Alcohol Increases *c-myc* mRNA and Protein in Skeletal and Cardiac Muscle

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The pathogenic mechanisms responsible for alcohol-induced muscle disease are unknown, although it is possible that increased proto-oncogene expression may be the causative process. Therefore, we investigated the responses of skeletal muscle c-myc protein and mRNA to a standard acute ethanol dosage regimen (75 mmol/kg/body weight [BW]) for 2.5 to 24 hours. Comparative studies were made on the heart. Acute ethanol administration in vivo led to an increase in *c-myc* proto-oncogene mRNA in rat skeletal and cardiac muscle. The changes in *c-myc* mRNA were mirrored by increases in the c-myc protein as demonstrated by immunohistochemistry. The changes in the c-myc protein were localized to the myonuclei, with no corresponding changes seen in the interstitial cell nuclei. This is the first report of altered proto-oncogene expression in muscle in response to ethanol. Increased *c-myc* mRNA and protein may reflect adaptive changes, a stress response, or another uncharacterized cellular adaptation.

EXCESSIVE INGESTION OF alcohol causes alcoholic myopathy, and this disease is one of the most common muscle disorders with a prevalence of 2,000 cases per 100,000 population.[2-6] The myopathy preferentially affects type II fibers.[2-6] In both man and rats, the disease is characterized by reductions in the rate of muscle protein synthesis and total protein content.[2-5] Alcoholic muscle disease is more prevalent than other alcohol-related pathologies, such as cirrhosis, peripheral neuropathy, or fetal alcohol syndrome (for example, see Bingol et al[7] and Estruch et al[8]). Although the causative agent is known, the precise sequence of events between alcohol ingestion and skeletal muscle damage is poorly characterized. However, it is possible that metabolic lesions resulting in muscle injury may be initiated at the molecular level. Candidate processes include the abnormal expression of proto-oncogenes.

Proto-oncogenes are genetic elements that regulate cellular development and proliferation.[9-11] Excessive expression can lead to abnormal growth of cells.[9-11] Thus, the proto-oncogenes, *c-myc* and *c-fos*, are involved in the molecular control of hypertrophy in skeletal[12] and cardiac[13] muscle. The response is rapid and transient, implying that the proto-oncogenes trigger other genes in a cellular cascade, such as ribosome biogenesis.[14,15] However, the functions of proto-oncogenes are multifaceted and include the induction of apoptosis,[9,16] the repression of cell adhesion,[17,18] and perturbations in cellular metabolism, such as the activation of genes involved in glycolysis or amino acid transport.[18] The most well-characterized proto-oncogene is *c-myc*. The myc protein forms heterodimers with the protein, Max and, after binding to DNA, activates gene expression.[18-20]

Recent attention has focused on studies reporting a possible link between increased proto-oncogene expression, particularly c-myc, and alcohol-induced pathologies.

Tissues in which increases in c-myc have been reported after exposure to alcohol include hepatic,[21,22] embryonic,[15] and neurologic tissues.[23-25] However, to date, there have been no studies of changes into c-myc mRNA or protein expression in muscle exposed to ethanol in vivo. This issue was addressed by analysis of skeletal and cardiac muscle of ethanol-dosed rats. A variety of muscle types were investigated concomitantly with a well-characterized dosing regimen.[26-29]

MATERIALS AND METHODS

Materials

Male Wistar rats were obtained from Charles River (Margate, Kent, UK). General molecular biology grade chemicals were purchased from Sigma-Aldrich Company (Poole, Dorset, UK) and Fisher Scientific (UK) (Loughborough, Leics, UK). RNAzol B was purchased from Biotecx Laboratories (Houston, TX). Agarose and molecular size markers were obtained from Gibco-BRL (Life Sciences International, Paisley, Scotland, UK). Nylon filter membrane for RNA transfer was obtained from DuPont (UK) (Stevenage, UK). [^{32}P] Deoxycytidine triphosphate (dCTP) and the Megaprime random priming kit were obtained from Amersham International (Little Chalfont, Bucks, UK).

The human anti-myc monoclonal antibody was obtained from Cambridge Research Biochemicals (Zeneca, Runcorn, Cheshire, UK). Streptavidin-peroxidase and biotin-labeled antimouse immunoglobulin G (IgG) were purchased from Amersham International. Hydrogen peroxide, butanol, and DPX mounting solution for the slides were from BDH Laboratory Supplies (Poole, UK). All other chemicals were obtained from Sigma-Aldrich Company.

Animals

Rats were housed in wire bottomed cages in a constant temperature and humidity-controlled animal house with free access to commercial pelleted diet and tap water until treatment. Animals were killed by decapitation at the relevant time.

Acute Ethanol Experiment

Rats were divided among the following groups, each of equal mean body weight (BW) (≈ 120 g). Study 1, fed rats: (1) fed + saline 2.5

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hours (0.15 mol/L NaCl, injected intraperitoneal (IP) 2.5 hours before sacrifice); (2) fed + ethanol 2.5 hours (75 mmol/kg BW; injected IP 2.5 hours before sacrifice); (3) fed + ethanol 6 hours; (4) 24 hours starved + saline 2.5 hours.

Study 2, 24-hour starved rats: (1) 24-hour starved + saline 2.5 hours; (2) 24-hour starved + ethanol 2.5 hours; (3) 24-hour starved + ethanol 24 hours.

RNA Extraction and Analysis

The heart, gastrocnemius, soleus, and plantaris muscles were rapidly dissected out, then the tissues were frozen in liquid nitrogen. Atria were removed from the hearts. Total cellular RNA was extracted from the tissues using RNAzol B according to the manufacturer's instructions. An aliquot of 20 μg of each RNA sample was run out on a 2.2 mol/L formaldehyde, 1.2% (wt/vol) agarose gel and blotted by capillary transferral to a nylon membrane. After transfer, the RNA was fixed and prehybridized.[30] *c-Myc* mRNA and 18S rRNA cDNA probes were labeled with [^{32}P] dCTP using the Megaprime random priming kit (Amersham International). The probes were hybridized with the filters for 16 hours at 42°C inside rotating circular bottles (Techne (UK), Duxford, UK). After hybridization, the filters were washed twice with either 0.5 × SSC/1% (wt/vol) sodium dodecyl sulfate (SDS) at 65°C for 1 hour in the case of *c-myc* or 0.2 × SSC/1% (wt/vol) SDS at 65°C for 1 hour for 18S (1 × SSC = 0.15 mol/L NaCl/0.015 mol/L sodium citrate). Between probings, the labeled DNA was stripped off by immersing the filter in 0.01% (wt/vol) SDS at 95°C for 10 minutes.

For each probing, the amount of label in each lane was quantified using an InstantImager (Canberra-Packard, Pangbourne, UK). The *c-myc*/18S ratios were then calculated for each group. Data showed that 18S rRNA in relationship to muscle DNA was not altered in the gastrocnemius and heart in these groups (data not shown), ie, the amount of 18S rRNA per cell was not significantly altered by the treatments. Thus, ensuing changes in c-myc mRNA were not likely due to alterations in cell number.

Immunochemistry

Transverse sections of 5 μm from the gastrocnemius muscles and hearts were cut and transferred to glass slides. The sections were fixed using ice-cold acetone for 10 minutes. Immersing the slides in 3% (vol/vol) H_2O_2 in methanol for 30 minutes destroyed endogenous peroxidase activity in the sections. Nonspecific binding was blocked using sheep serum (Serotec, Oxford, UK) diluted 1:100 in phosphate-buffered saline (PBS) (Sigma-Aldrich Company) for 30 minutes. Sections were incubated with mouse anti-myc monoclonal antibody (Cambridge Research Biochemicals, Runcorn, UK) diluted 1:500 with PBS for 16 hours at 4°C in a humid chamber. Negative controls were similarly treated with mouse nonspecific IgG (Sigma-Aldrich Company). All sections were washed in PBS and then incubated with goat biotinylated antimouse IgG (Amersham International) diluted 1:200 in PBS for 1 hour. The sections were washed in PBS, then incubated with streptavidin-biotinylated horseradish peroxidase (Amersham International) diluted 1:200 in PBS. Peroxidase activity was revealed by incubation for 15 minutes with diaminobenzidine substrate (6 mg dissolved in 10 mL PBS and 10 μL H_2O_2). Immersing the slides in PBS stopped the reaction. Passing them successively through distilled water, 50% (vol/vol) ethanol, 70% (vol/vol) ethanol, 90% (vol/vol) ethanol, 100% (vol/vol) ethanol (twice) and xylene then dehydrated the sections (twice). The sections were then mounted using DPX (Merck, Lutterworth, Leics, UK) and examined and photographed with a Zeiss Axioscop microscope with attached camera. A Pulnix Kinetic TM-765 video camera with Cyclops 2.33 visual analysis software (Kinetic Imaging, Liverpool, UK) was used to quantify the c-myc staining. Depending on the nuclear density, 30 to 50 myonuclei were quantified using an area of cytoplasm as background, followed by a similar number of interstitial nuclei. Similarly, values for the myonuclear and interstitial staining were obtained for the nonimmune controls. There was no significant difference between background values (data not shown). Myonuclei and interstitial cell nuclei were differentiated by using the phase-contrast switch of the microscope, which could be used to illuminate the interface between the cell membranes. The myonuclei were enclosed within the subsarcolemmal regions of the myofibrils and were more flattened than the interstitial nuclei.

Statistical Analysis

After checking the normality of the data, statistical analysis for unpaired data was performed by Student's *t* test using a pooled estimate of variance for multiple comparisons. Data are expressed as mean ± SEM.

RESULTS

Changes in c-myc mRNA

Northern blotting showed *c-myc* at low levels in the gastrocnemius muscle and heart (Fig 1) . A large increase in the *c-myc* mRNA was observed 2.5 and 6 hours after ethanol administration in the gastrocnemius muscle of the fed rats. There was also a large increase in *c-myc* mRNA in the heart 2.5 hours after ethanol administration.

Quantitative analysis of the data from Northern blotting experiments was performed to correct for any potential loading differences by expression of *c-myc* mRNA levels per the amount of 18S rRNA (Fig 2). In the gastrocnemius of the fed rat, the *c-myc*/18S ratio increased by 82% ($P < .05$) 2.5 hours after ethanol administration and 146% ($P < .01$) 6 hours after ethanol administration. Starvation for 24 hours increased the *c-myc*/18S ratio by 109% ($P < .05$). In the starved rat, the *c-myc*/18S ratio increased by 54% ($P < .05$) 2.5 hours after ethanol administration. The *c-myc*/18S ratio increased by 131% ($P < .05$) 24 hours after ethanol administration in the starved rats. In the heart, the *c-myc*/18S ratio was increased by 78% 2.5 hours after ethanol administration ($P < .01$), but was not significantly increased after 6 hours. Starvation for 24 hours caused a statistically significant increase in the *c-myc*/18S ratio of the heart (57%; $P < .05$). In the starved rat, there was an 88% increase in the cardiac *c-myc*/18S ratio 2.5 hours after ethanol administration ($P < .001$), again showing that the ethanol-mediated increase of *c-myc* occurred in addition to the increase caused by starvation. There was a 65% increase in cardiac *c-myc*/18S ratio 24 hours postinjection ($P < .01$). In the type I fiber-predominant soleus, only the 2.5-hour ethanol group in the starved rats showed a statistically significant increase in the *c-myc*/18S ratio (+59%; $P < .05$). In the type II fiber-predominant plantaris, there was a 53% increase 2.5 hours after ethanol administration ($P < .05$) and a 58% increase 6 hours after ethanol administration ($P < .05$) in the fed rats. *c-Myc* mRNA levels were increased by 191% ($P < .001$) in the plantaris in response to 24-hour starvation. In the starved rat, the *c-myc*/18S ratio was increased by 47% 2.5 hours after ethanol administration ($P < .05$) and 84% 24 hours after ethanol treatment ($P < .01$).

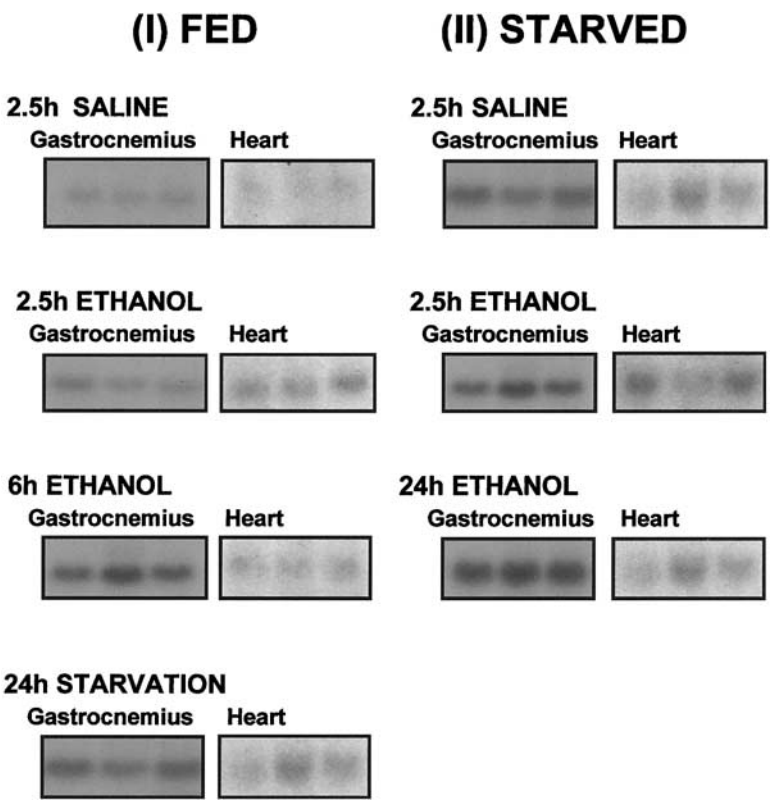

Fig 1. Representative autoradiographs showing *c-myc* mRNA levels in the gastrocnemius and heart after various ethanol treatments. Quantitative analysis of the data from Northern blotting experiments was performed to correct for any potential loading differences by expression of *c-myc* mRNA levels per the amount of 18S rRNA. All 3 lanes pertain to c-myc and are displayed as representative results. Numerical quantification of the lane intensities are displayed in Fig 2. Representative autoradiographs showing 18S rRNA not shown for brevity.

c-Myc Protein Immunohistochemistry

Levels of c-myc protein after ethanol administration were investigated in the gastrocnemius and heart by immunohistochemistry. This approach allowed distinction between myonuclei and interstitial cell nuclei to be made. Visual inspection showed increased staining of myonuclei after ethanol administration (photomicrographs not shown for brevity), and this was confirmed by quantitative analysis (Fig 3). There was a 32% increase in gastrocnemius myonuclear c-myc protein 6 hours after ethanol injection ($P < .05$). In the gastrocnemius interstitial nuclei (Fig 3), there was no statistically significant increase in the quantity of c-myc protein in response to starvation or any of the ethanol regimes. This showed that the increases in c-myc protein were specific to the myonuclei.

In cardiac myonuclei, c-myc protein content increased 36% 2.5 hours after the ethanol injection ($P < .001$) and 35% 6 hours postinjection ($P < .001$; Fig 3). After 24 hours starvation, a 37% increase in the c-myc protein in the myonuclei was observed ($P < .001$). In the starved rat, neither 2.5 nor 24 hours ethanol treatment caused a detectable increase in the myonuclear c-myc protein. None of the treatments caused any significant increase in the c-myc protein within the cardiac interstitial cells (Fig 3).

DISCUSSION

In the present study, we used a standard dose of ethanol (ie, 75 mmol/kg BW) to examine the response of muscle c-myc to ethanol. Although there are various models of alcohol dosing (reviewed previously in Preedy et al[31]), we selected a well-characterized regimen, which mimics binge drinking. In this model, there is a rapid increase in blood alcohol levels, which then declines.[27] Thus, after administration, plasma levels of ethanol are approximatelely 450, 375, 290 185 mg/100 mL at 20 minutes, 1, 2.5, and 6 hours, respectively.[27] At 24 hours, there is no detectable ethanol in the blood, at which time point there are no fatalities.[27] The concentration of blood ethanol achieved in the first 6 hours are comparable to the levels observed in other rat studies.[32,33] These values are similar to those pathophysiologic levels reported in numerous clinical studies. For example, in severely intoxicated individuals in a hospital emergency department, blood alcohol concentrations ranged from 109 to 558 mg/100 mL.[34] In another study, mean levels of 301 mg/100 mL were recorded.[35] Thus, we feel our model is directly applicable to man.

The present results showed that both gastrocnemius and heart tissues reacted to the ethanol with a rapid increase in *c-myc* mRNA. The effect appeared faster, but shorter lasting, in the heart than the gastrocnemius. The changes in *c-myc* mRNA were largely mirrored by the alterations in myonuclear c-myc protein in both the gastrocnemius and the heart. However, in the heart, there was also a highly significant increase in the c-myc protein content of the myonuclei 6 hours postinjection. The lack of c-myc accumulation in the

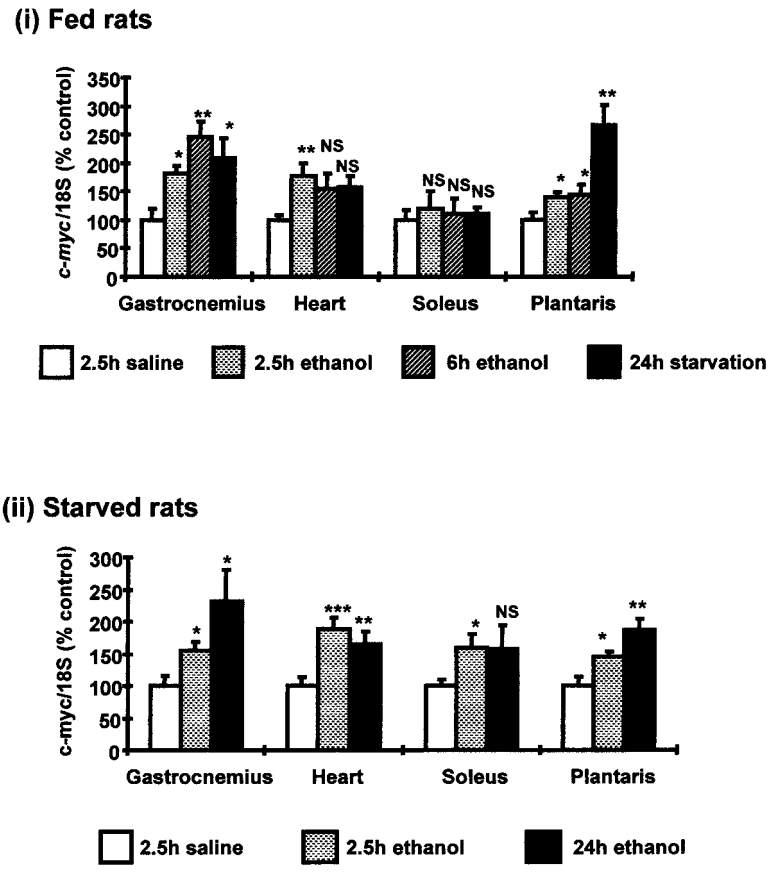

Fig 2. ***c-myc*** **mRNA in the various rat muscles after an injection of ethanol (75 mmol/kg BW, injected IP) or saline (0.15 mol/L NaCl). Statistical analysis (2.5-hour saline and all other corresponding groups) was by 1-way analysis of variance (ANOVA), followed by Student's *t* test using the pooled estimate of variance. *$P < .05$; **$P < .01$; NS, not significant; N = 5 to 6 for each group.**

interstitial cells showed that the initial increase in c-myc in the gastrocnemius and heart was an event specific to the muscle fibers themselves, not to fibroblasts or inflammatory cells in the interstitium. This contrasts with observed accumulation of c-myc protein in the heart after norepinephrine treatment, in which immunohistochemistry demonstrated that the protein was accumulating in nonmyocyte cells, such as the fibroblast.[36] However, increased protein could have come from increased translation of RNA, as well as protein stabilization, or both.

To determine whether the increases seen in skeletal muscle *c-myc* mRNA were fiber specific, *c-myc* mRNA levels were was investigated in the type I fiber-predominant soleus muscle and the type II fiber-predominant plantaris muscle. The results showed that ethanol significantly increased *c-myc* levels in the plantaris at all time points in both fed and starved rats. However, the only significant increase in soleus *c-myc* levels occurred in the starved rats dosed with ethanol for only for 2.5 hours. Thus, it appeared that ethanol preferentially increased *c-myc* mRNA levels in type II fiber-predominant muscles. This may be significant, as alcohol-induced myopathy causes a preferential reduction in the diameter of type II fibers.[37-40]

The increase in *c-myc* expression may represent an early event in a compensatory or adaptive mechanism. *c-myc* mRNA has been found to increase rapidly during cardiac and skeletal muscle hypertrophy.[12,13] Such changes should be considered in light of the observation that ventricular hypertrophy is a significant pathology in alcoholic patients.[41] In animals, acute ethanol dosage also induces cardiac growth,[42] possibly via activation of the sympathetic nervous system.[43] It is tempting to speculate that, in the present study, the increase in *c-myc* may be associated with an acute hypertrophic response as reported by others.[42,43]

With respect to skeletal muscle, other investigators have shown that chronic ethanol treatment of rats causes increase in the size of type I muscle fibers in the gastrocnemius.[44] However, the data in this present study reported here demonstrated that the statistically significant increase in *c-myc* mRNA levels occurred predominantly in the type II fiber-predominant plantaris, rather than the type I fiber-predominant soleus. Thus, it is possible to speculate that in cardiac, but not skeletal, muscle increases in *c-myc* mRNA and protein may be reflective of an initiation in cardiac hypertrophy. However, in terms of skeletal muscle, another interpretation is necessary for the increase in *c-myc* mRNA and protein.

It is possible that the change in *c-myc* expression may be a reaction to cellular stress. Eukaryotic cells contain a small set of normally silent genes coding for heat shock proteins, such as hsp70.[45] These may act as molecular chaperones to partially folded polypeptides to stop irreversible denaturation.[46,47] Alcohol, heavy metals, and oxidants activate

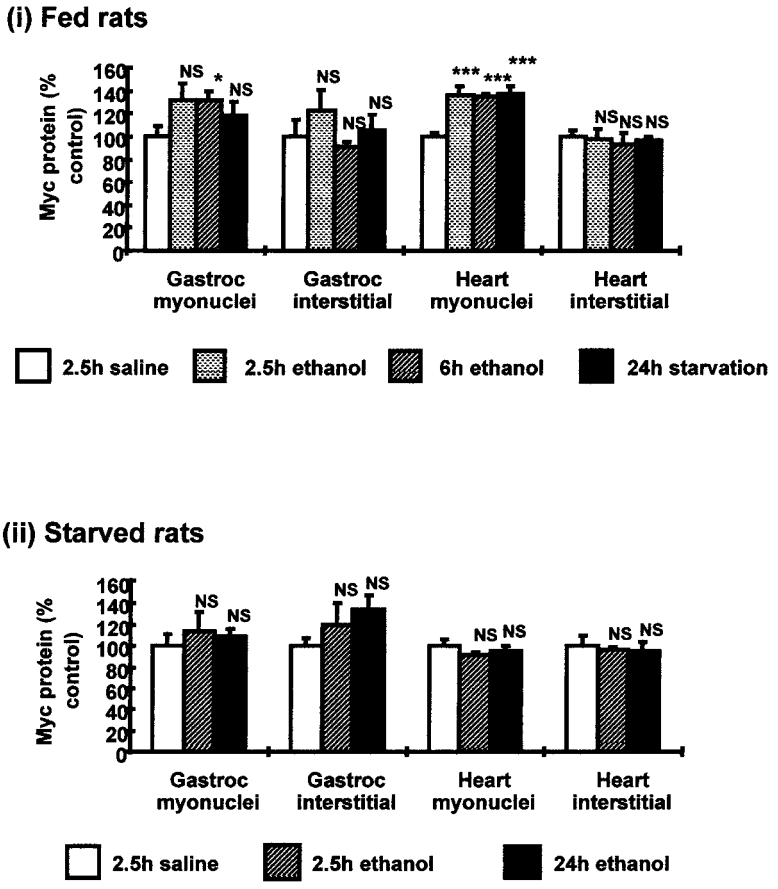

Fig 3. c-Myc protein in the rat gastrocnemius and heart myonuclei and interstitial nuclei after an injection of ethanol (75 mmol/kg BW, injected IP) or saline (0.15 mol/L saline). See Fig 2 for symbols and other details.

many of the same heat shock genes.[45] Several studies relate *c-myc* expression to the heat shock response. For example, in rat heart exposed to myocardial ischemia and reperfusion, expression of hsp70 and *c-myc* are increased.[48] The c-myc protein complex binds to sites in the hsp70 gene promoter region, indicating that c-myc may directly regulate hsp70 expression.[49]

Alternatively, it is possible that the increased c-myc expression may be related to other cellular or metabolic events. Recently, oligonucleotide microarray analysis has shown that myc protein induces a wide variety of genes encoding protein responsible for cell adhesion and cytoskeletal architecture.[20]

CONCLUSIONS

This is the first reported study into proto-oncogene expression in ethanol-exposed skeletal and cardiac muscle. Acute ethanol administration causes a rapid increase in *c-myc* mRNA and protein in skeletal and cardiac muscle, which occur predominantly in the myonuclei. This may represent part of an adaptive cellular defense mechanism against the insults of ethanol.

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