Introduction

Previous studies have demonstrated the important role of adipose tissue GLUT4 expression in determining insulin sensitivity. Adipocyte-specific GLUT4−/− mice develop insulin resistance and glucose intolerance[1], while mice with adipose-specific overexpression of GLUT4 have enhanced insulin sensitivity[2].

The effect of ethanol on adipose tissue GLUT4 expression is complex. When rats are given a normal diet, chronic ethanol exposure is reported to decrease GLUT4 expression[3, 4] and surface accessibility[3]. However, when rats are given a high-fat diet, chronic ethanol administration increases GLUT4 expression[6]. In addition, the mechanism of action of ethanol on GLUT4 is still obscure. In addition to the phosphoinositide 3-kinase (PI3K)-dependent pathway[7, 8], others have proposed that G protein[4, 9] and Cb1/TC10[10] pathways are involved in the effect of ethanol on GLUT4. Recently, AMP-activated protein kinase (AMPK) has been suggested to be a new target for ethanol, but the effect of ethanol on AMPK activation is now controversial. Several groups have reported that AMPK activity could be inhibited by ethanol in both hepatic cells[11, 12] and brain cells of mice at postnatal day 7[13, 14]. However, Hong-Brown reported that incubation of C2C12 myocytes with 100 mmol/L ethanol markedly increases AMPK phosphorylation and activity[15]. Our previous study demonstrated that long-term ethanol exposure restores AMPK activity in the adipose
tissue of high-fat diet-fed rats[6].

The AMPK heterotrimer consists of a catalytic α subunit and two regulatory β and γ subunits[16,17]. There are multiple isoforms of each subunit: α1 and α2; β1 and β2; and γ1, 2, and 3[18]. AMPK is activated by a mechanism that involves allosteric modification and phosphorylation of Thr172 in the α subunit[19]. Activated AMPK stimulates GLUT4 expression[20] and basal GLUT4 translocation in both skeletal muscle and adipocytes[21–24]. Moreover, studies in cardiac and skeletal muscle have found that AMPK regulates GLUT4 in a manner dependent upon a heterodimer of MEF2A and MEF2D (myocyte enhancer factor 2)[25–27], a complex that can bind to the human GLUT4 promoter[26] and thus regulate GLUT4 transcription.

In the present study, we investigated the effects of ethanol on the AMPK/MEF2/GLUT4 pathway in isolated rat and human primary adipocytes both in vitro and in vivo.

Materials and methods

Animal feeding

Thirty-six male Wistar rats (weight, 160–180 g; Laboratory Animal Center of Shandong University) were housed in individual cages in a temperature-controlled room (24 °C) on a 12-h light-dark cycle and fed pelleted commercial normal chow diet containing 10% fat, 70% carbohydrate, and 20% protein (total 4.5 kcal/g). Animal Center of Shandong University. After acclimatization for one week, the rats were divided into three groups and given edible ethanol (Ji-nan Baotu Spring Distillery, Shandong, China) at a single daily dose of 0.5 g/kg body weight (low dose, group L) or 5 g/kg (high dose, group H) or distilled water (controls, group C) at 8–9 am by gastric tube. The animal study was approved by the Shandong University Institutional Animal Care and Use Committee (Ji-nan, China).

Oral glucose tolerance test (OGTT)

OGTT was carried out after a 22-week ethanol treatment. Rats were fasted overnight; blood glucose was then measured in samples obtained by tail bleeding before administration of glucose (2 g/kg body weight) and at 30, 60, and 120 min after glucose. Blood glucose concentrations were determined using a One Touch SureStep Meter (Life Scan, Milpitas, CA). The area under the curve (AUC) was calculated to assess glucose tolerance.

Determination of plasma ethanol concentration

On the day of the study, two hours after ethanol administration, blood samples were obtained from the jugular sinus and were rapidly stored in tubes with seals. Plasma ethanol concentrations were determined using a dry chemical method (Johnson & Johnson, USA).

Tissue collection

All rats were allowed to recover from OGTT for three days before sacrifice. Rats were anesthetized by an intraperitoneal injection of sodium pentobarbital (0.1 mL/100 g BW) after a 10-h fast. Blood samples were obtained from the inferior vena cava for glucose and insulin determination. Epididymal and perirenal fat pads were rapidly removed and weighed. Ratios of epididymal and perirenal adipose tissue weight (g) to body weight (g) were calculated. Portions of the epididymal fat samples were fixed in 4% paraformaldehyde-0.2 mol/L phosphate-buffered saline (PBS, pH 7.4) for immunofluorescence and hematoxylin & eosin (H&E) staining analysis. The remaining tissues were frozen in liquid nitrogen for messenger RNA and protein analyses.

Biochemical analysis and evaluation of insulin sensitivity

Blood glucose was measured using the glucose oxidase method. Insulin was measured by radioimmunoassay (Northern Bioengineering Institute, China). HOMA-IR was calculated using the following formula: FPG (mmol/L)×FINS (mU/mL)/22.5[20].

AICAR injection

Six male Wistar rats weighing 170–180 g were randomly divided into two groups and injected subcutaneously with AICAR (an AMPK activator, 0.8 mg/g body weight, AICAR group) or a corresponding volume of 0.9% NaCl (control group). Two hours later, epididymal adipose tissues were obtained as described above for mRNA and protein analysis.

Isolation of rat and human adipocytes

Adipocytes were isolated from the epididymal fat pad of normal male Wistar rats (weighing 250–300 g) and from the omental adipose tissue of male patients aged 25–55 undergoing abdominal surgery at the Shandong Provincial Hospital (Jinan, China) in 2007. Patients with a history of ethanol ingestion or diabetes were excluded. All patients gave written informed consent for tissue donation before surgery. The human study was approved by the Ethics Committee of Shandong Provincial Hospital (Ji-nan, China).

All visible blood vessels were carefully removed from the fat tissue[29,30]. The fat pads were minced into millimeter-sized pieces, digested in Krebs-Ringer bicarbonate HEPES (KRBB) buffer (120 mmol/L NaCl, 4 mmol/L KH2PO4, 1 mmol/L MgSO4, 0.75 mmol/L CaCl2, 10 mmol/L NaHCO3, 30 mmol/L HEPES; pH 7.4) with 1 mg/mL collagenase type I, 1% (w/v) BSA, 2.5 mmol/L glucose, 100 μg/mL penicillin, 100 μg/mL streptomycin, and 1% (w/v) fungizone for 40–60 min in a 37 °C water bath with gentle agitation. After being filtered sequentially through 500- and 250-μm nylon mesh, the adipocyte cell suspension was centrifuged at 800×g at room temperature for 2 min. After several washes, cells were resuspended in KRBB buffer (pH 7.4) with 1% BSA and 2.5 mmol/L glucose, allowed to equilibrate for 30 min at 37 °C, and then used directly for the subsequent experiments. Cell concentration was adjusted to 1.0×106 cells/mL.

Cell culture and treatment

The isolated adipocytes were placed at a concentration of 1×107/100 mm per culture dish and incubated for 1 h in...
ethanol at a concentration of 100 mmol/L. These conditions were determined from preliminary experiments designed to select the optimal time and dose response for AMPK phosphorylation. In the preliminary experiments, we found that 20 mmol/L ethanol positively regulated AMPK α phosphorylation, yet 50 and 100 mmol/L treatments decreased the response, and the inhibitory effect of ethanol became more obvious following an increase in ethanol concentration (data not shown). Additionally, ethanol concentrations of 50–200 mmol/L are usually used in cell culture systems to observe the effect of high-dose ethanol.[31–33] Rat and human adipose cells were incubated at 37 °C for 1 h in the absence or presence of ethanol (100 mmol/L), AICAR (1 mmol/L), and compound C (a selective inhibitor of AMPK, 20 μmol/L). Compound C treatment was initiated 20 min before adding AICAR.

RNA extraction and RT-PCR
Total RNA was extracted from frozen epididymal adipose tissue and adipocytes using the standard Trizol RNA isolation method. The quality of RNA was checked using the DU640 nucleic acid analyzer (Beckman, USA). Reverse transcription of 4 μg of RNA was carried out according to the instructions of the Fermentas RevertAidTM First Strand cDNA Synthesis Kit (#K1622).

All primers were synthesized by the Shanghai Sangon Biotechnology Corporation (Shanghai, China) and the sequences are shown in Table 1. PCR amplification was carried out in a total reaction volume of 25 μL, including 2.5 μL PCR buffer (10×), 0.2 μL Taq polymerase, 2 μL dNTP (TaKaRa, 25 mmol/L), 2 μL MgCl2 (TaKaRa, 25 mmol/L), 2 μL primers (5×10−6 mol/L) and 2.5–3 μL of the cDNA (2.5 μL for AMPK α1, α2, and GLUT4; 3 μL for MEF2A). The PCR products were subjected to 1.5% agarose gel electrophoresis containing ethidium bromide and visualized by excitation under UV light, subjected to 1.5% agarose gel electrophoresis containing ethidium bromide and visualized by excitation under UV light, quantified using Alphaimager 2200. GAPDH was used as an internal control for quantity and quality.

Table 1. Sequences of primers and annealing temperatures.

| Gene          | Primers                                      | Annealing temperature (°C) | Product size (bp) | Accession number |
|---------------|----------------------------------------------|---------------------------|-------------------|------------------|
| AMPKα1        | sense: 5′-agg atc cat cag caa cta tgg-3′     | 56.4                      | 100               | NM_019142        |
|               | antisense: 5′-agg agg tca cgg atg agg-3′     |                           |                   |                  |
| AMPKα2        | sense: 5′-cat tgc aag gcc cct agt-3′         | 58.5                      | 100               | NM_023991        |
|               | antisense: 5′-gac tgt tgt tat ctt gtt ccc-3′|                           | 168               | NM_001014035     |
| MEF2A         | sense: 5′-agt gcc tgg agg gca gtt atc-3′     | 58.5                      | 447               | NM_030860        |
|               | antisense: 5′-agg agg tgt tgt gcg tgt-3′    |                           |                   |                  |
| MEF2D         | sense: 5′-gct gac atc ctc cag ggg-3′        | 58.5                      | 150               | XM_344448        |
|               | antisense: 5′-ggc ccc ctt cgg aca cta ct-3′|                           |                   |                  |
| GLUT4 (rat)   | sense: 5′-agt ctt tga gtt gtt cta aga-3′    | 57.6                      | 105               | NM_0102751       |
|               | antisense: 5′-ggc ccc cgg cct cct cca-3′   |                           |                   |                  |
| GAPDH (rat)   | sense: 5′-act tcc acc tga cgg gct ctc-3′    | 60.0                      | 138               | NM_001042        |
|               | antisense: 5′-tta cat gtt gaa acc cgg tc-3′ |                           |                   |                  |
| GAPDH (human) | sense: 5′-gag cca cat ctc tca gac ac-3′    | 60.0                      | 150               | NM_002046        |
|               | antisense: 5′-cat gtt gtt gga aat gaa gg-3′|                           |                   |                  |

Table 1. Sequences of primers and annealing temperatures.

Western blotting
Protein samples (60 μg) were resolved by SDS-PAGE (10% resolving gels for total AMPKα, phosphorylated AMPKα, total-MEF2, MEF2A, MEF2D, and GLUT4; 6% resolving gels for phosphorylated acetyl-CoA carboxylase, pACC) and transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). All membranes were incubated overnight at 4 °C with primary antibodies against total AMPKα (rabbit polyclonal antibody, 1:1000 dilution, Cell Signaling, Danvers, MA,USA), pAMPKα (rabbit polyclonal antibody, 1:1000 dilution, directed against both α1 and α2 isoforms of the enzyme phosphorylated at Thr172, Cell Signaling, Danvers, MA,USA), pACC (rabbit polyclonal antibody, Ser-79; 1:1000 dilution, Cell Signaling, Danvers, MA, USA), MEF2 (rabbit polyclonal antibody, 1:100 dilution, Santa Cruz, USA), MEF2A (rabbit monoclonal antibody, 1:1000 dilution, Abcam Ltd, Cambridgeshire, UK), MEF2D (goat polyclonal antibody,
1:200 dilution, Santa Cruz, USA), or GLUT4 (rabbit polyclonal antibody, 1:2500 dilution, Abcam Ltd, Cambridgeshire, UK). After incubation with secondary antibody (Zsbio, Ltd, China), immune complexes were detected using Amersham ECL Plus™ Western Blotting Detection Reagents (Amersham, UK), and immunoreactive bands were quantified using AlphaImager 2200. Expression of β-actin was measured as an internal loading control by reblotting the membranes with mouse anti-rat β-actin monoclonal antibody (1:10000 dilution, Abcam Ltd, Cambridgeshire, UK). The relative target protein levels were normalized to β-actin.

Immunofluorescence and hematoxylin and eosin (H&E) staining
The fixed epididymal adipose tissue was embedded in paraffin, and 5-µm sections were obtained. The glass-mounted sections were cleared from paraffin with xylene and rehydrated by sequential washings with graded ethanol solutions (70%-100%), subsequently incubated in 3% H2O2 in methanol for 10 min to quench the endogenous peroxidase activity, pre-treated in a microwave oven in sodium citrate buffer (pH 7.4) for 20 min with the temperature always kept at 95-98 °C, and then cooled at room temperature for 20 min to ensure recovery of protein spatial configuration. After being washed with PBS, the sections were blocked by 10% secondary antibody homologous sera (goat serum) in PBS for 2 h at room temperature, followed by overnight incubation with the primary antibody (rabbit anti-GLUT4, 1:300 dilution) in 5% goat serum in PBS at 4 °C in a moisture chamber. Negative controls for immunospecificity were included in all experiments, and the primary antibody was replaced by PBS or matching concentrations of normal rabbit or mouse serum. All sections were then incubated with a FITC-conjugated anti-rabbit secondary antibody (1:150 dilution) for 1 h at room temperature. After sections were mounted with DAPI in PBS, analysis and documentation were performed using a fluorescent microscope (Leica Microsystems GmbH, Wetzlar, Germany). The obtained sections were also stained with H&E and then examined under an optical microscope.

Statistical analyses
All of the experiments were repeated at least four times. All values are presented as means±SD. Data were analyzed using SPSS 11.5 software (SPSS, Inc, Chicago, IL). Statistical significance was assessed by one-way ANOVA. *P<0.05 was considered statistically significant.

Results
Characteristics of rats after 22-week treatment
As shown in Table 2, all three groups were of similar body weights at baseline. After 22 weeks of treatment, ethanol-fed rats showed lower body weights, and high-dose ethanol feeding led to a greater reduction in body weight than low-dose ethanol (393.8±56.5 vs 456.7±62.6, *P<0.05), but only the body weight of the high-dose ethanol group (group H) was statistically significant compared with that of the controls (393.8±56.5 vs 477.7±34.5, *P<0.01). Long-term high-dose ethanol administration significantly increased the ratio of epididymal adipose tissue to total body weight compared to both low-dose ethanol and distilled water consumption groups (1.2±0.3 vs 0.9±0.2, *P<0.05 vs group L; 1.2±0.3 vs 0.9±0.3, *P<0.05 vs group C) but had no significant influence on perirenal fat mass. By contrast, low-dose ethanol consumption did not significantly change the mass of adipose tissue in either site relative to the controls.

Although high-dose ethanol administration increased fasting plasma glucose levels, the value was not statistically significant. Compared to groups L and C, fasting serum insulin levels were markedly elevated in group H (28.4±6.1 vs 22.0±5.6, *P<0.05 vs group L; 28.4±6.1 vs 20.6±5.2, *P<0.05 vs group C). Similarly, HOMA indexes, which were used to evaluate insulin sensitivity, were significantly increased after high-dose ethanol administration (4.5±1.0 vs 3.4±1.0, *P<0.05 vs group L; 4.5±1.0 vs 3.2±1.2, *P<0.05 vs group C) but were not markedly elevated in group L (3.4±1.0 vs 3.2±1.2, *P>0.05) compared to the controls.

Long-term ethanol consumption induces glucose intolerance in rats
Long-term ethanol consumption, both in low and high dosages, increased glucose levels at the time points before and after the glucose load, but the increases were not significant. Deterioration in glucose tolerance (11% enhancement in glucose AUC, *P<0.05 vs controls) was observed in high-dose etha-
nol fed rats. Low-dose ethanol had no marked influence on glucose tolerance relative to the controls (Figure 1).

Figure 1. Long-term ethanol exposure induced glucose intolerance in rats. Thirty-six male Wistar rats were divided into three groups and received edible ethanol with a single daily dose of 0.5 or 5 g/kg or distilled water. Twenty-two weeks after, oral glucose tolerance test (OGTT) was carried out. Blood glucose levels were measured from samples obtained by tail bleeding before administration of glucose (2 g/kg body weight) by gavages, and 30, 60, 120 min after the glucose load (A). Area under the curve (AUC) was calculated to assess glucose tolerance (B). Values are given as means±SD (n=12 for the low-dose ethanol and control group, n=10 for the high-dose ethanol group).

Long-term ethanol feeding reduces GLUT4 expression in rat adipose tissue
In epididymal adipose tissue of ethanol-fed rats, GLUT4 mRNA levels decreased by 28% (group L, P<0.05) and 47% (group H, P<0.01), respectively (Figure 2A), and the corresponding protein levels decreased by 21% (group L, P<0.05) and 40% (group H, P<0.01), respectively, relative to the controls (Figure 2B). High-dose ethanol had a greater inhibitory effect on GLUT4 mRNA and protein expression than low-dose ethanol (both P<0.05 vs group L). To confirm the action of ethanol on GLUT4 expression, adipose tissue sections were stained by immunofluorescence. The specific immunofluorescence signal was absent in the negative control sample. Compared to non-ethanol-fed rats, weaker signals for GLUT4 expression were observed in the ethanol-fed rat adipose tissue, and the effect of ethanol was dose dependent (Figure 2C). These data show that ethanol feeding decreased GLUT4 expression in rat adipose tissue.

Ethanol suppresses MEF2 expression
With the description of a functional MEF2A/MEF2D heterodimer binding site in the rat and human GLUT4 promoters,[35,36] MEF2 is considered a transcriptional regulator of GLUT4. To explore whether MEF2 was involved in the inhibitory effect of ethanol on GLUT4 expression, we measured the expression of total MEF2 and its two isoforms, MEF2A and D, in adipose tissue exposed to ethanol. Figure 3A shows that while the MEF2A mRNA level was not affected by ethanol, the MEF2D isoform was reduced by 9% in group L (P>0.05) and 23% in group H (P<0.01) relative to the controls (Figure 3A and 3B). The total MEF2 protein levels were correspondingly decreased by 24% (P<0.05) and 33% (P<0.05) in groups L and H, respectively, versus the controls (Figure 3B). The difference in MEF2D mRNA between groups H and L was statistically significant (P<0.05, Figure 3A and 3B). Similarly, total MEF2 protein levels were affected by the different dose of ethanol (P<0.05, Figure 3B). We hypothesized that the ability of ethanol to decrease MEF2D mRNA expression might contribute, at least partially, to a decline in MEF2D protein. To test this, we measured the nuclear and cytoplasmic expression of MEF2D.
in adipocytes. Our results show that ethanol treatment decreased MEF2D protein expression and there was a notable difference between groups H and L, not only in whole cell extracts but also in nuclear and cytoplasmic extracts (Figure 3C). Moreover, for an analysis of MEF2A isoform, although the total MEF2A protein expression in group L and H was of no statistical significance relative to the control, the nuclear MEF2A level in group H was markedly lower than that in group L and C (Figure 3C).

Figure 3. Effect of long-term ethanol administration on MEF2 expression in rat adipose tissue. After rats were fed with edible ethanol at the daily dosage of 0 (control), 0.5, or 5 g·kg⁻¹·d⁻¹ for 22 weeks, by using RT-PCR and Western blotting, we determined MEF2 expression both at mRNA (A) and total protein (B) levels. We also measured nuclear, cytoplasmic, and total proteins of MEF2A and D, two isoforms of MEF2 (C) in adipose tissue. MEF2A, D mRNA levels were normalized by GAPDH and MEF2 protein levels were normalized by β-actin. Values are given as means±SD (n=12 for the low-dose ethanol and control group, n=10 for the high-dose ethanol group). *P<0.05, **P<0.01 vs controls. †P<0.05 vs the low-dose ethanol group.

Figure 4. Long-term ethanol exposure impaired activation, but not expression of AMPK in rat adipose tissue. After feeding rats with ethanol at the daily dosage of 0, 0.5, or 5 g·kg⁻¹·d⁻¹ for 22 weeks, we determined mRNA levels of AMPK α1 and α2 isoforms by RT-PCR (A), protein levels of total-AMPK (T-AMPK) and phosphorylated-AMPK (pAMPK) by Western blotting (B). mRNA expression was normalized by GAPDH, and protein expression was normalized by β-actin. Values are given as means±SD (n=12 for low-dose ethanol and control group, n=10 for high-dose ethanol group). *P<0.05, **P<0.01 vs controls. †P<0.05 vs the low-dose ethanol group.

Long-term ethanol feeding impairs AMPK activation but not expression

Recently, AMPK has been suggested as a new target for ethanol. Studies in skeletal muscle have shown that AMPK regulates GLUT4 via MEF2. To explore whether the decreased MEF2 and GLUT4 expression after ethanol treatment in adipose tissue was associated with the inhibition of AMPK, we measured the expression of AMPKα1 and α2 mRNA and total AMPKα and pAMPKα protein in rat adipose tissue.

Our results show that mRNA expression of the AMPKα1 and α2 isoforms in ethanol-fed rats was not statistically different from that in control rats (Figure 4A). Consistent with this result, total AMPKα protein was also not affected by long-term ethanol feeding (Figure 4B). However, in ethanol-fed groups, the bands of phosphorylated AMPK protein detected by Western blotting were fainter than those in the control group (21% decrease in group L, P<0.05; 41% decrease in group H, P<0.01). Moreover, a much fainter phosphorylated AMPKα band was found in group H compared to group L (P<0.05, Figure 4B), indicating that ethanol feeding impairs AMPK activation and that the impairment effect correlates with the ethanol dosage.
Activated AMPK positively regulates MEF2 and GLUT4 expression in rat adipose tissue

Activated AMPK has been reported to regulate GLUT4 expression via MEF2 in skeletal muscle. In the present study, we found consistent changes in pAMPKα, MEF2, and GLUT4 in adipose tissue after long-term ethanol exposure, which prompted us to hypothesize that in adipose tissue, inhibition of AMPK activation might also result in decreased MEF2, which subsequently decreases GLUT4 expression. To test our hypothesis, rats were injected with AICAR. AICAR injection led to a marked increase in AMPKα phosphorylation in rat adipose tissues. As an indicator of AMPK activation, pACC was enhanced accordingly, indicating that the phosphorylation of AMPKα was followed by increased activity. Following the activation of AMPK, MEF2 protein level was elevated. As a result, GLUT4 mRNA levels were increased and, subsequently, its protein level increased. Our data show that activation of AMPK up-regulated MEF2 expression, with subsequent increases in GLUT4 expression in adipose tissue in vivo (Figure 5).

Inhibition of AMPK activity by ethanol leads to decreased MEF2 and GLUT4 expression in rat and human adipocytes

To investigate the action of ethanol on the AMPK/MEF2/GLUT4 pathway, isolated rat and human adipocytes were treated with ethanol. Compared to the controls, ethanol decreased pAMPKα protein by 39% (P<0.05) and 56% (P<0.05) in rat and human adipocytes, respectively; decreased pACC expression by 49% (P<0.05) and 56% (P<0.01), respectively; and decreased MEF2 levels by 32% (P<0.05) and 45% (P<0.05), respectively. As a result, the mRNA and protein levels of GLUT4 were diminished by 52% (P<0.05) and 40% (P<0.05) in rat adipocytes and by 40% (P<0.01) and 60% (P<0.05) in human adipocytes (Figure 7), respectively. Taken together, these results suggest that the impairment of GLUT4 expression by ethanol is most likely due to the decline of AMPK activity and subsequent inhibition of MEF2 expression.

Discussion

Epidemiological studies suggest that chronic light or heavy ethanol consumption leads to insulin resistance, whereas moderate ethanol consumption results in increased insulin sensitivity. In the present study, we found that long-term high-dose ethanol consumption led to insulin resistance in rats, which was consistent with previous studies. However, in low-dose ethanol-fed rats, insulin sensitivity as measured by the HOMA index was not statistically different from that of the controls. Consistent with our HOMA insulin sensitivity results, oral glucose tolerance was significantly impaired after high-dose ethanol feeding but did not change after low-dose ethanol consumption. The discrepancy between epidemiological studies and our findings is uncertain but may be due to the following reasons: (1) the HOMA index and OGTT, which were used in our study, are not as sensitive as the hyperinsulinemic euglycemic clamp and intravenous glucose tolerance test (IVGTT) used in the former studies; and (2) the development of insulin resistance and glucose intolerance induced by low-dose ethanol may take longer than 22 weeks and may be related to a threshold total accumulated dose of ethanol.

The mechanisms for the increase in insulin resistance or glucose intolerance induced by ethanol have not been well established. GLUT4 is a key molecule for glucose utilization in adipose tissue and skeletal muscle. The factors that can inhibit GLUT4 expression and/or translocation may lead to a disturbance of glucose utilization. Consistent with previous research, we found that long-term ethanol consumption decreased GLUT4 expression at both the mRNA and the protein levels in rat adipose tissue. The inhibitory action of ethanol on GLUT4 expression in adipose tissue might be a key step in ethanol-induced insulin resistance, because changes in GLUT4 expression in adipose tissue are considered a key factor in determining whole body insulin sensitivity and may be more important than the changes in both skeletal muscle and...
Inhibition of GLUT4 mRNA expression prompted us to investigate the mechanism by which ethanol regulates GLUT4 transcription. MEF2 is a transcription factor that plays a key role in skeletal muscle differentiation. It contains a known nuclear localization sequence that encompasses amino acids 472–507 in the primary sequence, indicating that MEF2 can be translocated to the nucleus. Further studies indicate that MEF2 colocalizes to the nucleus with histone deacetylase 4. Since the identification of a functional MEF2 binding site located between -522 and -420 in the rat and human GLUT4 promoters, it is believed that MEF2 is a transcriptional regulator of GLUT4, because the truncation or deletion of this binding sequence in the GLUT4 promoter decreases GLUT4 mRNA expression.

In the present study, we found that chronic ethanol consumption decreased MEF2A and MEF2D expression at both the mRNA and the protein levels in rat adipose tissue. The inhibition of MEF2 expression by ethanol may help us to understand the decrease in GLUT4 mRNA expression. The reduced nuclear MEF2A and MEF2D levels could have resulted in the decreased formation of the MEF2A/MEF2D heterodimer. Subsequently, the heterodimers that bind to the GLUT4 promoter would also be decreased, which in turn would result in impaired GLUT4 expression. Based on our present results, we could not determine which isoform of MEF2 was most important for GLUT4 gene expression. However, based on other reports, it seems that MEF2D is more important than MEF2A in regulating GLUT4 expression in adipose tissue.

**Figure 6.** Activated-AMPK was a positive regulator for MEF2 and GLUT4 in rat and human adipocytes. Primary adipocytes were isolated from epididymal fat pad of normal male Wistar rats and omental adipose tissue of male patients who received polyp intestinal surgery. The adipose cells were incubated at 37 °C for 1 h in the absence or presence of AICAR (1 mmol/L) and compound C (20 μmol/L). Compound C was supplied for 20 min prior to the addition of AICAR. GLUT4 mRNA expression was measured using RT-PCR. Protein expression of pAMPKα, pACC, MEF2, and GLUT4 was determined by Western blotting. The data presented are based on the results of at least four separate experiments. N, control; A, AICAR; C, compound C; A+C, AICAR plus compound C. *P<0.05, **P<0.01 vs controls.
vent the GLUT4 downregulation that occurs in insulin-deficient states\textsuperscript{[46]}. The reason for the discrepancy between their results is still unclear, but the different tissues utilized might be a possible explanation. The relationships between the two isoforms of MEF2 and GLUT4 need further exploration.

AMPK has been demonstrated to be an upstream regulator for MEF2 and can increase MEF2 expression in skeletal muscle, resulting in up-regulation of GLUT4 transcription\textsuperscript{[47, 48]}. The existence of this mechanism in adipose tissue after ethanol-treatment has not been studied. We investigated the expression of AMPKα1 and α2 mRNA, total AMPKα, and pAMPKα protein in ethanol-treated rat adipose tissue. Our results show that feeding rats ethanol resulted in a reduction in pAMPKα level without any significant effect on AMPKα1/2 mRNA or total AMPKα protein level, suggesting that ethanol affects the activation, but not expression of AMPKα. Activated AMPK can be translocated to the nucleus from the cytosol\textsuperscript{[48]}, which could be the reason that MEF2 is affected at a transcriptional level in the nucleus. As an indicator of pAMPK activation, the decline in pACC further confirmed the decreased activation of AMPK after long-term ethanol feeding.

Although low-dose ethanol showed no significant influence on insulin sensitivity or glucose tolerance, the expression of AMPK, MEF2, and GLUT4 in rat adipose tissue was significantly reduced. Based on these findings, it is possible to conclude that changes at the molecular level may take place before any pathological consequences and that, with time, the negative effect of ethanol on insulin sensitivity will gradually become more significant.

In skeletal muscle, activated AMPK has been demonstrated to increase GLUT4 protein level and translocation\textsuperscript{[21, 49, 50]} via MEF2\textsuperscript{[25, 27, 48]}. However, the findings in adipose tissue are controversial. Under basal conditions (no insulin stimulation), several studies have reported that the activation of AMPK by AICAR accelerates GLUT4 translocation and increases glucose uptake\textsuperscript{[23, 24]}, whereas others have shown that glucose uptake is suppressed by activated AMPK in adipocytes, probably due to decreased translocation of GLUT4\textsuperscript{[51]}.

To test whether an AMPK/MEF2/GLUT4 pathway existed in adipose tissues and to observe the regulation of MEF2

\begin{figure}
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\caption{Ethanol-treatment impaired AMPK/MEF2/GLUT4 pathway in rat and human adipocytes. Rat and human primary adipocytes were isolated as previously described (Materials and Methods). The cells were incubated at 37 °C for 1 h in the absence or presence of ethanol (100 mmol/L) and AICAR (1 mmol/L). GLUT4 mRNA expression was measured using RT-PCR. Protein expression of pAMPK α, pACC, MEF2, and GLUT4 was determined by Western blotting. The data presented are based on the results of at least four separate experiments. N, control; E, ethanol; A, AICAR; E+A, ethanol plus AICAR. \( ^{b}P<0.05, ^{c}P<0.01 \) vs controls.}
\end{figure}
and subsequent GLUT4 expression by AMPK in vivo and in vitro, rats were subcutaneously injected with AICAR, and adipocytes isolated from rat and human adipose tissues were treated with or without AICAR or compound C. Our results show that AICAR increased pAMPKα levels in both adipose tissue and adipocytes. Parallel to the changes in pAMPKα, the level of pACC was also enhanced. Because ACC is a cytosolic protein and an indicator of AMPK activation, the increased pACC level reflects enhanced AMPK activation in the cytosol. As a downstream molecule of AMPK in the nucleus, MEF2 expression was elevated after AICAR treatment. Therefore, from these results, we conclude that enhanced activation of AMPK increased MEF2 expression.

MEF2 is reported to be a transcriptional regulator of GLUT4 in skeletal muscle. Therefore, we wanted to know whether increased MEF2 expression would lead to a rise in GLUT4 mRNA expression in adipocytes. As expected, both GLUT4 mRNA and protein levels were enhanced in AICAR-treated rat and human adipocytes. In adipocytes treated with the AMPK inhibitor compound C prior to AICAR for 20 min, the augmentation effects of AICAR on activated AMPK, MEF2, and GLUT4 levels were inhibited to a level close to normal. These findings indicate that GLUT4 expression in adipocytes is positively regulated by activated AMPKα via MEF2.

To further confirm the action of ethanol on the AMPK/MEF2/GLUT4 pathway observed in vivo, primary adipocytes isolated from rat and human adipose tissue were incubated with ethanol. AMPK activation and MEF2 expression were inhibited by ethanol. Accordingly, mRNA and protein expression of GLUT4 were suppressed. The changes in this pathway in vitro further confirmed the findings we observed in vivo. However, the in vitro incubation time was only 1 h, which was not long enough to mimic the chronic effects of ethanol. We incubated cells for only 1 h for the following reasons. In our preliminary experiments, the longest survival time of isolated mature adipocytes was no more than 24 h. Moreover, in the preliminary experiments, we incubated the rat adipocytes with 100 mmol/L ethanol for different times (data not shown). The maximum inhibitory effect on AMPK phosphorylation was observed when adipocytes were treated with 100 mmol/L ethanol for 1 h. The inhibitory effect of ethanol was not enhanced when the incubation was prolonged to 120 min. Furthermore, ethanol is a volatile substance. With decreases of ethanol concentration in the medium, the inhibitory effect of ethanol was weakened. Therefore, to maintain cell activity and mimic the effects of high-dose ethanol in vitro, one hour was chosen for the in vitro experiments.

In an earlier study, we reported that the addition of ethanol to a high fat diet ameliorated the effects of the high fat diet and increased GLUT4 expression. In the present study, we found that ethanol decreased GLUT4 expression. The findings clearly indicate that the effect of ethanol on GLUT4 expression is more complex than previously thought. A comparison of the two experiments suggests some possible reasons for the discrepancy. First, although the animals in both studies received the same total daily ethanol dosage, the frequencies of ethanol intake were different. Previously, rats received ethanol twice daily, while here they received it only once daily. The differences in ethanol intake led to different plasma ethanol concentrations (87±24.9 mg/dL in the present study, 10.8±3.6 mg/dL in the earlier experiment), resulting in different outcomes even though the average daily ethanol consumption was similar[52, 53]. Administration of ethanol once daily at 0.5 and 5 g/kg might mimic the effects of light and heavy ethanol consumption, respectively, whereas twice daily administration at the dosage of 5 g/kg might mimic the effects of moderate ethanol consumption. Second, it is possible that a counteractive effect may exist between ethanol and a high-fat diet. For example, ethanol attenuates fatty acid-induced apoptosis in neonatal rat cardiomyocytes[54]. Diets rich in saturated fatty acids protect against alcoholic liver injury[55-60]. Furthermore, administration of saturated fat has been shown to reverse established alcoholic liver injury in rats and improve nearly all liver pathological changes, despite continued ethanol administration[61, 62]. Based on these data, our present findings suggest that the adverse effects of a high-fat diet might be countered by the addition of ethanol, with a restoration of GLUT4 expression, but that such an effect would not be observed when rats were fed a normal diet.

In conclusion, we demonstrate that an AMPK/MEF2/GLUT4 pathway exists in adipose tissue and that activated AMPK up-regulates GLUT4 expression via MEF2. Inhibition of this pathway by long-term ethanol feeding, at least in part, contributes to the impairment of GLUT4 expression in adipose tissue, thus decreasing insulin sensitivity and glucose tolerance.

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Author contribution
Li FENG and Yong-feng SONG did most of the work in this research, such as feeding animals, isolating adipocytes, performing experiments, and writing article. Qing-bo GUAN provided help for the experiment designing. Hong-jun LIU helped the first author collect human adipose tissues for adipocytes isolation. Bo BAN and Hai-xin DONG helped the first author determine plasma ethanol concentration. Xiao-lei HOU helped the first author prepare the animals. Kok-Onn LEE critically edited the manuscript. Jia-jun ZHAO and Ling GAO are corresponding authors, they designed the experiment and
supply essential support of technology and fund.

**Abbreviations**

MPK, AMP-activated protein kinase; MEF2, myocyte enhancer factor 2; GLUT4, glucose transporter 4; GAPDH, glyceraldehyde-3-phosphate dehydrogenase

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