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Chronic ethanol feeding impairs AMPK and MEF2 expression and is associated with GLUT4 decrease in rat myocardium

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Abbreviations: AICAR, 5-amino-4-imidazolecarboxamide ribonucleotide; AMPK, AMP-activated protein kinase; GLUT4, glucose transporter 4; MEF2, myocyte enhancer factor 2

Abstract

Chronic and heavy alcohol consumption is one of the causes of heart diseases. However, the effects of ethanol on insulin sensitivity in myocardium has been unclear. To investigate the effects of ethanol on the expression of AMP-activated protein kinase (AMPK), myocyte enhancer factor 2 (MEF2) and glucose transporter 4 (GLUT4), all of which are involved in the regulation of insulin sensitivity, in the myocardium, we performed three parts of experiments *in vivo* and *in vitro*. I: Rats were injected with 5-amino-4-imidazolecarboxamide ribonucleotide (AICAR, 0.8 mg·kg^{-1}) for 2 h. II: Rats received different dose (0.5, 2.5 or 5 g·kg^{-1}·d^{-1}) of ethanol for 22-week. III: Primary neonatal rat cardiomyocytes were isolated and treated with or without 100 mM ethanol or 1 mM AICAR for 4 h. The cardiac protein and mRNA expression of AMPKα subunits, MEF2 and GLUT4 were observed by western-blotting and RT-PCR, respectively. Serum TNFα levels were assessed by ELISA. The results showed chronic ethanol exposure induced insulin resistance. Ethanol decreased the mRNA levels of *AMPKα1* and *α2*, the protein levels of total- and phospho-AMPKα in cardiomyocytes. Similarly, ethanol showed inhibitory effects on both the mRNA and protein levels of MEF2A and 2D, and GLUT4 in a dose-response-like fashion. Correlation analysis implied an association between phospho-AMPKα and MEF2A or MEF2D, and between the levels of MEF2 protein and GLUT4 transcription. In addition, ethanol elevated serum TNFα level. Taken together, chronic ethanol exposure decreases the expression of AMPKα and MEF2, and is associated with GLUT4 decline in rat myocardium.

Keywords: AMP-activated protein kinases; ethanol; glucose transporter type 4; myocardium; myocyte-specific enhancer-binding factor 2

Introduction

Ethanol exposure is associated with impaired glucose homeostasis. The consumption of heavy amounts of alcohol increases serum glucose levels (Fowman, 1988), impairs glucose intolerance and induces insulin resistance (Risinger and Cunningham, 1992). Chronic and heavy alcohol consumption has been strongly hintly as an independent risk factor for the development of type 2 diabetes in some populations (Tsumura *et al*., 1999; Wei *et al*., 2000; Feng *et al*., 2008; Zhao *et al*., 2009), even raising the risk and mortality of coronary heart diseases (Onat *et al*., 2009). However, the mechanism by which ethanol disrupts glucose homeostasis, especially in heart, the recognized target organ of diabetes, remains obscure.

Glucose transporter (GLUT) 4, the predominant glucose transporter in the heart, skeletal muscle and adipose tissue, is important for the regulation of glucose homeostasis. However, the effect of ethanol on GLUT4 expression, and its corresponding mechanism, is complex and unclear. There is considerable evidence that activation of AMP-activated protein kinase (AMPK) is involved in GLUT4 translocation and glucose uptake. In cardiac myocytes specifically, increased glucose uptake induced by the translocation of GLUT4 requires the activation of AMPK (Russell *et al*., 1999). The induction of myocardial GLUT4 translocation via AMPK phosphorylation by resveratrol and niacin-bound chromium treatments in diabetic rats has further confirmed the regulatory effect of

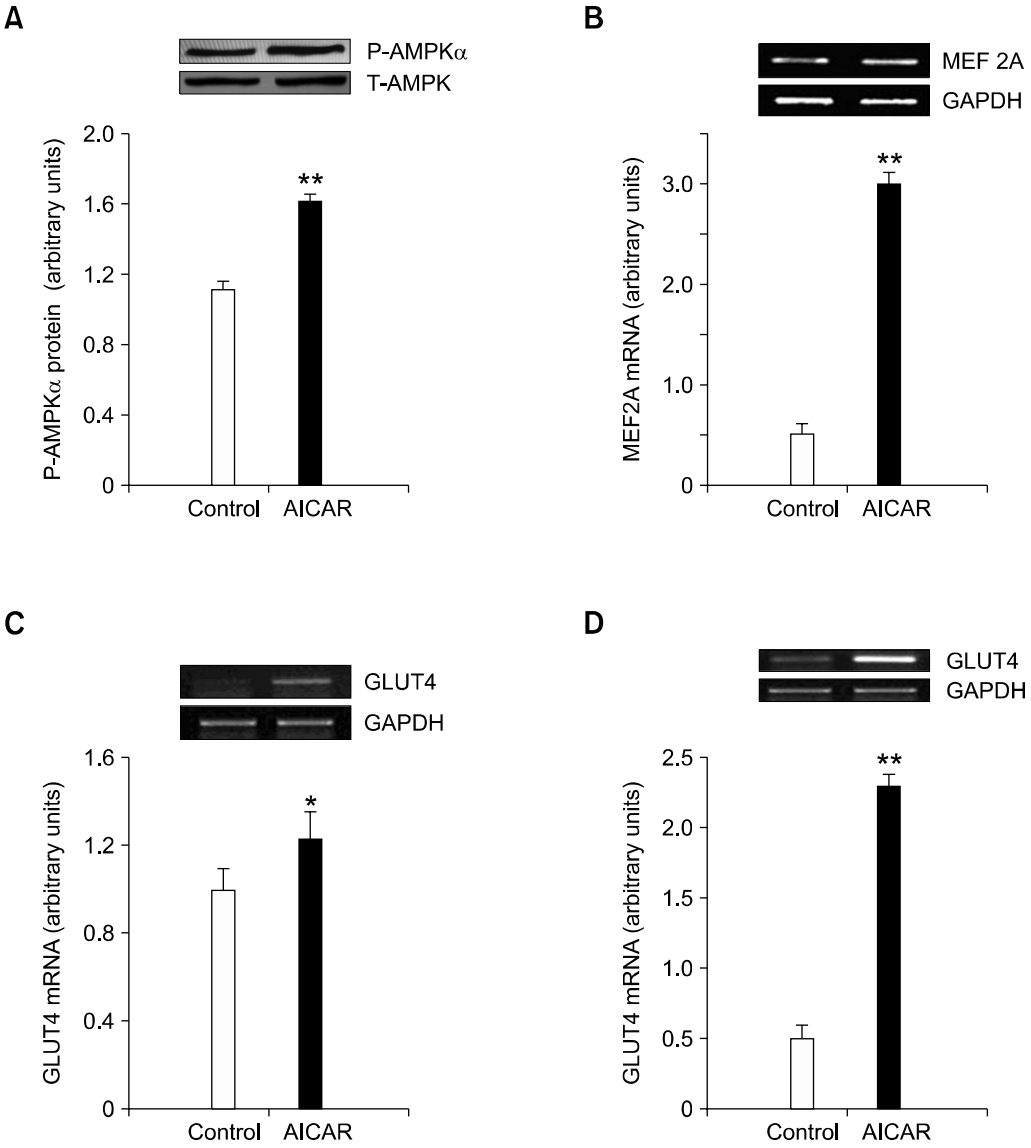

Figure 1. Effects of AICAR on cardiac P-AMPKα, MEF2A and GLUT4 expression. Male Wistar rats or primary cardiomyocytes were treated with AICAR. The P-AMPKα protein in cardiomyocytes (A), MEF2A mRNA in myocardium (B), GLUT4 mRNA in cardiomyocytes (C) and myocardium (D) were examined by western blotting and RT-PCR, respectively. Data show a representative gel of P-AMPKα protein, MEF2A mRNA and GLUT4 mRNA products, and a bar graph of data quantitated as relative expression (a.u.) normalized by T-AMPK or GAPDH (mean ± SD) (n = 5). $^{**}P < 0.01$, $^{*}P < 0.05$ compared with control group.

AMPK on GLUT4 (Penumathsa *et al*., 2008, 2009). Analysis of the *GLUT4* gene promoter revealed that a conserved binding site for the myocyte enhancer factor (MEF) 2 transcription factor is essential for human skeletal muscle GLUT4 expression (Thai *et al*., 1998). In support of this, MEF2 DNA binding activity was substantially reduced in nuclear extracts isolated from both cardiac and skeletal muscle of STZ-induced diabetic mice, and this reduction correlated with change of GLUT4 gene expression (Thai *et al*., 1998). Furthermore, this regulation required the activation of AMPK, which is considered necessary for MEF2 transcription (Thai *et al*., 1998; Ju *et al*., 2005). GLUT4 could also be regulated by some inflammatory factors, such as TNFα, which markedly suppressed GLUT4 expression in 3T3-L1 adipocytes (Rotter *et al*., 2003). To date, it is far less clear whether ethanol is able to affect the expression of AMPK, MEF2 and GLUT4 in myocardium.

In the present study, we first tested, *in vivo*, whether the activated AMPK could indeed affect the expression of MEF2 and GLUT4 in the

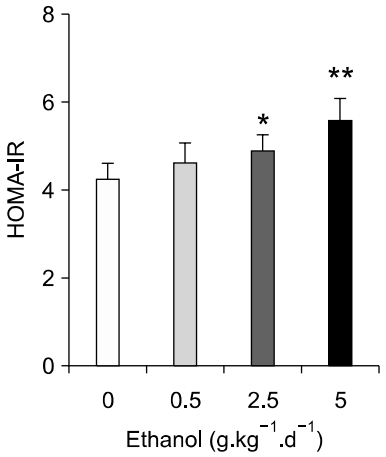

Figure 2. Effect of ethanol on insulin sensitivity in rats. Male Wistar rats were fed with different dose of ethanol (0.5, 2.5 or 5 g·kg^{-1}·d^{-1}) for 22 weeks. Fasting blood samples were collected on the day before sacrifice to measure blood glucose levels and insulin concentrations. Insulin resistance was estimated by the HOMA-IR method and defined as fasting serum insulin (μU/ml) × FPG (mM)/22.5. $^{**}P < 0.01$, $^{*}P < 0.05$ compared with control group.

myocardium. We then investigated, both *in vivo* and *in vitro*, the influences of long-term ethanol consumption on myocardial GLUT4 and whether this is associated, to a certain extent, with AMPK and MEF2 changes.

Results

GLUT4 expression was positively regulated by the activated AMPK in rat myocardium

In order to explore the association among AMPK, MEF2 and GLUT4 in the myocardium, we examined their cardiac expression levels in rats and cardiomyocytes treated with AICAR. Our data showed in cardiomyocytes, AICAR significantly stimulated the phosphorylation of AMPKα by 70.25% over the basal level ($P < 0.01$) (Figure 1A). *In vivo*, AICAR resulted in a 4.9-fold up-regulation of the mRNA levels of MEF2A (Figure 1B) compared with the controls, with no significant change in the levels of MEF2D mRNA. In addition, AICAR caused a 0.24-fold ($P < 0.05$) and 4.5-fold ($P < 0.01$) increase in the level of GLUT4 mRNA in cardiomyocytes (Figure 1C) and myocardium of the rats (Figure 1D) respectively. These results suggest, therefore, that the expression of GLUT4 in myocardium can be stimulated by AMPK activation and the subsequent upregulation of MEF2A.

Long-term ethanol administration induced insulin resistance

Homeostasis model assessment of insulin resistance (HOMA-IR) has been validated as a surrogate for insulin resistance in non-diabetic

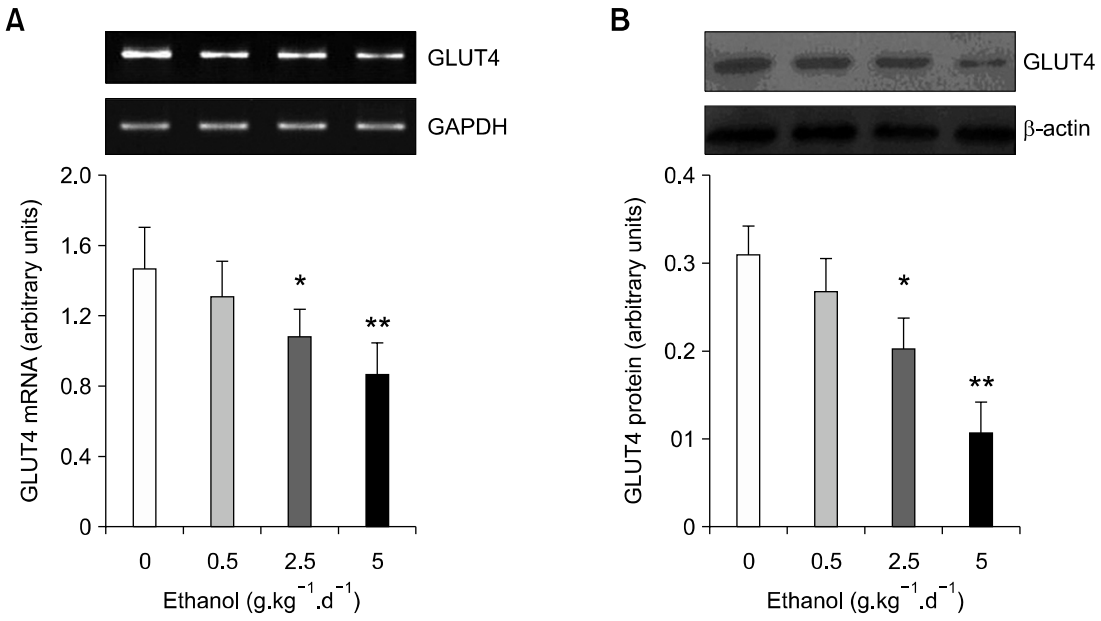

Figure 3. Effect of ethanol on GLUT4 expression. Left ventricular muscle was from the same rats as mentioned in Figure 2. GLUT4 mRNA and protein analysis were performed by RT-PCR (A) and western blotting (B), respectively. The mean density of bands was quantified using Alpha Imager 2200 software, with GAPDH/β actin as the internal control. $^{**}P < 0.01$, $^{*}P < 0.05$ compared with control group.

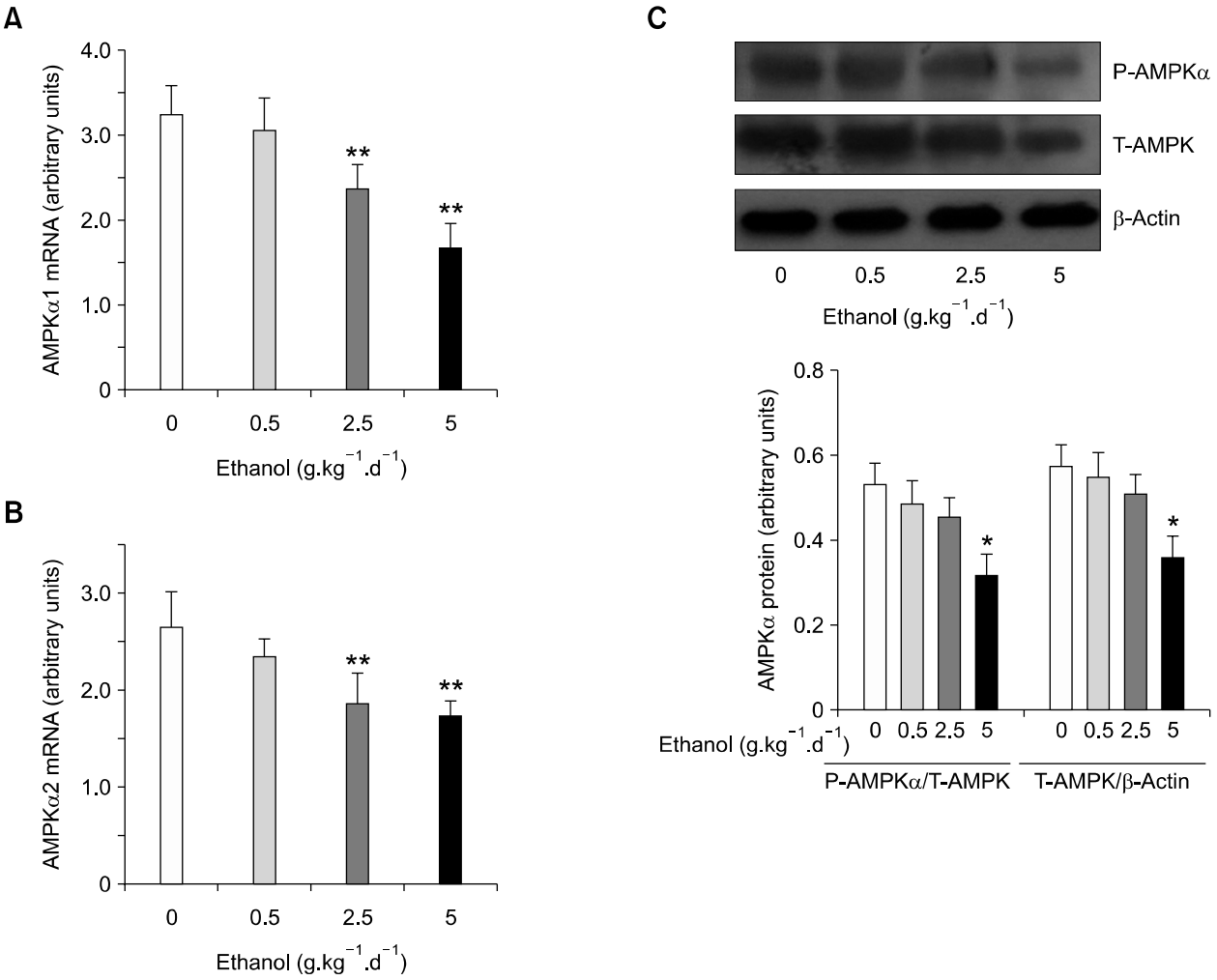

Figure 4. Effect of ethanol on AMPK expression. Tissues were prepared the same as in Figure 2. AMPKα1 (A) and α2 (B) mRNA, total- and phosphorylated- AMPKα protein (C) levels were measured by RT-PCR and western blotting, respectively. The quantification processes were similar with those in Figure 3. $^{**}P < 0.01$, $^{*}P < 0.05$ compared with control group.

children, with studies showing correlations as high as 0.91 with clamp or FSIVGTT measures (Yeckel *et al*., 2004; Keskin *et al*., 2005). Here we found that after 22-week ethanol treatment, the rat HOMA-IR values in L-, M- and H-groups were 4.59, 4.87 and 5.58, respectively (Figure 2). In addition, the HOMA-IR values for M- ($P < 0.05$) and H- ($P < 0.01$) groups were significantly increased when compared with the control group (4.21). Therefore, long-term ethanol administration induced insulin resistance in rats.

Long-term ethanol administration reduced GLUT4 expression in rat myocardium

RT-PCR and western blotting were used to investigate the effect of chronic ethanol intake on the expression level of GLUT4 within myocardium. GLUT4 expression, at both the mRNA and proteins levels, was decreased in all three chronic ethanol feeding groups. The GLUT4 mRNA levels were decreased by 10.3%, 26.3% ($P < 0.05$), and 41% ($P < 0.01$) in L-, M- and H- groups, respectively, relative to the control group (Figure 3A). Similar results were seen at the protein level, which was diminished in a dose-dependent manner by 3.98%, 30.13% ($P < 0.05$), and 63.91% ($P < 0.01$) with the administration of low-, medium- and high-dose of ethanol, respectively, compared with the pair-fed group (Figure 3B).

Long-term ethanol administration suppressed AMPKα mRNA and protein expression and activation in the myocardium of rats

The effects of ethanol on the mRNA levels of the

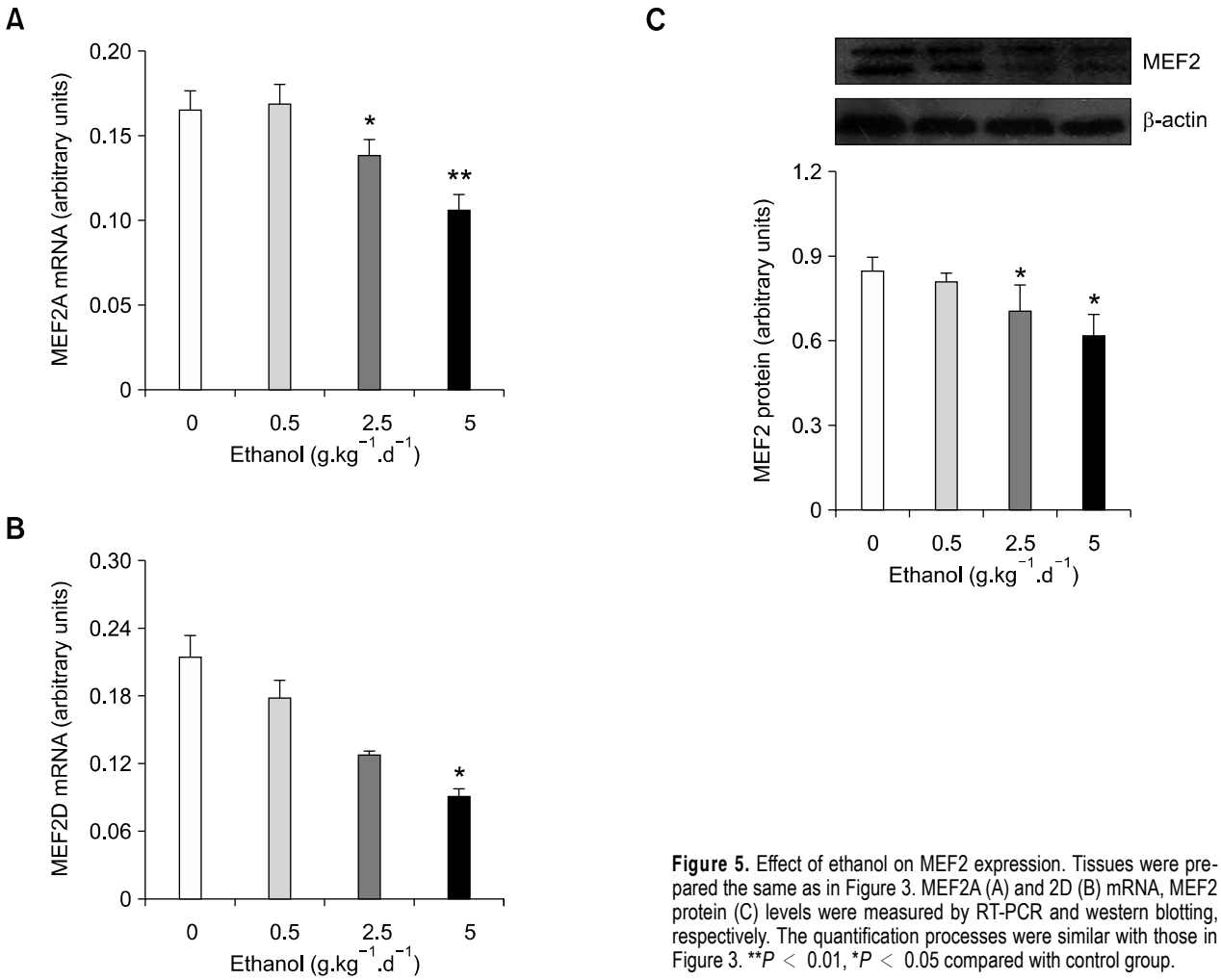

Figure 5. Effect of ethanol on MEF2 expression. Tissues were prepared the same as in Figure 3. MEF2A (A) and 2D (B) mRNA, MEF2 protein (C) levels were measured by RT-PCR and western blotting, respectively. The quantification processes were similar with those in Figure 3. $^{**}P < 0.01$, $^{*}P < 0.05$ compared with control group.

AMPKα1 and α2 isoforms were examined next. Compared with the controls (Figure 4A), low-, medium- and high-dose of ethanol lowered the mRNA levels of AMPKα1 by 15%, 36.9% ($P < 0.01$) and 49.5% ($P < 0.01$), respectively. The levels of AMPKα2 mRNA were attenuated by 11.8%, 30.5% ($P < 0.01$) and 34.5% ($P < 0.01$) with low-, medium- and high-dose of ethanol treatment, respectively (Figure 4B). Thus, ethanol administration caused a decrease in the mRNA levels of both the AMPKα1 and α2 isoforms, in a dose-dependent manner.

In addition, there was a stronger down-regulation of AMPKα1 than of AMPKα2, which implied that ethanol has a greater inhibitory effect on AMPKα1 gene transcription.

The effect of ethanol on the levels of total AMPK (T-AMPK) α protein was also examined. Compared with the control group, animals treated with ethanol showed a marked decrease in the expression of T-AMPKα protein in myocardium by 0.79%, 10.89%, and 37.72% ($P < 0.05$) in the L-, M- and H-groups, respectively (Figure 4C).

Since the phosphorylation of the Thr-172 residue within the activation loop of the AMPKα catalytic domain is essential for the activation of both the α1 and α2 subunits of AMPK, the activation state of total AMPKα was assessed by determining the phosphorylation of AMPKα using a specific phosphor-Thr-172 antibody in western blotting analysis. Ethanol exposure caused decline in the level of P-AMPKα, with 3.11%, 8.25% and 32.56% ($P < 0.05$) by low-, medium- and high-dose, respectively (Figure 4C).

Long-term ethanol administration inhibited mainly the MEF2A isoform in rat myocardium

The mRNA levels of MEF2A and 2D isoforms were examined in all groups. Compared with the control, the mRNA level of the MEF2A isoform diminished 17.24%, 40.80% ($P < 0.05$) and 57.68% ($P <$

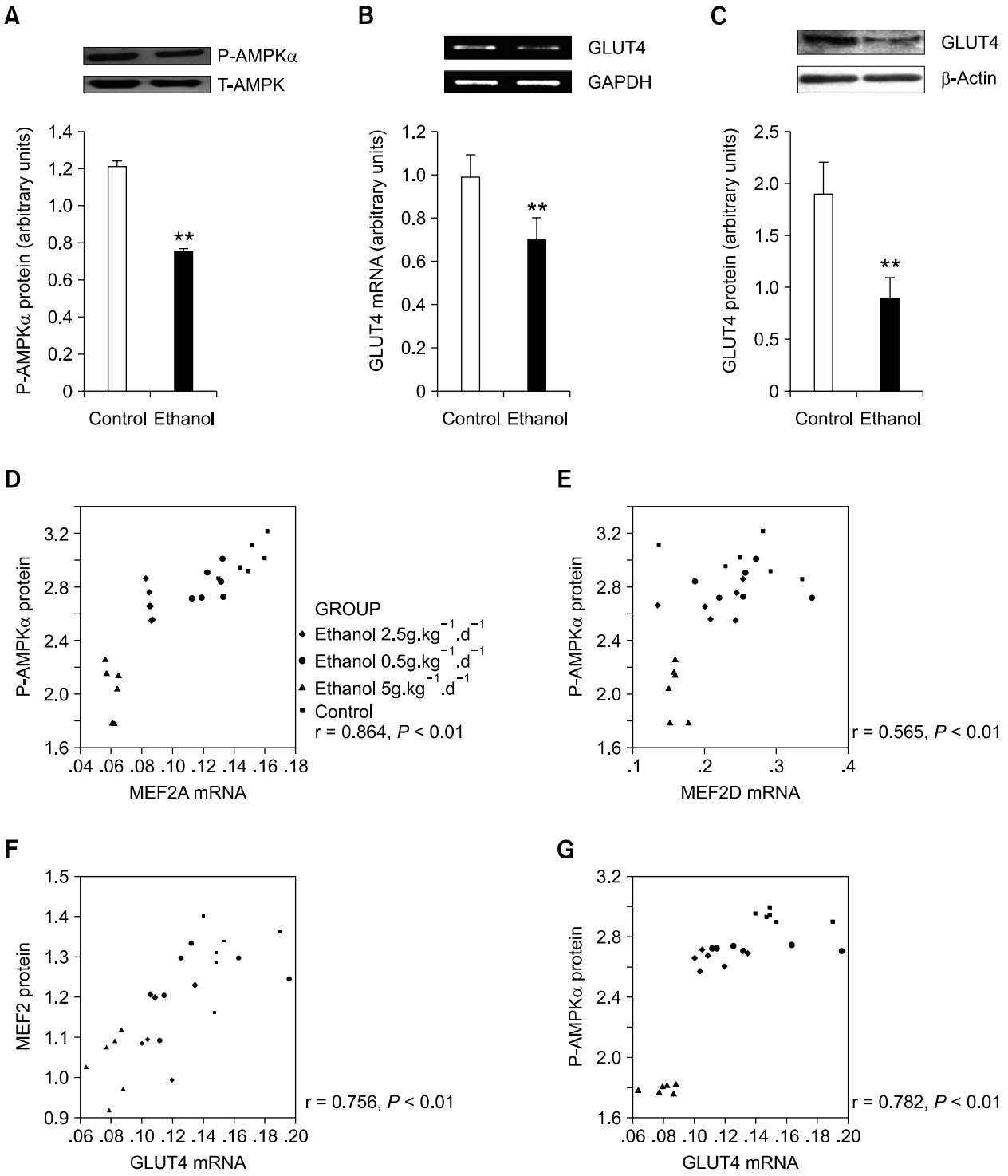

Figure 6. Effect of ethanol on the expression of P-AMPK and GLUT4 *in vitro*. Primary neonatal Wistar rat cardiomyocytes were prepared and incubated for 4 h in the presence of 100 mM ethanol. P-AMPKα protein (A), GLUT4 mRNA (B) and protein (C) were assayed by western blotting and RT-PCR, respectively. An association analyses were made between P-AMPKα protein and MEF2A (D) or MEF2D (E) mRNA, MEF2 (F) or P-AMPKα (G) protein and GLUT4 mRNA. $^{**}P < 0.01$, $^{*}P < 0.05$ compared with control group.

0.01) in L-, M- and H-groups, respectively (Figure 5A). The MEF2D mRNA levels were decreased by 16.17% and 35.79% ($P < 0.05$) in M- and H-groups, respectively, with no change in low dose

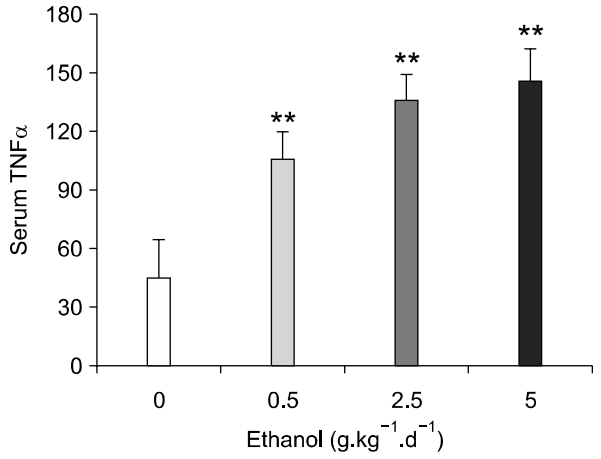

Figure 7. Effect of ethanol on serum TNFα level. Rat blood samples were prepared as mentioned in Figure 2. Serum TNFα level was quantified using ELISA. Values represent mean ± SD. ***P* < 0.01, compared with control group.

group (Figure 5B). These results showed that the inhibitory effect of ethanol, at the mRNA level, was greater on the MEF2A isoform than on the MEF2D isoform.

MEF2 protein levels were also measured. With an increase in the ethanol dosage, MEF2 protein expression was gradually decreased by 4.98 %, 13.46% ($P < 0.05$), and 21.27% ($P < 0.05$) in the L-, M- and H- groups, respectively (Figure 5C).

Ethanol decreases AMPK and impairs GLUT4 expression in the myocardium of rats

To further investigate the effect of ethanol on AMPK activation and GLUT4 expression, isolated rat cardiomyocytes were incubated with 100 mM ethanol for 4 h. Compared with the controls, ethanol decreased the levels of P-AMPKα in neonatal cardiac myocytes by 60.04% ($P < 0.01$) (Figure 6A). At the same time, both the mRNA and protein levels of GLUT4 were impaired and reduced 30.43% (Figure 6B) and 51.23% (Figure 6C), respectively (both $P < 0.01$), by ethanol.

In addition, the association between inhibited expression of P-AMPKα protein and reduction in the level of MEF2 gene transcription was further confirmed by correlation analyses. There were positive association between the P-AMPKα protein and the mRNA levels of MEF2A or MEF2D (r = 0.864 and $P < 0.01$ for MEF2A, r = 0.565 and $P < 0.01$ for MEF2D) (Figures 6D and 6E).

Additional correlation analyses were performed in order to clearly understand the relationship between the mRNA level of GLUT4 and the levels of MEF2 or P-AMPKα. GLUT4 expression appeared to be closely associated with that of MEF2 (Figure 6F) and P-AMPKα (Figure 6G) in the hearts of rats under chronic ethanol exposure (r = 0.756 and $P < 0.01$ for MEF2, r = 0.782 and $P < 0.01$ for P-AMPK). These data strongly suggest that the ethanol-induced down-regulation of GLUT4 expression had most likely association with the decline in AMPK activity and the subsequent inhibition of MEF2 expression.

Ethanol elevates TNFα levels in the serum of rats

The effect of chronic ethanol exposure on serum TNFα levels was assessed by ELISA. Enhancement in serum TNFα levels was achieved by long term ethanol feeding. Concretely, low-, medium- and high-dose of ethanol produced 1.2-, 2.1-, 2.2-fold (all $P < 0.01$) augmentation in serum TNFα levels, respectively, when compared with normal controls (Figure 7).

Discussion

The effect and mechanism of action of ethanol on insulin sensitivity differ according to the tissue type, the duration of exposure and other experimental conditions. Currently, the majority of relevant studies focused on liver, adipose tissue and skeletal muscle, with very few on heart. Given that the existence of a diabetic cardiomyopathy has been supported by animal models, epidemiological findings and clinical studies, we chose to explore the effect of ethanol on insulin sensitivity in the heart. There were expression level changes of both AMPK and MEF2 that were associated with GLUT4 in myocardium. In addition, chronic ethanol feeding induced insulin resistance and down-regulation of AMPK, MEF2 and GLUT4, and upregulation of TNFα.

GLUT4 is involved in the rate-limiting step in glucose utilization, and most likely represents the major mechanism by which the heart increases glucose uptake under insulin stimulus (Fischer *et al*., 1997). The metabolic disturbances that are induced by the depletion of GLUT4 at an early stage characterize and initiate diabetic cardiomyopathy. This impaired cardiac function can be normalized in transgenic db/db-hGLUT4 mice (Semeniuk *et al*., 2002). In addition, transient insulin resistance in adult rat cardiomyocytes is related to the impairment of GLUT4 translocation (Rosenblatt-Velin *et al*., 2004). In our present study, we determined that long-time ethanol consumption induced insulin resistance and decreased the expression of cardiac GLUT4 at the mRNA and

protein levels. Our results, together with those from previous studies (Tuma *et al*., 1991; Rachdaoui *et al*., 2003), may partially explain the alcohol related coronary heart diseases among people with type 2 diabetes mellitus.

AMPK has been proposed to be a key regulator of glucose metabolism in skeletal muscle and a therapeutic target for the treatment of type 2 diabetes. In the heart, the activation of AMPK has been recently reported to stimulate the translocation of GLUT4 and to increase glucose uptake (Russell *et al*., 1999; Li *et al*., 2004). Thus, we first investigated the effects of AICAR on myocardial AMPK and GLUT4. AICAR stimulated the protein expression of P-AMPKα in cardiomyocytes and the mRNA expression of GLUT4 in both cardiomyocytes and myocardium. These data, in consistent with preliminary studies (Russell *et al*., 1999; Li *et al*., 2004), indicated that the increase in GLUT4 transcription within the myocardium might, at least partially, be mediated by AMPK activation. Next, we determined that ethanol decreased P-AMPK expression both *in vivo* and *in vitro*. There are two α isoforms that are capable of serving as the AMPK catalytic subunit, with AMPKα2 having a minimum effect on glucose metabolism in the heart relative to AMPKα1 (Sambandam and Lopaschuk 2003; Fujii *et al*., 2005). Our studies indicated that ethanol had a stronger inhibitory effect on the AMPKα1 isoform than on the AMPKα2 isoform. In addition, we demonstrated that ethanol administration inactivated AMPK in the myocardium, which was associated with the reduction in the expression of myocardial GLUT4. This observation may be further supported by the tight association between the down-regulation of the P-AMPKα subunit and the decrease in GLUT4 mRNA expression after ethanol treatment.

Recent studies have shown that there is a MEF2-binding site in the GLUT4 promoter that is essential for GLUT4 expression in skeletal muscle (Thai *et al*., 1998; Ojuka *et al*., 2002). These findings motivated us to examine the effect of AICAR or ethanol on MEF2 expression in myocardium. We found that the MEF2 and GLUT4 levels elevated when stimulated by AICAR, while descended with ethanol consumption. Our data provided further support for the previous finding that reduced MEF2 expression was correlated with reduced GLUT4 expression in the myocardium (Thai *et al*., 1998). There was also a parallel decline in the level of MEF2 and GLUT4 under the state of chronic ethanol treatment, demonstrating the close relationship, at least to some extent, between the MEF2 protein levels and transcription of the GLUT4 gene. It is the shortage of the present study not to provide more evidence for this phenomenon.

Another important observation is that MEF2A mRNA increased more obviously than MEF2D with AICAR injection. Similarly, ethanol administration decreased myocardial MEF2A and MEF2D mRNA expression, with a stronger effect on MEF2A than on MEF2D. In addition, an ethanol-treated state resulted in the P-AMPKα protein having a more powerful relationship with the MEF2A isoform ($r = 0.864$, $P < 0.01$) than with the MEF2D isoform ($r = 0.565$, $P < 0.01$). This observation can be partially accounted for by the fact that MEF2A has a stronger effect on the regulation of glucose uptake in myocardium (Mora *et al*., 2001).

Chronic alcohol feeding is known to enhanced expression of pro-inflammatory cytokines including TNFα (Leudemann *et al*., 2005; Wahl *et al*., 2007). TNFα on the other hand exerts similar inhibitory effect on the expression of GLUT4 (Rotter *et al*., 2003). Here we still observed elevated serum TNFα level in ethanol treated rats, indicating that ethanol may exert negative role on GLUT4 by stimulating inflammatory effects.

In conclusion, we clearly demonstrated that long-term ethanol exposure decreased the expression of AMPK and MEF2 expression in the myocardium of rats, which was associated with the decreased expression of GLUT4. Further studies are needed to determine the precise intracellular mechanisms responsible for these down-regulations.

Methods

Animal preparation

Male adult (180 ± 5 g, aged 6-8 weeks) and neonatal (1-3 days old) Wistar rats (Experimental Animal Center of Shandong University, Jinan, China) were used. All rat experiments were approved by the Animal Care and Use Committee of Shandong Provincial Hospital (Jinan, China).

Experiment I-AICAR (an AMPK activator) injection

Six rats were injected subcutaneously with 0.8 mg 5-amino-4-imidazolecarboxamide ribonucleotide (AICAR)/g body weight (Bolster *et al*., 2002) or a corresponding volume of 0.9% NaCl (each $n = 3$). The left ventricular muscle was quickly removed at 2 h post-injection.

Experiment II-Chronic ethanol feeding

Forty-eight male rats were randomly allocated into control group, L group (a low dose of ethanol, 0.5 $g \cdot kg^{-1} \cdot d^{-1}$), M group (a medium dose of ethanol, 2.5 $g \cdot kg^{-1} \cdot d^{-1}$) and H group (a high dose of ethanol, 5 $g \cdot kg^{-1} \cdot d^{-1}$). Each group

had 12 rats. Animals in control group received distilled water. Edible ethanol (Jinan Baotu Spring Distillery, Shandong, China) was infused intragastrically and the indidividual dose was adjusted weekly, after the measurement of body weight.

At the end of 22 weeks, on the day before sacrifice, fasting blood samples were collected from all animals. The following day, the rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (50 $mg \cdot kg^{-1}$). The left ventricular muscle was quickly removed for subsequent analyses.

Experiment III-Isolation of rat primary ventricular cardiomyocytes and treatment

Cardiac myocytes were selected for these experiments, as they are the only cells in the heart that express GLUT4 (Young *et al*., 1997) and they account for the majority of glucose transport in the heart.

Ventricular cardiomyocytes were isolated from 1- to 3-day-old Wistar rats. Briefly, neonatal rat ventricles were dispersed in a series of incubations at 37°C in 0.125% tripsin. To reduce fibroblast contamination to $< 5\%$, dispersed cells were preplated for 30 min, and the unattached cells were replated on culture flask. Cells were cultured in DMEM (Sigma, St. Louis) containing 20% FBS (GIBCO, Rockville) and 100 U/ml penicillin/streptomycin. After 24 h, plating medium was replaced with DMEM supplemented with 0.5 μg/ml mytomycin. The isolated cardiomyocytes preparations were $> 95\%$ pure as revealed by staining with cardiac-specific sarcomeric actin (Abcam, UK) antibody.

The purified myocytes were suspended in the fresh culture medium supplemented with 5% FBS and seeded into fibronectin-coated culture plates and were incubated in a humidified 5% CO2-air incubator.

Cardiomyocytes were seeded into fibronectin-coated culture plates and grown in DMEM containing 5% FBS. Cells within 48-72 h after culture initiation were incubated for 4 h in the absence or presence of 1 mM AICAR or of 100 mM ethanol. Protein and mRNA levels for genes of interest were then analyzed.

Evaluation of insulin sensitivity

Blood glucose levels and insulin concentrations were measured by the glucose oxidase method and the radioimmunity method, respectively. Insulin resistance was estimated by HOMA-IR and defined as fasting serum insulin (μU/ml) × fasting plasma glucose (FPG) (mmol/L)/ 22.5. The HOMA method has been shown to be a useful measure for assessing insulin resistance across a wide range of glucose levels (McAuley *et al*., 2001).

RNA isolation and RT-PCR

Total RNA from myocardium or cardiomyocytes was extracted using Trizol (Invitrogen, Grand Island). cDNA was synthesized from 2 μg of total RNA using the TaKaRa RT kit (Japan). The specific primers designed against the rat AMPKα1 and α2 isoforms, MEF2A and MEF2D isoforms, GLUT4 as well as GAPDH, a housekeeping gene were verified by NCBI Blast. All the primers were synthesized by Shanghai Sangon Biotechnology Corporation (Shanghai, China).

PCR amplifications were performed in a total volume of 25 μl containing 3 μl of cDNA above, 0.5 μM of each primer, 2 mM Mg^{2+} and 1 U Taq DNA polymerase. The primer sequences and reaction conditions used in this study are listed in Supplemental data Table S1. The subsequent products were visualized with gel electrophoresis (1.5%). Relative band intensities were determined using Alpha Imager 2200 (Alpha Innotech) software. The levels of target mRNA were normalized to the signal obtained for GAPDH mRNA expression.

Western blot analysis

Left ventricular muscle samples were ground to powder with liquid nitrogen. For total AMPKα (T-AMPKα), MEF2 and GLUT4 protein analysis, the powder was homogenized in a RIPA buffer including protease inhibitors (cocktail kit, Pierce). For the phospho-AMPKα (P-AMPK) protein analysis, the powder was homogenized in a RIPA buffer containing a phosphatase inhibitor for preserving the phosphorylation state of the enzymes.

Myocardium or cardiomyoctye lysates (50 μg for GLUT4, 80 μg for AMPK and MEF2) were subjected to 10% SDS-PAGE and transferred onto nitrocellulose membranes (PVDF membrane for P-AMPK) (Millipore, MA). The membrane was blocked with 5% (8% for GLUT4) nonfat dried milk for 1 h (1.5 h for GLUT4 and 0.5 h for P-AMPK) and then incubated overnight with the specific antibodies to total AMPKα (direct against both α1 and α2-isoforms, Cell Signaling Technology, Danvers), phospho-AMPKα (direct against both α1 and α2 isoforms of the enzyme phosphorylated at Thr172, Cell Signaling), MEF2 (Santa Cruz Biotechnology, CA) or GLUT4 (Abcom). Blots were probed with the appropriate horseradish peroxidase-conjugated anti-rabbit antibody and visualised by the ECL method. The mean density of each band was measured using Alphaimager 2200 software, with β actin serving as the internal control.

ELISA

TNFα levels in the serum were tested according to the manufacturer's instructions using a TNFα assay kit (R&D systems, Minneapolis). Briefly, samples and a diluted series of standards were incubated with a mouse monoclonal antibody to TNFα and TNFα conjugate in a 96-well plate at room temperature for 3 h. After washing thoroughly, substrate solution and stop solution were added, and the optical density of each well at 450 nm and corrected at 540 nm was determined.

Statistical analysis

Each experiment was repeated three to six times. All values are presented as mean ± SD. Statistical significance was assessed by One-Way ANOVA. Correlation analyses were performed to explore the relationship among AMPK, MEF2 and GLUT4 using a two-tailed Pearson's correlation

coefficient (r). The data were analyzed using SPSS 15 software, with a *P*-value of less than 0.05 considered to be significant.

Supplemental data

Supplemental Data include a table and can be found with this article online at http://e-emm.or.kr/article/article_files/SP-42-3-06.pdf.

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