—Original—

Ethanol Dose- and Time-dependently Increases α and β Subunits of Mitochondrial ATP Synthase of Cultured Neonatal Rat Cardiomyocytes

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Mitochondria are target subcellular organelles of ethanol. In this study, the effects of ethanol on protein composition was examined with 2-dimensional electrophoresis of protein extracts from cultured neonatal rat cardiomyocytes exposed to 100 mM ethanol for 24 hours. A putative β subunit of mitochondrial ATP synthase was increased, which was confirmed by Western blot. The cellular protein abundances in the α and β subunits of ATP synthase increased in dose (0, 10, 50, and 100 mM)- and time (0.5 hour and 24 hours)-dependent manners. The DNA microarray analysis of total RNA extract demonstrated that gene expression of the corresponding messenger RNAs of these subunit proteins did not significantly alter due to 24-hour ethanol exposure. Therefore, protein expression of these nuclear-encoded mitochondrial proteins may be regulated at the translational, rather than the transcriptional, level. Alternatively, degradation of these subunit proteins might be decreased. Additionally, cellular ATP content of cardiomyocytes scarcely decreased following 24-hour exposure to any examined concentrations of ethanol. Previous studies, together with this study, have demonstrated that protein abundance of the α subunit or β subunit or both subunits of ATP synthase after ethanol exposure or dysfunctional conditions might differ according to tissue: significant increases in heart but decreases in liver and brain. Thus, it is suggested that the abundance of subunit proteins of mitochondrial ATP synthase in the ethanol-exposed heart, being different from that in the liver and brain, should increase dose-dependently through either translational upregulation or decreased degradation or both to maintain ATP production, as the heart requires much more energy than other tissues for continuing sustained contractions.
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Key words: ethanol, cardiomyocyte, ATP synthase, Western blot, DNA array

Introduction

Mitochondria are subcellular organelles targeted by ethanol; giant mitochondria are sometimes observed in the heart and liver after alcohol intake[1,2]. After abstaining from alcohol, however, most patients with alcoholic cardiomyopathy show improvements of myocardial ultrastructure and of such symptoms as dilated cardiomyopathy[2]. Our previous studies of cultured mouse cardiomyocytes have shown that exposure to ethanol induces the fusion and enlargement of mitochondria and increases their membrane potential[3,4], all in a dose- and time-dependent manner[5]. In contrast, the mitochondrial membrane potential of rat cardiomyocytes tends to decrease dose-dependently at 24 hours, despite increasing significantly just after the cardiomyocytes are exposure to 100 mM ethanol[6].

The ultrastructure of giant mitochondria of cultured mouse cardiomyocytes exposed to higher concentrations of ethanol do not appear to be deteriorated or swollen but, instead, appear to contain more cristae than do the mitochondria of alcoholic patients and animals[3,4]; the *in vivo* observation likely deteriorates consequence of secondary humoral and neuronal effects of ethanol[3,4]. The giant mitochondria observed in cultured cardiomyocytes were speculated to be an adaptive form accompanied by increased mitochondrial proteins in response to ethanol,

which is generally believed to inhibit protein synthesis[6]. In addition, our previous studies have also shown increases in glycogen granules[3] and the production of reactive oxygen species[5], which indicate ethanol-induced alteration in cellular energy metabolism.

In the present study, we examined whether ethanol induces quantitative variation on myocardial proteins, including mitochondrial proteins. Two-dimensional (2-D) electrophoresis of neonatal rat ventricular cardiomyocytes exposed to 100 mM ethanol have indicated quantitative increases in several kinds of mitochondrial protein; for the present study we selected a ethanol concentration of 100 mM because protein content per cell and mitochondrial membrane potential of the cultured rat cardiomyocytes were most significantly altered for 100 mM ethanol in our previous study[6]. To confirm such 2-D electrophoresis results as increases in protein abundance, we performed Western blot analyses and other analyses related to mitochondrial functions.

Materials and Methods
Culture of Cardiomyocytes and Exposure to Ethanol

Ventricular cardiomyocytes were isolated from neonatal rats by a modification of a previously described method[6]. Briefly, hearts removed from 1-day-old Sprague-Dawley rats were dispersed by repeated digestion with 0.06% of collagenase (Worthington Biochemical Corp., Lakewood, NJ, USA) in Ads buffer (130 mM NaCl, 5.4 mM KCl, 0.8 mM NaH_2PO_4, 0.8 mM $MgSO_4$, 5.5 mM glucose, and 20 mM N-2 hydroxyethylpiperazine ethane sulphonate [HEPES], pH 7.4). After each digestion, except the first one, dispersed cells were suspended in newborn calf serum (Sigma-Aldrich Inc., St. Louis, MO, USA), centrifuged, and resuspended in new newborn calf serum. The combined cell suspension was rinsed twice with culture medium (68% M199 [Sigma-Aldrich Inc.], 17% Dulbecco's minimum essential medium [DMEM] [GIBCO™ Invitrogen Corp. Grand Island, NY, USA], 10% heat inactivated horse serum [GIBCO], 5% heat inactivated fetal bovine serum [GIBCO], 100 U/mL penicillin/0.1 mg/mL streptomycin, and 0.1% cytosine β-D-arabinofuranoside), resuspended in fresh culture medium, and preincubated in culture dishes (Nunclon, Nunc, Roskilde, Denmark) for 1 hour in a CO_2 incubator. After preincubation cardiomyocytes were plated in a gelatin-coated dish (φ35 mm; Nunclon, Nunc) at a concentration of $1×10^6$ cells/well for protein extraction and total RNA extraction, or gelatin-coated 96-well plates (Nunclon, Nunc) at a concentration of $5×10^3$ cells/well for determination of cellular ATP con-

tent. One or 2 days after the isolation, the medium was changed to serum-free medium (DMEM : M199=4 : 1) for 24-hour serum starvation before ethanol exposure. The rats were used as approved by "The Animal Ethics Committee of The University of Western Australia" in accordance with "The Australian Code of Practice for the Care and Use of Animals for Scientific Purposes" (National Health and Medical Research Council, 7th Edition, 2004) and by "The Regulations of Animal Experimentation of Nippon Medical School," which are based on "The Guidelines of the International Committee on Laboratory Animals, 1974."

Exposure to ethanol was started by replacing the serum-free medium with 21 mM HEPES-buffered serum-free medium (DMEM : M199=4 : 1) containing ethanol (0, 10, 50, and 100 mM). The plastic culture plates and dishes were covered with a sheet of sterile sticky plate seal (Sumitomo Bakelite Co. Ltd., Tokyo, Japan) to prevent the ethanol from evaporating[6]. The dishes for Western blot and DNA microarray analyses were put into a sealed glass container with an ethanol solution having the same concentration as the serum-free ethanol medium[2]. Then, cardiomyocytes were incubated in a CO_2-free environment at 37°C for 0.5 hour or 24 hours.

Sample Preparation for 2-D Electrophoresis—Protein Extraction

Cardiomyocytes cultivated in culture dishes (φ35 mm) and exposed to 0 or 100 mM ethanol were rinsed once with cold Ads buffer, removed from the bottom of the dishes by trypsinization (0.05% trypsin-ethylenediaminetetraacetic acid [GIBCO] in Ads buffer), and washed twice with cold Ads buffer by centrifugation. The final pellet in an Eppendorf tube was stored at −80°C. The frozen pellet was added with 100 μL of cold 20% (w/v) trichloroacetic acid in acetone, vortexed, and left at −80°C for more than 2 hours for fixation. The fixed pellet was warmed at room temperature for 5 minutes and centrifuged for 30 minutes at 10,000 rpm and 4°C. The pellet was washed 3 times with 1.5 mL each of cold acetone by vortexing and centrifugation (10,000 rpm, 5 minutes, 4°C), and completely dried with a Savant™ SpeedVac™ Concentrator (SVC100H; Thermo Fisher Scientific, Waltham, MA, USA). The dried pellet was then added with 200 μL of multiple surfactant solution (MSS)/Tris buffer (6 M urea, 2 M thiourea, 2.5% [w/v] 3-[(3-cholamindopropyl) dimethylammonio]-1-propanesulfonate, Tris/HCl [pH 8.5]), homogenized with a plastic pestle in an Eppendorf tube, and solubilized by overnight incubation at room temperature. On the following day the homogenate

was centrifuged for 60 minutes at 10,000 rpm and 4°C, and the obtained supernatant (=protein extract of cardiomyocytes) was aliquoted and stored at −80°C. Protein concentration was determined with the Bradford method[7] using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA).

2-D Gel Electrophoresis

The 2-D electrophoresis was performed as described previously[8,9]. Briefly, cardiomyocyte protein samples (150 µg protein in 150 µL of MSS/Tris buffer), Tris-free MSS (150 µL), 6 mM Tri-n-butyl phosphine (0.5 µL) and 2% (v/v) Carrier ampholyte (6 µL of IPG Buffer [pH 3−10]; GE Healthcare Life Sciences, Buckinghamshire UK) were applied to ready-to-use Immobiline™ DryStrip gel strips (linear pH gradient, 3−10; length, 18 cm; GE Healthcare) and subjected to an isoelectric focusing unit (Ettan™ IPGphor™ Isoelectric Focusing System; GE Healthcare) in groups of 2 experimental conditions (exposures to 0 mM [control] and 100 mM ethanol). First, strips were rehydrated for 10 hours at a constant voltage of 200 V at 20°C. Then, the strips were exposed to a linear increase to 1,000 V over 4 hours followed by 8,000 V until a total of 450,000 V-h was reached (for focusing). Before the second dimension separation, strips were equilibrated for 30 minutes at 25°C on an orbital shaker in 10 mL of equilibration buffer (6 M urea, 2% [w/v] sodium dodecylsulphate [SDS], 0.3 M Tris-HCl buffer [pH 8.8], 20% [v/v] glycerol, 2.5% [w/v] acrylamide, 7.4 mM Tri-n-butyl phosphine, 243 mM iodoacetamide). Following equilibration, strips were embedded in a 0.5% (w/v) agarose solution containing a trace amount of bromophenol blue on top of a 12.5% SDS-polyacrylamide gel (0.375 M Tris/HCl [pH 8.8], 0.1% [w/v] SDS, 12.5% [w/v] acrylamide, 0.545% [w/v] bis-acrylamide, 0.1% [w/v] ammonium persulphate, and 0.075% [v/v] N, N, N', N'-tetramethylethylenediamine [TEMED]). The SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in a Protean® II Multicell (Bio-Rad Laboratories) at 9 mA per gel for 16 hours (until the bromophenol blue front was approximately 0.5 cm from the bottom of the gel). After electrophoresis, gels were immediately fixed by soaking twice in 7% (v/v) acetic acid in 50% (v/v) methanol for 30 minutes each and rinsed twice in deionized water for 10 minutes each. The gels were stained in dark with SYPRO® Ruby Protein Gel Stain (Bio-Rad Laboratories) with gentle agitation at room temperature for more than 4 hours, washed twice in wash solution (7% [v/v] acetic acid in 10% [v/v] methanol) for 30 minutes each, and washed twice in deionized water for 5 minutes each.

Stained gels were scanned at a pixel size of 100 µm with a Typhoon™ TRIO Scanner (GE Healthcare), and the obtained gel images were analyzed using ImageMaster™ 2D Platinum Software (version 5.0; Amersham Biosciences, Uppsala, Sweden).

Sample Preparation for Western Blot—Protein Extraction

Cardiomyocytes cultivated in dishes (φ 35 mm) and exposed to 0, 10, 50, or 100 mM ethanol were rinsed twice with cold phosphate-buffered saline (PBS) (−) buffer, put on ice, added with 1.5 mL of cold 20% (v/v) trichloroacetic acid in deionized water, and incubated for 1 hour on ice. Fixed cardiomyocytes in each dish were removed by scraping with a scraper, transferred to an Eppendorf tube, and centrifuged at 3,000g for 5 minutes. The pellet was washed 3 times by addition of cold acetone and centrifugation, completely dried with a Savant™ DNA110 SpeedVac™ (Thermo Fisher Scientific), and stored at −80°C. Protein extraction from the dried pellet and determination of protein concentration were performed in almost the same way as for the protein sample for 2-D electrophoresis. Protein extract was aliquoted and stored at −80°C.

Western Blot

Protein extract was added with one-fifth volume of sample buffer (6×) (0.35 M Tris/HCl [pH 6.8], 10% [w/v] SDS, 30% [v/v] glycerol, 9.3% dithiothreitol, and 0.012% bromophenol blue) and heated at 95°C for 3 minutes. The heated protein sample was loaded and separated on stacking gel (0.125 M Tris/HCl [pH 6.8], 0.1% SDS, 4.39% acrylamide, 0.11% bis-acrylamide, 0.1% ammonium persulfate, and 0.2% TEMED) on top of a 7.5% polyacrylamide gel (0.375 M Tris/HCl [pH 8.8], 0.1% SDS, 7.31% acrylamide, 0.19% bis-acrylamide, 0.1% ammonium persulfate, and 0.075% TEMED), and transferred to a polyvinylidene difluoride (PVDF) membrane (Hybond-LFP, GE Healthcare). Membranes were blocked in 3% ECL Advance Blocking Reagent (GE Healthcare) in Tris (20 mM, pH 7.6)-buffered saline-Tween 20 (TBS-T) with gentle agitation for 1 hour at room temperature and washed with TBS-T. Membranes were incubated overnight at 4°C in 3 fluorescent dye-conjugated primary antibodies in TBS-T, washed with TBS-T, and washed with TBS. Protein bands were detected with a Typhoon™ 9400 Scanner (GE Healthcare) and quantified with ImageQuant TL software (GE Healthcare). The fluorescent dye-conjugated primary antibodies used were as follows: purified mouse anti-ATP synthase α (BD Biosciences, San Jose, CA, USA) was conjugated with Cy3 using the Am-

ersham Cy3 mAb Labelling Kit (GE Healthcare); purified mouse anti-ATP synthase β (BD Biosciences) was conjugated with Cy5 using Amersham Cy5 mAb Labelling Kit (GE Healthcare); and anti-β-actin (AnaSpec Inc., Fremont, CA, USA) was conjugated with fluorescein using Fast-Link Fluorescein Labeling Kit (Abnova Corp., Taipei, Taiwan).

Sample Preparation for Gene Expression Analysis─Total RNA Isolation

At the end of ethanol exposure cardiomyocytes were washed once with cold PBS(−) buffer, removed from the bottom of the dish by trypsinization, and washed with cold PBS(−) by centrifugation.

Tris (2-carboxyethyl) phosphine (280 μg/sample) was added to the final cell pellet, vortexed, and stored at −80°C. Total RNA was extracted from the cell pellet using NucleoSpi® RNA XS (Macherey-Nagel GmbH & Co. KG, Düren, Germany). The purity of the total RNA extract was confirmed by applying it to RNA Chips (Agilent RNA 6000 Pico Kit, Agilent Technologies, Santa Clara, CA, USA) and analyzing with 2100 Bioanalyzer (G2939A, Agilent).

Gene Expression Analysis

The total RNA extract was labeled with Cy3 fluorescent dye with the Low Input Quick Amp Labeling Kit (Agilent) and purified with Absolutely RNeasy Mini Kit (Qiagen, Venlo, Netherlands). The Cy3-labeled total RNA was hybridized to SurePrint G3 Rat GE 8×60K Microarray (Agilent) using Gene Expression Hybridization Kit (Agilent), washed with Gene Expression Wash Buffer Kit (Agilent), and dried. The fluorescence of Cy3 of Microarray was scanned with Agilent G2565CA Microarray Scanner System (Agilent) and digitalized with Feature Extraction 11.5 software (Agilent). Digitalized signal values were normalized to correct differences between DNA arrays and signal baselines and transformed to fold changes calculated as the ratio of expression values of ethanol groups (10 mM, 50 mM, and 100 mM) against that of the 0 mM group with GeneSpring (version 12.6.1, Agilent) software. Then, the fold changes of messenger (m) RNA expression of 3 sets of ethanol groups were analyzed with Ingenuity Pathways Analysis (Qiagen) software.

ATP Assay

Cardiomyocytes cultured in a 96-well plastic plate were exposed to ethanol in 100 μL of 21 mM HEPES-buffered serum-free medium. Cellular ATP content was assayed by detecting luciferin-luciferase chemiluminescence reaction. Twenty-four hours after ethanol exposure

was started, the plate was placed at room temperature for 30 minutes. To each well 100 μL of "Cell" ATP Assay Reagent (Wako Pure Chemical Industries, Ltd., Osaka, Japan) containing D-luciferin and luciferase was added and mixed well in dark on a microplate shaker for 1 minute. After the plate had been incubated in the measuring chamber of a plate reader (Wallac 1420 ARVOMX/Light, PerkinElmer Inc., Turku, Finland) for 10 minutes at 23°C, chemiluminescence was measured at 23°C.

Statistics

Student's t-test was used for statistical analysis of 2-D electrophoresis protein spots. A 2-factor analysis of variance (2-way ANOVA) for time and ethanol group and a 1-factor ANOVA (1-way ANOVA) of Western blot data were performed with the standard statistics software "StatView," Version 4.02 (Abacus Concepts, Inc., Berkley, CA, USA). A multiple comparison was performed with the Fisher's protected least significant difference as a post hoc test. Differences with values of $p<0.05$ were considered to be statistically significant.

Results

2-D Gel Electrophoresis

Figure 1 (a) shows one of the SYPRO™ Ruby fluorescence images of 2-D electrophoresis gel of protein extract from the control cardiomyocytes. **Figure 1 (b)** shows cropped spots with their ID automatically numbered by ImageMaster™ 2D Platinum Software. When the fluorescence intensity of each spot was expressed as the percentage of volume of total fluorescence intensity of all cropped spots on the gel, the percentage of volume of 3 cropped spots (ID: 29,050, 29,059, and 29,181) in the 100 mM ethanol group increased significantly compared with that in the control group (**Table 1**; $p<0.05$, Student's t-test, n=5).

In reference to a HEART-2DPAGE website (http://userpage.chemie.fu-berlin.de/˜pleiss/bild1.html) 2 large spot clusters on the upper-left corner of the gel (**Fig. 1b**) might be inferred to be α-actin and mitochondrial ATP synthase β subunit (ID: 29,181). Similarly, at least one of the other spots (ID: 29,050 and 29,059) might be inferred to be ATP synthase α subunit.

Western Blot

Cardiomyocyte protein extracts obtained from the same primary culture in groups of 4 experimental conditions (exposures to 0, 10, 50, and 100 mM ethanol) were separated by SDS-PAGE, and the fluorescent image of the PVDF membrane stained with 3 kinds of fluorescent dye-conjugated primary antibodies for 3 proteins (α subunit
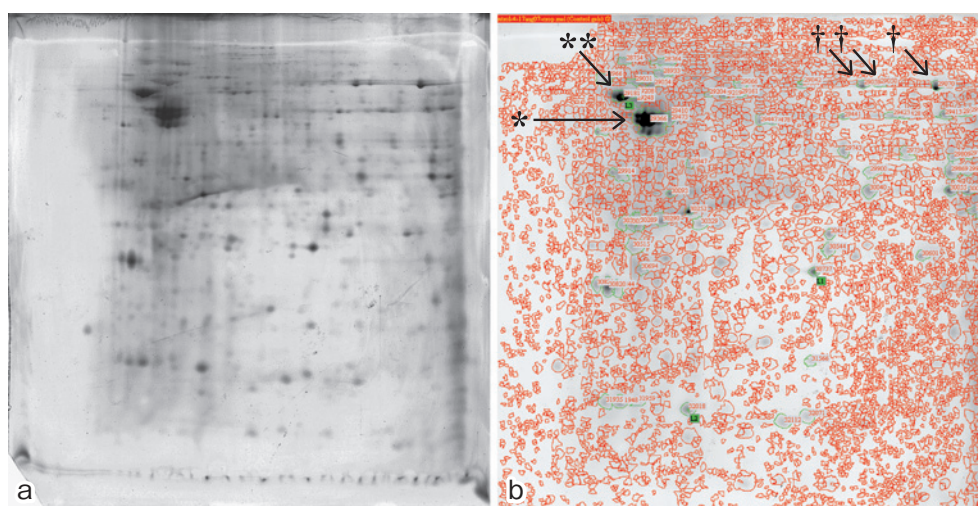
Fig. 1   Scanned gel image of two-dimensional (2-D) electrophoresis of protein extract from cultured rat cardiomyocytes

(a) The 2-D electrophoresis acrylamide gel of control cardiomyocyte protein extract was stained by SYPRO™ Ruby and scanned with Typhoon™ Scanner. (b) Scanned gel fluorescence image was analyzed using ImageMaster™ 2D Platinum software to obtain a cropped image. For many spots IDs were numbered automatically. Spots of α actin (*) and mitochondrial ATP synthase β subunit (**) were inferred in reference to a HEART-2DPAGE website (http://userpage.chemie.fu-berlin.de/~pleiss/bild1.html). Similarly, at least 1 of 3 unknown spots (†) might be inferred to be mitochondrial ATP synthase α subunit.

Table 1   SYPRO™ Ruby fluorescence intensity of 2-D electrophoresis spots of protein extract from rat cardiomyocytes following 24-hour exposure to 0 and 100 mM ethanol

Spot Number	0 mM ethanol		100 mM ethanol		p value
	mean	SD	mean	SD	
29050	0.447	0.091	0.639	0.110	0.017
29059	0.550	0.264	0.985	0.281	0.035
29181	4.442	0.980	5.886	0.865	0.038

Fluorescence intensity of each protein spot was expressed as percentage of volume of total fluorescence intensity of all cropped spots on the gel; means and SD of protein spots of 5 pairs of 2-D electrophoresis gels. Student's *t*-test was performed between 0 and 100 mM groups.

β-subunit

α-subunit

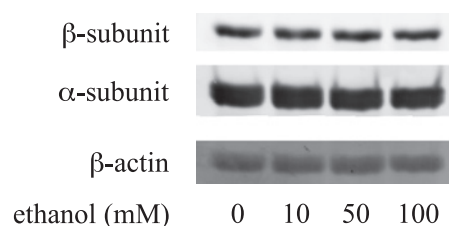β-actin

ethanol (mM)   0   10   50   100

Fig. 2   Representative Western blot of protein extract from cultured rat cardiomyocytes exposed to ethanol (0, 10, 50, and 100 mM) for 24 hours. Western blot was performed with standard sodium dodecylsulphate-polyacrylamide gel electrophoresis. The blotted polyvinylidene difluoride membrane was incubated simultaneously with 3 kinds of fluorescent dye-conjugated primary antibodies for 3 proteins (α subunit and β subunit of mitochondrial ATP synthase and β actin) and detected with Typhoon™ Scanner, and the obtained fluorescent image was quantified with ImageQuant TL software.

and β subunit of mitochondrial ATP synthase and β-actin) was obtained (**Fig. 2**). Fluorescence intensities of the α and β subunits of mitochondrial ATP synthase were normalized by using that of β actin on the same lane as a reference. **Figure 3** represents relative fold changes of protein abundances calculated as the ratios of normalized fluorescence band intensities of the α subunit (a) and β subunit (b) of mitochondrial ATP synthase of ethanol-exposed cardiomyocytes against that of the corresponding control; means and SD of 3 independent primary cultures. Two-way ANOVA analysis demonstrated

that after 24-hour exposure to ethanol protein abundances in both subunits of ATP synthase increased dose-dependently, although there were no significant alterations in those protein abundances at 0.5 hour (**Fig. 3**). There were also statistical significances between 10 mM and 100 mM ethanol groups and between 0 mM and 100 mM groups in both subunit protein abundances (**Fig. 3**).
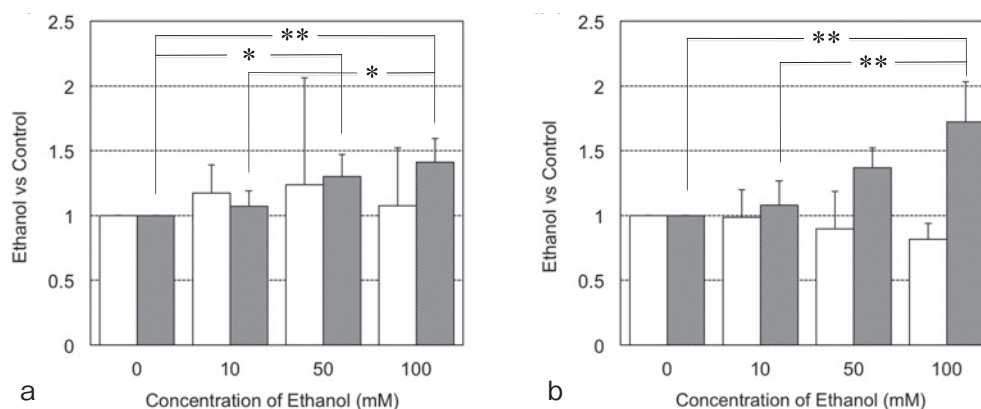
Fig. 3 Relative fold change of protein abundance in α subunit (a) and β subunit (b) of cardio-myocyte mitochondrial ATP synthase normalized against that in β actin between 0.5-hour (open) or 24-hour (shaded) ethanol exposure and corresponding control; means and SD of 3 independent primary cultures. Two-way and 1-way analysis of variance demonstrated that protein abundance of both subunits of ATP synthase increased dose- and time-dependently. *, $p<0.05$; **, $p<0.01$.

Table 2 Cellular ATP content of cardiomyocytes following 24-hour ethanol exposure

ethanol concentration (mM)	normalized value	
	mean	SD
0	1	-
10	0.929	0.086
50	0.950	0.187
100	1.054	0.350

Cellular ATP content was assayed by detecting luciferin-luciferase chemiluminescence reaction. Normalized values were means and SD of 3 independent experiments. In each experiment an average of chemiluminescence intensity values of each ethanol group was normalized to that of the corresponding control (0 mM) (each group has 3 to 6 intensity values).

Gene Expression Analysis and ATP Assay

Gene expression on DNA microarray of the ATP synthase α subunit and β subunit of cardiomyocytes after 24-hour exposure to ethanol showed no significant alteration by analysis with GeneSpring and Ingenuity Pathways Analysis softwares. Fold changes calculated as the ratio of expression values of ethanol groups (10, 50, and 100 mM) against that of 0 mM group were as follows: α subunit, −1.071, −1.077, and −1.087; β subunit, −1.038, −1.132, and −1.144.

Cellular ATP content of cardiomyocytes did not alter following 24-hour exposure to any concentrations of ethanol (**Table 2**).

Discussion

We performed 2-D electrophoresis to examine how etha-nol exposure alters cellular protein abundance in cultured rat cardiomyocytes. This study is novel because standard 2-D electrophoresis isoelectric focusing/SDS-PAGE studies focusing on ethanol effects have exclusively focused on using tissues sampled from animals chronically fed with ethanol: heart[10–12], liver[12–14], and kidney[12].

Following 24-hour exposure to 100 mM ethanol, the putative β subunit of mitochondrial ATP synthase (Complex V) was found to have increased in protein abundance (**Fig. 1**). This observation was confirmed by Western blot, which demonstrated that both the α subunit and β subunit proteins of the ATP synthase increased in a dose- and time-dependent fashion (**Fig. 3**). Because ethanol does not increase the proliferation of cultured rat cardiomyocytes[4], the increase in the subunit proteins is likely a result of an increase in the cellular abundance of these proteins. There is a possibility that giant mitochondria that appear at higher concentrations of ethanol[3,4] would have more of these subunits than do normal mitochondria, but this remains to be established. In addition, the increase in the α and β subunits might reflect a general increase in Complex V. Increases in other subunits of Complex V were likely not detected because of their lower abundance relative to the α and β subunits; the Complex V enzyme consists of 3 copies of each subunit of α and β and only 1 copy of other subunits[15].

No 2-D electrophoresis studies of ethanol-exposed hearts have described alterations in the abundances of the α and β subunits of ATP synthase. However, 5 studies with 2-D electrophoresis have reported the effects of

various agents and conditions other than ethanol on the abundance of the heart ATP synthase. Exposure to endothelin 1 for 48 hours induced cardiac hypertrophy and increased the abundance of the β subunit of ATP synthase in HL-1 cells[16] but did not change its abundance in neonatal rat cardiomyocytes[8]. In iron-overloaded, hypertrophied rat heart mitochondria both the α and β subunits of ATP synthase were increased[17]. Valdecoxib, a nonsteroidal anti-inflammatory drug, increased abundance in the β subunit of ATP synthase of neonatal rat cardiomyocytes[18]. In contrast, cardiac resynchronization therapy for canine hearts with dyssynchronous failure decreased both the α and β subunits of ATP synthase of adult heart mitochondria[16]. Additionally, proteomic analysis by liquid chromatography-tandem mass spectrometry of isolated heart mitochondria from mice with cardiomyocyte deletion of insulin receptors elucidated increases in both the α and β subunits of ATP synthase[20]. Together, these studies have demonstrated that a variety of conditions, including ethanol, as we have shown, can affect the abundance in the α and β subunits of ATP synthase of cardiomyocytes or hearts.

Quantitative alteration of ATP synthase subunits in tissues other than the heart has been found in 5 studies. Studies with 2-D electrophoresis of liver mitochondria from rats chronically exposed to ethanol have shown the abundances of both the α and β subunits of ATP synthase were decreased[14] or unchanged[13]. Protein abundance of the β subunit of ATP synthase was shown by 2-D electrophoresis to decrease in the skeletal muscle of patients with diabetes[21]. Liquid chromatography-tandem mass spectrometry analysis has shown that in the brains of fetal mice exposed to ethanol for 1 week *in utero*, the α subunit of ATP synthase was decreased[22]. In cell lines derived from neuroblastoma, kidney, and adipocytes, insulin-like growth factor 1 was shown with Western blot to increase the β subunit of ATP synthase[23]. Taken together with the present study, it appears that the abundance of the α subunit or β subunit or both subunits of ATP synthase is affected by dysfunction in a range of tissues, such as the heart, liver, and brain.

In contrast to the protein abundance measures, the present DNA microarray analysis of cultured cardiomyocytes demonstrated that the corresponding mRNAs of these subunit proteins do not increase but, instead, slightly decrease due to ethanol exposure. Therefore, protein expression of these nuclear-encoded mitochondrial subunits may be regulated to increase, not at a transcriptional level but at a translational level. Alternatively, degradation of these subunit proteins might be decreased[19]. It remains to be established whether either mechanism or both mechanisms are involved in the protein increments of heart ATP synthase subunits.

Despite the dose-dependent increase in protein abundance of the ATP synthase subunits (**Fig. 3**), the cellular ATP content of rat cardiomyocytes did not change in any examined concentrations of ethanol (**Table 2**). In other studies, inconsistent results have been obtained for the effects of ethanol on the ATP content of tissues or cells. In the hearts of dogs and rats chronically fed with ethanol, myocardial ATP content was reported to be decreased[24-26] or unchanged[27,28] at estimated ethanol concentrations of 10 to 30 mM[29,30]. Similar findings have been obtained in studies with mitochondria from the liver[31,32] and brain[33], where the maximum blood ethanol concentration is presumed to be no more than 30 mM. Similarly, in the liver acutely perfused with ethanol, the ATP content was lowered with about 60 mM of ethanol but not with about 10 mM of ethanol[34] but was shown to decrease with 10 to 70 mM ethanol when evaluated with ^{31}P nuclear magnetic resonance[35,36]. Taken together, the ATP content in tissues, such as heart, liver, and brain, may decrease or not be affected by ethanol concentrations of 30 mM or less, but the ATP content appears to be maintained in the heart but not in the liver or brain by ethanol concentrations greater than 30 mM.

Most myocardial ATP is produced by mitochondrial ATP synthase (=Complex V). The effects of ethanol on the enzymatic activity of oligomycin-sensitive ATPase (=Complex V) of the heart have been examined, but inconsistent results have been reported: the activity decreased or was unchanged[25,37,38]. The ATPase activity of rat liver mitochondria was decreased after chronic feeding with ethanol[39,40]. A tissue-specific difference in the enzymatic activity of ATP synthase in ethanol was clearly demonstrated by 2 studies using 3 tissues—heart, liver and brain—simultaneously obtained from ethanol-fed adult rats[41] and from neonatal rats maternally exposed to ethanol[42]. Significant increases were evident in heart, but decreases occurred in the liver and brain. These tissue-specific differences in enzymatic activity may be a consequence of changes in the protein abundance of ATP synthase.

Clearly, both rat heart[43] and the human heart[44] require much more energy per tissue weight than do the liver and brain for continuing sustained contractions, although the reason for the ethanol-induced discrepancy in abundance of ATP synthase among heart, liver, and brain re-

mains to be determined. A possible explanation for the increased abundance of subunit proteins of the heart mitochondrial ATP synthase at ethanol concentrations over 30 mM is either translational upregulation or decreased degradation to maintain ATP production. However, the exact mechanism whereby ethanol increases the 2 subunits of heart mitochondrial ATP synthase remains unclear. Such an increase of the particular protein under deteriorated situations is likely an example of the typical cellular adaptations. In addition, it should be noted that such an adaptive response at protein level does not necessarily arise through upregulation of mRNA transcription.

Conflict of Interest: The authors declare no conflict of interest.

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