic stresses such as those imposed by starvation [25,26].

Previous reports on HSPs in alcohol-exposed tissues have produced conflicting results. HSP 27 mRNA decreases in livers of rats immediately dosed with alcohol [27]. However, increases in HSPs have been demonstrated in alcohol-exposed cardiac tissue in vitro and in vivo, and hepatic tissue in vivo [28-31]. Although this disparity is difficult to explain, there may be differences in the pathophysiology of the models used. These studies were conducted in well-characterized models of alcohol administration that causes marked perturbations in muscle biochemistry. The heterogeneity in response between the HSPs measured is not surprising given their differing functions and that they are induced by different stimuli. The statistically significant changes seen in male rats upon long-term alcohol feeding were in HSP 27 (which is both inducible), with little change in HSP 60 and HSP 90 (both of which are constitutive cell proteins, albeit with potential to be upregulated) [12,21]. The fact that HSP 27 is largely inducible may explain why it is these HSPs that alter in long-term alcohol feeding.

4.3. Sex susceptibility

Although long-term alcohol feeding caused a reduction in muscle HSP 27 mRNA in male rats, this reduction was not seen in female rats and this resulted in a significant interaction for sex. Paradoxically, female rats exhibit increased HSP 90 mRNA expression in long-term studies, whereas there is no change seen in male rats, again resulting in a significant interaction for sex. We are unable to explain this observation from a biological perspective, but consideration needs to be given to the fact that females are particularly susceptible to alcohol-induced muscle damage (both clinically and in terms of biochemical alterations) [6,32]. Differences in response of male and females rats may have their origins in differing endocrine responses to alcohol [33,34]. For example, stimulation of the hypothalamic-pituitary-adrenal axis induces adrenal HSP 70 mRNA [35].

4.4. Cyanamide predosing

We also injected rats with cyanamide, an acetaldehyde dehydrogenase inhibitor which markedly suppresses both the low and high K_m acetaldehyde dehydrogenase activities (in the order of 70%-100%) [36]. Blood acetaldehyde levels in identical conditions have been reported previously; concentrations increase from about 5 nmol/mL in saline-treated controls to 30 nmol/mL in ethanol-injected rats, which is potentiated to approximately 2500 nmol/mL in cyanamide + ethanol–injected rats [37]. In muscle, corresponding values are 51, 62, and 830 nmol acetaldehyde per gram, respectively, whereas blood ethanol levels are less than 1, 33, and 46 mmol/L, respectively [37]. Although investigating the effect of grossly elevated acetaldehyde on muscle is insufficient to establish acetaldehyde as a mediator of alcohol-induced changes in HSP mRNA (as this requires use of combinations of the alcohol dehydrogenase inhibitor 4-methylpyrazole and cyanamide), it does allow examination of the effects of this toxic metabolite which has numerous effects of its own [38-40]. For example, a combined cyanamide + ethanol dosing protocol results in much greater perturbations in muscle protein synthesis and oxidative stress than ethanol alone [38-40]. Thus, any change in HSP mRNA in the muscle of rats dosed immediately with ethanol with or without cyanamide must reflect the fact that HSP mRNAs are relatively refractory in skeletal muscle compared to other tissues or metabolic situations.

Surprisingly, cyanamide per se increased HSP 60 mRNA, independent of the effect of ethanol. Cyanamide imparts its actions by binding catalase to aldehyde dehydrogenase–nicotinamide adenine dinucleotide complex to form a catalase–aldehyde dehydrogenase–nicotinamide adenine dinucleotide hybrid in peroxisomes and mitochondria [41]. Alternately, the generation of cyanide, a classical tissue toxin, may contribute to aldehyde-dehydrogenase inhibition [42]. Both the inhibition of catalase or cellular poisoning by cyanide would be expected to increase oxidative stress and/or HSP mRNA, although it is unclear why HSP 60 mRNA alone is affected by cyanamide. However, further interpretational experiments in this area are really beyond the realms of the present alcohol toxicity studies.

4.5. Concluding remarks

Long-term alcohol exposure significantly decreased HSP 27 mRNA in male rats compared to controls. An effect of sex was also demonstrated with no change in HSP 27 mRNA in females, and an increase in HSP 90 mRNA in female rats not seen in males. Starvation also factors into HSP expression; the rise in HSP 27 mRNA seen in

starvation is blunted by administration of alcohol. However, there was no effect of immediate alcohol administration on any HSP mRNA despite raised acetaldehyde levels. Thus, alcohol-induced alterations in HSP mRNAs are selective and sex-specific and may contribute to, or reflect, the biochemical lesions seen in alcohol-exposed muscle. Although there were small changes in HSP mRNA levels, these were not translated into significant changes in the HSP protein levels. Other dietary components such as dietary fat content, the type of lipid, and, indeed, the presence of preexisting liver disease have all been shown to modulate the effect of alcohol on tissues and may impact on alcohol-induced changes in muscle HSP mRNA.

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