Long-term moderate ethanol consumption restores insulin sensitivity in high-fat-fed rats by increasing SLC2A4 (GLUT4) in the adipose tissue by AMP-activated protein kinase activation

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Abstract

The sole effect of either saturated fatty acid or moderate ethanol consumption on SLC2A4 (GLUT4) expression is widely reported but the combined effects of them remain obscure. Here, we observed their combined effects on SLC2A4 expression, explored the underlying mechanism mediated by AMP-activated protein kinase α (PRKAA2) and myocyte enhancer factor 2 (MEF2) both in vivo and in vitro. In the in vitro experiments, 36 male Wistar rats, divided into three groups, were fed with normal diet, high-fat (HF) diet, or HF diet plus ethanol for 22 weeks. We measured the expressions of total-PRKAA2 (T-PRKAA2), phosphorylated-PRKAA2 (pPRKAA2, activated form of PRKAA2), MEF2, and SLC2A4 in epididymal adipose tissues. In the in vitro experiments, primary adipocytes, isolated from normal Wistar rats, were incubated in the presence or absence of palmitate, ethanol, and compound C (an PRKAA2 inhibitor) for 1 h. Thereafter, T-PRKAA2, pPRKAA2, MEF2, and SLC2A4 expressions were measured. We found that both HF diet and in vitro exposition to palmitate impaired SLC2A4 expression in rat adipocytes with a parallel reduction in PRKAA2 activation and MEF2 expression. This impairment was reversed by ethanol administration. We further demonstrated that ethanol-mediated PRKAA2 activation stimulates MEF2 and SLC2A4 expressions in adipocytes, as evidenced by compound C blockade of these effects. In summary, long-term moderate ethanol consumption reversed the adverse effect of saturated fatty acid on SLC2A4 expression in adipocytes, which was likely to be a result of PRKAA2 activation and subsequent up-regulation of MEF2 and SLC2A4 expressions.

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Introduction

The high-fat (HF) diet with a high ratio of saturated fatty acid is considered as a risk factor for insulin resistance, while moderate ethanol drinking was reported to have beneficial effect on insulin sensitivity (Kiechl et al. 1996, Wei et al. 2000, Kao et al. 2001, Wannamethee et al. 2002). It is known that the lifestyle of HF diet plus ethanol drinking is being widely adopted in some parts of the world. What effect does this lifestyle has on insulin sensitivity? Wilkes found that long-term ethanol feeding (35% calories from ethanol) in a HF diet decreased SLC2A4 (GLUT4) translocation to the plasma membrane (Wilkes et al. 1996), thus resulting in insulin resistance in rat adipocytes. However, similar studies on consuming HF diet plus ethanol are too few to clearly understand their effects on insulin sensitivity although this issue is of most importance to help people with a healthy lifestyle and dietary habits.

Expression of SLC2A4 in adipose tissue is now recognized to play an important role in determining an individual's insulin sensitivity (Minokoshi et al. 2002). For example, adipocyte-specific SLC2A4−/− mice developed insulin resistance and glucose intolerance (Minokoshi et al. 2003), while mice with adipose-specific overexpression of SLC2A4 had enhanced insulin sensitivity (Shepherd et al. 1993). These studies manifested that impaired SLC2A4 expression in adipose tissue might be the primary step of an individual's insulin resistance, namely earlier than that in both skeletal muscle and liver (Abel et al. 2001, Minokoshi et al. 2003, Yang et al. 2005).

Besides the insulin-dependent pathway, SLC2A4 expression was also regulated by AMP-activated protein kinase (PRKAA2, Jessen et al. 2003), a fuel gage for glucose and lipid metabolism. But the connection of PRKAA2 activation to SLC2A4 expression was not well understood. Mora & Pessin (2000) reported that activated PRKAA2 stimulates SLC2A4 expression through up-regulating MEF2, a transcription factor that plays a key role in skeletal muscle differentiation (Wei et al. 2000). Since a functional MEF2 binding site that locates between −522 and −420 of rat SLC2A4 promoter was found (Liu et al. 1994), it is believed that MEF2 was a transcription regulator of SLC2A4. Moreover, such a regulation was independent of the insulin presence, but required the activation of PRKAA2 in skeletal muscle.

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Several studies demonstrated that long-chain saturated fatty acids inhibited PRKAA2 in skeletal muscle and distinct cell types like endothelial cells and pancreatic β cells (Tong et al. 2006, Wu et al. 2007), thus leading to glucose intolerance (Liu et al. 2006, Sriwiatkamol et al. 2006, Tanaka et al. 2007). Additionally, it was demonstrated that the activation of PRKAA2 reversed insulin resistance caused by free fatty acids (FFAs) in skeletal muscle (Olsen & Hansen 2002). These observations corroborated the idea that in models of insulin resistance associated with obesity and increased circulating levels of FFAs, impairment of PRKAA2 contributed to a reduction in insulin sensitivity. In the present study, we reveal that ethanol improves insulin sensitivity in insulin-resistant rats fed with a HF diet by means of PRKAA2 activation in the adipose tissue.

Previous study found that ethanol increased AMP-to-ATP ratio, a trigger for PRKAA2 activation, via inhibiting ATP synthase activity in mitochondria (Cunningham et al. 1990), and increasing the intracellular AMP level in ethanol-treated liver cells (Jing & Ismaisl-Beigi 2006). Based on these findings, PRKAA2 has recently been considered as a possible target of ethanol (You et al. 2004, Hong-Brown et al. 2007). Although a whole host of reports showed that saturated fatty acids inhibit PRKAA2 activity, another study revealed surprisingly that saturated fatty acids plus ethanol could stimulate PRKAA2 activity, which would potentially increase insulin sensitivity in neonatal rat cardiomyocytes (Spargara et al. 2004) and mice liver (You et al. 2005). Up to now, no data are available on how HF diet plus ethanol works on PRKAA2 in adipose tissue, an important organ for insulin sensitivity.

In this study, we explored the combined effects of saturated fatty acids and ethanol on SLC2A4 expression in adipocytes both in vivo and in vitro, and hypothesized that the PRKAA2/MEF2 pathway integrated a novel mechanism by which ethanol ameliorated HF-induced reduction of SLC2A4 expression in adipose tissue. This mechanism might reflect in putative improvement of the insulin resistance associated with HF diet.

Materials and Methods

Animal grouping by diet

Thirty-six male Wistar rats (weight, 160–180 g; age, 4–6 weeks) were purchased from the laboratory Animal Center of Shandong University. After acclimatization for 1 week, the rats were randomly allocated into three experimental groups (n = 12 in each group): normal diet group (N), HF diet plus ethanol group (HF+E), and the pair-fed HF diet group. Both diets were purchased from the Laboratory Animal Center of Shandong University (Jinan, China). On a caloric basis, the HF diet consisted of 59% fat from lard (a representative food full of saturated fatty acid), 24% carbohydrate, and 17% protein (total 5.95 kcal/g), whereas the normal diet contained 10% fat, 70% carbohydrate, and 20% protein (total 4.24 kcal/g). Rats in group HF+E received edible ethanol (Jinan Baotu Spring Distillery, Shandong, China) twice at a total daily dosage of 5 g/kg and rats in other groups received distilled water by gastric tubes. Body weights were monitored and ethanol volumes were adjusted every week. All the treatments lasted for 22 weeks.

During the period of treatment, rats were housed in individual cages in a temperature-controlled room (24 °C) on a 12 h light:12 h darkness cycle. Water was available ad libitum. The animal study was approved by the Shandong University Institutional Animal Care and Use Committee (Jinan, China).

Oral glucose tolerance test (OGTT)

OGTT was carried out after a 22-week-feeding period. After fasting overnight, rats received glucose solution (2 g/kg body weight) by gastric tubes. Then blood glucose levels were measured from samples obtained by tail bleeding at 0, 30, 60, and 120 min after the glucose load. Blood glucose concentrations were determined using a One Touch SureStep Meter (Life Scan, Milpitas, CA, USA). Area under the curve (AUC) was calculated to assess the glucose tolerance.

Tissue collection

All rats were allowed to recover from OGTT for 3 days before being killed. Rats were anesthetized by an i.p. injection of sodium pentobarbital (0.1 ml/100 g BW) after a 10 h fasting. Blood samples were obtained from inferior vena cava for chemical analysis, such as glucose, insulin, adiponectin, and FFAs. Epididymal and perirenal fat pads were rapidly removed and weighed for calculating relative adipose tissue weight to body weight. Parts of epididymal fats were fixed in 4% w/v paraformaldehyde-0.2 M PBS (pH 7.4) for immunofluorescence and hematoxylin & eosin (H&E) staining analysis. The remaining tissues were frozen in liquid nitrogen for mRNA and protein analyses.

Biochemical analysis and evaluation of insulin sensitivity

Blood glucose levels and insulin concentrations were measured by the glucose oxidase method and RIA (Northern Bioengineering Institute, Beijing, China) respectively. Adiponectin concentrations in both adipose tissue and serum were respectively measured by an ELISA kit (adiponectin: Bioneer-trans Pharmaceutical Biotechnology Co., Ltd, Franklin, IN, USA), and then total adiponectin contents in adipose tissue of each rat were calculated according to adipose tissue weight. Total FFA levels were also determined by an ELISA kit (Adipose Diagnostic Laboratories, Inc, Shanghai, China) HOMA-IR was calculated by the following formula: fasting plasma glucose (FPG) (mmol/l) × FINS (µg/ml)/22.5 (Matthews et al. 1985).

Isolation of primary adipocytes and in vitro treatment

Adipocytes were isolated from the epididymal fat pads of normal male Wistar rats (weighing 160–180 g) as described in the references (Tanti et al. 2001, Wu et al. 2003). In brief, excised adipose tissues from five to six rats were pooled together and

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visible blood vessels were removed. Then the minced adipose tissues were digested in Krebs-Ringer bicarbonate HEPES (KRHB) buffer with 1 mg/ml collagenase type I, 1% (wt/vol) BSA, 2.5 mM glucose, 100 μg/ml penicillin, 100 μg/ml streptomycin, and 1% (vol/vol) fungizone for 40–60 min at 37 °C. Subsequently, the cell suspension was filtered sequentially through 500 and 250 μm nylon mesh and centrifuged at 800 g at room temperature for 2 min. After washing twice in KRHB buffer (pH 7.4) with 1% BSA and 2.5 mM glucose, the cells were resuspended, equilibrated with wash buffer for 30 min at 37 °C, and then were seeded in 100 mm culture dishes at 1 million each. Before analysis or preparation of cell lysates, the adipose cells were incubated at 37 °C for 1 h in the absence or presence of palmitate (0-4 mM), ethanol (20 mM), and compound C (a selective inhibitor of PRKAA2, 20 μM). Compound C treatment was initiated 20 min before adding ethanol.

RNA extraction and RT-PCR

Total RNA was extracted from epididymal adipose tissue using the standard Trizol RNA isolation method. The quality of RNA was checked by the DU640 nucleic acid analyzer (Beckman, Los Angeles, CA, USA). Reverse transcription of 4 μg RNA was carried out according to the instructions of the Fermentas RevertAid First Strand cDNA Synthesis Kit (#K1622).

All the primers were synthesized by Shanghai Sangon Biotechnology Corporation (Shanghai, China) and the sequences were shown in Table 1. PCR amplification was carried out in a total reaction volume of 25 μl including 2.5 μl PCR buffer (10X), 0.2 μl Taq polymerase, 2 μl dNTP (TaKaRa, 2.5 mM), 2 μl MgCl2 (TaKaRa, 25 mM), 2 μl Primers (5 × 10^-6 mol/l), and 2.5–3 μl of the cDNA above (2.5 μl for PRKAA1, PRKAA2, and SLC2A4, 3 μl for MEF2A, MEF2D). The PCR products were submitted to 1.5% agarose gel electrophoresis containing ethidium bromide, visualized by excitation under u.v. light, and quantified using a Bio-Rad imager. The amplification of GAPDH for each sample was performed and used as an internal control for quantity and quality. The relative target gene levels were normalized with GAPDH.

Western blotting

Total proteins were extracted from either adipose tissue or isolated adipocytes using RIPA lysis buffer supplemented with 1 mM phenylmethylsulphonyl fluoride and the protein concentration was measured with Lowry Protein Assay Kit (Bio-Rad). Protein extracts (60 μg) were resolved by SDS-PAGE (10% resolving gels for T-PRKAA2, pPRKAA2, MEF2, and SLC2A4, 6% resolving gels for phosphorylated-CoA carboxylase, pACACA) and transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). All membranes were blocked with 5% non-fat dried milk in tris-buffered saline (TBS; 25 mM Tris, 135 mM NaCl, 2.5 mM KCl)/1% Tween-20 (TBST) for 1 h at room temperature, and then incubated overnight at 4 °C with polyclonal rabbit antibodies of T-PRKAA2 (1:1000 dilution, Cell Signaling, Danvers, MA, USA), pPRKAA2 (1:1000 dilution, direct against both PRKAA1 and PRKAA2 isoforms of the enzyme phosphorylated at Thr172, Cell Signaling), pACACA (Ser-79; 1:1000 dilution, Cell Signaling), MEF2 (1:100 dilution, Santa Cruz Biotechnology, Santa Cruz, CA, USA), or SLC2A4 (1:2500 dilution, Abcam Ltd, Cambridge, UK). After incubation with a second antibody (Zsbio Ltd, Beijing, China), immune complexes were detected by Amersham enhanced chemiluminescent method (ECL) Western blotting detection reagents (Amersham) and immunoreactive bands were quantified by Alphaimager 2200. Expression of β-actin, as internal control, was verified through reblotting the same membranes with mice anti-rat β-actin monoclonal antibody (1:10 000 dilution, Abcam Ltd). The relative target protein levels were normalized with β-actin.

Immunofluorescence and H&E staining

After fixing in paraformaldehyde for 36 h, epididymal adipose tissues were embedded in paraffin, and 5 μm sections were

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers</th>
<th>Annealing temperature (°C)</th>
<th>Product size (bp)</th>
<th>Accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRKAA1</td>
<td>Sense: 5’-agg atc cat cag caa cta tgc-3’</td>
<td>56-4</td>
<td>100</td>
<td>NM019142</td>
</tr>
<tr>
<td></td>
<td>Antisense: 5’-agg agg tca cgg atg agg-3’</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PRKAA2</td>
<td>Sense: 5’-cat tgt gtc aag gcc cct agt-3’</td>
<td>58-5</td>
<td>100</td>
<td>NM023991</td>
</tr>
<tr>
<td></td>
<td>Antisense: 5’-gac tgt tgg fat ctc gct tcc-3’</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MEF2A</td>
<td>Sense: 5’-agt ggc tgg agg gca gct tgt ctc-3’</td>
<td>58-5</td>
<td>168</td>
<td>NM001014035</td>
</tr>
<tr>
<td></td>
<td>Antisense: 5’-tgg agg tgt cgg tgt gct tggg-3’</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MEF2D</td>
<td>Sense: 5’-ggt gac atc atc ctc ttc gg-3’</td>
<td>58-5</td>
<td>447</td>
<td>NM030860</td>
</tr>
<tr>
<td></td>
<td>Antisense: 5’-agg ccc tgt ctc agt aaa ct-3’</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SLC2A4</td>
<td>Sense: 5’-agg ctc tga ggt agt gct ttc-3’</td>
<td>57-6</td>
<td>150</td>
<td>NM012751</td>
</tr>
<tr>
<td></td>
<td>Antisense: 5’-cag cga ggc aag gct aga-3’</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>Sense: 5’-tgg tgt acc tca tgt ctc ac-3’</td>
<td>105</td>
<td></td>
<td>XM344448</td>
</tr>
<tr>
<td></td>
<td>Antisense: 5’-cag caa ctc agg gcc tct ct-3’</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 1 Sequences of primers and annealing temperatures
obtained. The sections were incubated with the primary antibody (rabbit anti-SLC2A4, 1:300 dilution, Abcam Ltd) at 4 °C overnight, and subsequently in a fluorescein isothiocyanate (FITC) conjugated anti-rabbit secondary antibody (1:150 dilutions) at room temperature for 1 h. Nuclei were stained with 4, 6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA, UK). Analysis and photo-documentation were performed using a fluorescent microscope (Laica Microsystems GmbH, Wetzlar, Germany). All images were acquired using the same intensity and photodetector gain to allow quantitative comparisons of the relative protein levels among groups. Moreover, the same sections described above were also stained with H&E and imaged under an optical microscope.

**Correlation analysis**

We made the correlation analysis between adiponectin contents in epididymal adipose tissues and sera by SPSS, Chicago, IL, USA, 11.5 software.

**Data analysis**

Each experiment was repeated at least thrice. All values were given as mean ± S.D. Data were analysed by SPSS 11.5 software (SPSS, Inc.) The LSD statistical test was used for post hoc comparison after the ANOVA. \( P<0.05 \) was considered statistically significant.

**Results**

**Characterization of rats in three diet groups after 22-week treatment**

At the baseline, three groups destined for normal diet, HF diet with or without ethanol were of similar body weights. After a 22-week treatment, HF diet alone increased the relative epididymal and perirenal adipose tissue weights by 40.9% (\( P<0.05 \) versus N) and 80.5% (\( P<0.01 \) versus N) compared with normal diet respectively. Whereas the addition of long-term moderate ethanol to HF diet reduced both adipose tissue weights by 21.4% (\( P>0.05 \) versus HF) and 30.3% (\( P<0.01 \) versus HF) in relation to HF diet alone respectively. Coincident with adipose tissue weights, the body weight in group HF was increased by 13.2% compared with that in group N (\( P<0.01 \) versus N) and then was reduced by 8.4% (\( P<0.05 \) versus HF) after long-term ethanol administration in relation to that in group HF (Table 2).

HF diet increased fasting glucose level by 28.1% (\( P>0.05 \) versus N), fasting insulin concentration by 28.3% (\( P<0.05 \) versus N), and HOMA value by 69.8% (\( P<0.01 \) versus N) in relation to normal diet, which indicated that insulin resistance was presented in HF–diet-fed rats. However, in rats fed with HF diet plus ethanol, fasting glucose, fasting insulin, and HOMA values were decreased by 7.8, 19.7, and 28.2% in comparison with that in group HF (Table 2) respectively, indicating an improved insulin sensitivity that appeared after long-term ethanol consumption.

The adiponectin contents in adipose tissue and serum concentrations of adiponectin were reduced by 46.67% (\( P<0.01 \) versus N) and 36.05% (\( P<0.05 \) versus N) respectively, in the animals treated with HF diet alone compared with that in normal diet-fed rats, and elevated by 74.48 and 37.64% (both \( P<0.01 \) versus HF; Table 2) in group HF+E in relation to that in group HF. Correlation analysis results showed an intimate correlation between tissue contents and serum levels of adiponectin (\( r=0.572, \ P<0.01 \)), indicating that the elevated serum adiponectin levels after additional ethanol feeding might be due to the enhancement of adiponectin synthesis in epididymal adipose tissues.

The serum FFAs levels were 3.36-fold in group HF and 2.19-fold in group HF+E over that in group N (both \( P<0.01 \) versus N). Although an addition of ethanol to HF diet did not restored the FFA concentrations to the normal levels, we found that long-term ethanol administration in the setting of HF diet decreased the serum FFAs levels by 34.79% in relation to HF diet only (\( P<0.01 \) versus HF).

**Table 2** Characterization of the rats in three diet groups.

<table>
<thead>
<tr>
<th></th>
<th>N (n=12)</th>
<th>HF (n=12)</th>
<th>HF+E (initial: n=12; final: n=9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW (g)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initial</td>
<td>220.25±13.1</td>
<td>219.5±14.97</td>
<td>224.62±19.54</td>
</tr>
<tr>
<td>Final</td>
<td>477.67±34.47</td>
<td>540.67±58.36</td>
<td>495.33±43.98</td>
</tr>
<tr>
<td>Epididymal fat mass (% of BW)</td>
<td>0.93±0.28</td>
<td>1.31±0.33*</td>
<td>1.03±0.45</td>
</tr>
<tr>
<td>Perirenal fat mass (% of BW)</td>
<td>1.28±0.49</td>
<td>2.31±0.45*</td>
<td>1.61±0.71*</td>
</tr>
<tr>
<td>FBG (mmol/l)</td>
<td>4.09±1.65</td>
<td>5.24±1.01</td>
<td>4.83±1.37</td>
</tr>
<tr>
<td>FINS (mIU/l)</td>
<td>20.58±5.24</td>
<td>26.4±5.58*</td>
<td>21.2±3.36*</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>3.74±1.21</td>
<td>6.35±2.33*</td>
<td>4.56±1.86*</td>
</tr>
<tr>
<td>Serum adiponectin (μg/ml)</td>
<td>25.6±3.13</td>
<td>16.5±3.68*</td>
<td>22.71±6.48*</td>
</tr>
<tr>
<td>Total adiponectin contents in epididymal adipose tissue (μg)</td>
<td>88.75±20.34</td>
<td>47.33±18.89*</td>
<td>2.58±10.95*</td>
</tr>
<tr>
<td>FFA (μmol/l)</td>
<td>50.67±20.77</td>
<td>170.44±22.63*</td>
<td>111.14±47.43*</td>
</tr>
</tbody>
</table>

N, normal diet group; HF, high-fat diet group; HF+E, high-fat diet plus ethanol group. *\( P<0.05 \), †\( P<0.01 \) versus group N; ‡\( P<0.05 \), §\( P<0.01 \) versus group HF.
Long-term ethanol consumption improved high-fat-diet-induced glucose intolerance in rats. Rats were fed with normal diet (N), high-fat (HF) diet, and high-fat diet plus ethanol (HF+E). After a 22-week-feeding period, oral glucose tolerance test (OGTT) was carried out. (A) Blood glucose levels were measured from samples obtained by tail bleeding before administration of glucose (2 g/kg body weight), and 30, 60, and 120 min after glucose load. (B) Area under the curve (AUC) was calculated to assess glucose tolerance. Values were given as means ± s.d. (n = 12 in groups N and HF, n = 9 in group HF+E).

**Figure 1** Long-term ethanol consumption improved high-fat-diet-induced glucose intolerance in rats. Rats were fed with normal diet (N), high-fat (HF) diet, and high-fat diet plus ethanol (HF+E). After a 22-week-feeding period, oral glucose tolerance test (OGTT) was carried out. (A) Blood glucose levels were measured from samples obtained by tail bleeding before administration of glucose (2 g/kg body weight), and 30, 60, and 120 min after glucose load. (B) Area under the curve (AUC) was calculated to assess glucose tolerance. Values were given as means ± s.d. (n = 12 in groups N and HF, n = 9 in group HF+E).

**Long-term ethanol consumption prevented glucose intolerance induced by HF diet**

Compared with normal diet, either HF diet alone or HF diet plus ethanol had no significant effect on fasting and postprandial (30, 60, 120 min) glucose levels (Fig. 1A). As shown in Fig. 1B, the AUC (9.72 ± 0.58 for group N versus 10.68 ± 1.07 for group HF, P < 0.05) revealed that 22-week HF-diet treatment induced glucose intolerance in rats, while addition of ethanol to HF diet restored glucose tolerance to normal (10.4 ± 0.72 for group HF+E versus 9.72 ± 0.58 for group N, P > 0.05).

**SLC2A4 expression in both mRNA and protein levels was recovered after long-term ethanol feeding in the setting of HF diet**

In rat adipose tissue, Slc2a4 mRNA level was diminished by 65-06% (P < 0.01 versus N) in group HF in comparison with that in group N (Fig. 2A). Although a 11-20% reduction of Slc2a4 mRNA expression was also observed after the treatment with the HF diet plus ethanol in relation to the normal diet, but when compared with the HF diet only, the combination of HF diet and ethanol increased Slc2a4 mRNA level by 154-15% (P < 0.01 versus HF). Consistent with the changes in Slc2a4 mRNA expression, SLC2A4 protein expression was also reduced by 59-98% after 22-week HF-diet feeding in relation to normal diet (P < 0.01 versus N) and recovered to nearly normal levels by the addition of ethanol (Fig. 2B).

In immunofluorescence microscopy observation, weak signals were present for the rats fed with HF diet alone, while the stronger signals were observed for the animals fed with a combination of HF diet and ethanol (Fig. 2C). Taken together, long-term ethanol consumption ameliorated both Slc2a4 gene transcription and mRNA translation in the setting of the HF diet.

The inhibition of pPRKAA2 caused by HF diet in rat adipose tissue was lessened by long-term ethanol consumption

To explore the underlying mechanism for the protective effect of ethanol on SLC2A4 expression, we measured the expression and activation levels of PRKAA2, a catalytic subunit of PRKAA2 heterotrimer (Kemp et al. 1999, Carling 2004), in rat adipose tissue. Prkaa1, Prkaa2 mRNA levels and T-PRKAA2 protein expression remained unchanged in all the groups (Fig. 3A and B), indicating that both ethanol and HF diet had no significant effect on the transcription and expression of PRKAA2. As we know, the phosphorylation on Thr172 site in PRKAA2 heterotrimer (Kemp et al. 1999, Carling 2004), in rat adipose tissue. Prkaa1, Prkaa2 mRNA levels and T-PRKAA2 protein expression remained unchanged in all the groups (Fig. 3A and B), indicating that both ethanol and HF diet had no significant effect on the transcription and expression of PRKAA2. As we know, the phosphorylation on Thr172 site in PRKAA2 heterotrimer (Kemp et al. 1999, Carling 2004), in rat adipose tissue. Prkaa1, Prkaa2 mRNA levels and T-PRKAA2 protein expression remained unchanged in all the groups (Fig. 3A and B), indicating that both ethanol and HF diet had no significant effect on the transcription and expression of PRKAA2. As we know, the phosphorylation on Thr172 site in PRKAA2 heterotrimer (Kemp et al. 1999, Carling 2004), in rat adipose tissue. Prkaa1, Prkaa2 mRNA levels and T-PRKAA2 protein expression remained unchanged in all the groups (Fig. 3A and B), indicating that both ethanol and HF diet had no significant effect on the transcription and expression of PRKAA2. As we know, the phosphorylation on Thr172 site in PRKAA2 heterotrimer (Hawley et al. 1996). HF diet reduced the ratio of pPRKAA2 to T-PRKAA2 to 39-08% of that in group N (P < 0.01 versus N) and ethanol addition to HF diet recovered the ratio to 88-87% of that in group N (P < 0.01 versus HF; Fig. 3B). To test whether phosphorylation of PRKAA2 was followed by its activation, we measured pACACA, a substrate for PRKAA2. As expected, HF diet plus ethanol led to a 42% (P < 0.01 versus HF) increase in ACACA phosphorylation compared with HF diet that caused a 50% (P < 0.01 versus N) decrease in relation to normal diet (Fig. 3C). These changes indicate that both ethanol and HF diet affect the activation but not expression of PRKAA2, which was in concert with other group’s finding (Srivijitkamol et al. 2006). Furthermore, in the setting of HF diet, consumption of ethanol leads to an improvement of PRKAA2 activation.

**Long-term ethanol feeding improved MEF2 expression in adipose tissue of rat fed with HF diet**

Because MEF2 is a downstream molecule of PRKAA2 and is necessary to increase Slc2a4 mRNA expression (Thai et al. 1998, Santalucia et al. 2001), we next evaluated whether it was involved in the mechanism by which ethanol and HF diet modulate SLC2A4 expression. The mRNA levels of two MEF2 isoforms (A and D) and protein expression of total MEF2 were diminished by 20-53% (P < 0.01 versus N) after the 22-week HF diet treatment. Consistent with the changes in MEF2 expression, MEF2 protein expression remained unchanged in all the groups (Fig. 3A and B), indicating that both ethanol and HF diet had no significant effect on the transcription and expression of PRKAA2. As we know, the phosphorylation on Thr172 site in PRKAA2 heterotrimer (Hawley et al. 1996). HF diet reduced the ratio of pPRKAA2 to T-PRKAA2 to 39-08% of that in group N (P < 0.01 versus N) and ethanol addition to HF diet recovered the ratio to 88-87% of that in group N (P < 0.01 versus HF; Fig. 3B). To test whether phosphorylation of PRKAA2 was followed by its activation, we measured pACACA, a substrate for PRKAA2. As expected, HF diet plus ethanol led to a 42% (P < 0.01 versus HF) increase in ACACA phosphorylation compared with HF diet that caused a 50% (P < 0.01 versus N) decrease in relation to normal diet (Fig. 3C). These changes indicate that both ethanol and HF diet affect the activation but not expression of PRKAA2, which was in concert with other group’s finding (Srivijitkamol et al. 2006). Furthermore, in the setting of HF diet, consumption of ethanol leads to an improvement of PRKAA2 activation.
MEF2 were measured by RT-PCR and western blotting methods respectively. In mRNA level, Mef2a isoform was unchanged, while Mef2d isoform was markedly reduced in group HF (40.32% of that in group N, \( P < 0.01 \) versus N) and elevated in group HF+E (85.23% of that in group N, \( P < 0.05 \) versus HF, Fig. 4A). In parallel with the changes of Mef2d mRNA, the total protein expression of MEF2 was diminished in group HF (31.68% of that in group N, \( P < 0.01 \) versus N) and recovered in group HF+E (75.24% of that in group N, \( P < 0.01 \) versus HF, Fig. 4B).

Ethanol treatment prevented impairment effect of palmitate on PRKAA2 activation, subsequently restored MEF2 and SLC2A4 expression in isolated primary adipocytes

Ethanol treatment prevented palmitate inhibition of PRKAA2 phosphorylation and restored MEF2 and SLC2A4 levels in isolated primary adipocytes. We incubated adipocytes in the presence or absence of palmitate and ethanol in order to observe the sole and combined effects of free fatty acids and ethanol on PRKAA2 activation, and MEF2 and SLC2A4 expressions. As shown in Fig. 5, the weak bands representing pPRKAA2, pACACA, MEF2, or SLC2A4 were detected in cells treated with palmitate alone. A stronger staining for these proteins was observed when the cells were treated with a combination of palmitate and ethanol.

Furthermore, we incubated the isolated primary adipocytes with ethanol plus compound C, attempting to test whether the protective effect of ethanol was due to the activation of PRKAA2. Our result showed that ethanol at the concentration of 20 mM indeed enhanced PRKAA2 phosphorylation, as well as the expression of its downstream molecules, pACACA and MEF2. Consequently, the expression of SLC2A4, a transcriptional target of MEF2, was also enhanced. However, when the cells were treated with compound C, a selective PRKAA2 inhibitor, prior to ethanol, the enhanced bands were no longer evident (Fig. 5). Thus, we assume that the effect of ethanol on MEF2 and SLC2A4 expressions is dependent on PRKAA2 activation.

**Discussion**

In this study, we found that long-term moderate ethanol consumption reverses the adverse effect of saturated fatty acid on

**Figure 2** Long-term ethanol exposure increased SLC2A4 expression in adipose tissue of high-fat-diet-fed rats. Feeding rats with normal diet (N), high-fat (HF) diet, and HF diet plus ethanol for 22 weeks, we determined (A) Slc2a4 mRNA levels by RT-PCR and (B) protein levels by western blotting. The mRNA levels were normalized by GAPDH and the protein levels were normalized by \( \beta \)-actin. (C) The immunofluorescence microscopy (×200) was adopted to measure SLC2A4 protein expression as well. Nuclei of adipocytes were stained by DAPI. Hematoxylin and eosin (HE) stain (×200) was performed to observe the appearance of adipocyte (C). Values were given as means±s.d. (n=12 in groups N and HF, n=9 in group HF+E). \(^*^ P<0.01\) versus group N; \(^{##} P<0.01\) versus group HF.
SLC2A4 expression in rat adipose tissue. Increased PRKAA2 activation and subsequent up-regulation of MEF2 expression might be one potential mechanism underlying this event.

Epidemiological and experimental studies suggested that light-to-moderate ethanol consumption had beneficial effect on insulin sensitivity (Kiechl et al. 1996, Wei et al. 2000, Kao et al. 2001, Wannamethee et al. 2002, Furuya et al. 2003). But how to define the dosage of ethanol is still controversial. After pooling articles searching in PubMed between 1966 and July 2004, a meta-analysis gave a definition of light, moderate, and heavy drinkers as those who consume ethanol <6, 6–48, and >48 g/d respectively (Koppes et al. 2005). Here, we fed rats with ethanol at the dosage of 5 g/kg per d, which was equal to a dosage of 48 g/d for a person whose body weight was 60 kg, so as to mimic the moderate effect of ethanol in the human body.

We found that the concomitant consumption of ethanol and a HF diet prevents the weight gain associated with the diet. Based on the results in Table 1, this seems to be due in part to decreased adipose tissue mass. Increased body weight, especially from fat tissue, plays an important role in the development of insulin resistance, in part through altered adiponectin secretion. The reduction in body weight in this group (HF + E) may play a role in improved insulin sensitivity.

Here, we found that long-term ethanol administration improved insulin sensitivity induced by HF diet. A decrease in the circulating FFAs after ethanol addition to HF diet might be a contribution to insulin sensitivity because FFAs were reported to play important role in insulin resistance (Boden & Chen 1995, Belfort et al. 2005, Lee et al. 2006). The reduction of total FFA levels might result from increased PRKAA2 activation. In this study, we found that moderate ethanol treatment (5 g/kg per d in vivo, 20 mM in vitro) successfully restored the PRKAA2 activation in HF-diet-fed rat adipose tissue to a level close to normal. Activated PRKAA2 could phosphorylate and inhibit ACACA, a rate-limiting enzyme in fatty acid synthesis. Inhibited ACACA might reduce malonyl-coenzyme A, and thereby permitted fatty acid transporting into and oxidizing in the mitochondrion. Thus, the decreased FFA serum levels after long-term ethanol treatment are likely to reduce lipotoxicity induced by HF diet and, by extension, improve insulin sensitivity.

How does ethanol stimulate PRKAA2 activation, via direct or indirect pathways? According to other reports and parts of our results, we proposed several possible mechanisms: 1) ethanol increases the AMP-to-ATP ratio, the stimuli of PRKAA2, via the following possible mechanisms. First, in vivo, during the process of ethanol transforming into acetaldehyde and acetic acid, NAD can be oxidized to NADH. Resulting from the decreased amounts of NAD+, the NADH oxidation respiratory chain is affected, thus ATP generation is reduced. Secondly, ethanol can also inhibit ATP synthase activity in mitochondria. As a result, ATP

Figure 3 Long-term ethanol administration reversed the impairment of PRKAA2 activation induced by high-fat diet in rat adipose tissue. Feeding rats with normal diet (N), high-fat (HF) diet, and HF diet plus ethanol (HF + E) for 22 weeks, we determined (A) the mRNA levels of PRKAA1 and PRKAA2 isoforms by RT-PCR and (B) the protein levels of T-PRKAA2, pPRKAA2, and pACACA by western blotting. The mRNA expression was normalized by GAPDH and the protein expression was normalized by β-actin. Values were given as means ± S.D. (n = 12 in groups N and HF, n = 9 in group HF + E). **P<0.01 versus group N; ##P<0.01 versus group HF.
production was decreased (Cunningham et al. 1990). Thirdly, ethanol enhances intracellular AMP levels as well (Jing & Ismail-Beigi 2006). As it is described above, the AMP/ATP ratio is increased, which leads to PRKAA2 activation. 2) We found moderate ethanol consumption increased the adiponectin contents in both adipose tissue and serum, which was coincided with the previous studies (Sierksma et al. 2004, Beulens et al. 2006, 2007). Adiponectin was considered to be an activator of PRKAA2 (Yamauchi et al. 2002). Thus, the elevated adiponectin level might contribute to PRKAA2 activation by ethanol. 3) Besides the mechanisms mentioned above, direct effect of ethanol on PRKAA2 activation still cannot be excluded, which can be observed in our experiments in vitro.

We found that long-term moderate ethanol supplement restored the impairment of SLC2A4 expression induced by HF diet in adipose tissue. However, contrary to our findings, Wilkes et al. (1996) demonstrated that chronic ethanol feeding in a HF diet decreased total SLC2A4 protein in rat adipocytes. The reason for the discrepancy in the SLC2A4 expression between their result and ours is still unclear, but the different dosages of ethanol in the two studies might be the main explanation. The rats were received 35% calories from ethanol in their experiments, whereas only 11% calories from ethanol in ours. Furuya et al. (2003) reported that only a certain dosage range of alcohol (about 9% calories from ethanol) can improve insulin sensitivity. So far, SLC2A4 regulation by PRKAA2 is not completely understood. In skeletal muscles, activated PRKAA2 has been demonstrated to increase SLC2A4 protein expression or translocation (Kurth-Kraczek et al. 1999, Buhl et al. 2001, Koistinen et al. 2003). However, the conclusion about this issue in adipose tissue is controversial. Under basal conditions (namely, no insulin stimulation), several studies reported the activation of PRKAA2 by 5-aminomidazole-4-carboxamide
ribonucleoside (ATIC)-accelerated SLC2A4 translocation (Salt et al. 2000, Yamaguchi et al. 2005), whereas others expressed a different opinion, namely that translocation of SLC2A4 was suppressed by activated PRKAA2 in adipocytes (Gaidhu et al. 2006). In our study, we demonstrated a positive regulation of PRKAA2 on SLC2A4 in adipocytes under basal conditions.

The precise mechanism of PRKAA2 affecting SLC2A4 was not fully elucidated yet. Holmes et al. (2005) found that PRKAA2 could regulate SLC2A4 expression via MEF2. This finding was derived from the evidence that a functional MEF2 binding site located between −466 and −457 of rat SLC2A4 promoter. In the present study, we found that when HF-induced reduction of PRKAA2 activity occurred, both Mef2d mRNA and total MEF2 protein levels were also decreased, and the level of Slk2a4 gene transcription was coincidently declined. On the contrary, when ethanol enhanced the PRKAA2 activity in adipose tissues, the expressions of MEF2D and SLC2A4 became enlarged. The findings in vitro were reconfirmed by the in vivo experiments. Palmitate inhibited the activation of PRKAA2, subsequently MEF2 and SLC2A4 expressions in adipocytes, which were largely reversed by ethanol. Importantly, all of the ethanol-induced reversing effects could be counteracted by compound C, a selective PRKAA2 inhibitor, which suggested that, in adipocytes, there existed a regulation of SLC2A4 by PRKAA2, and that PRKAA2 was indeed a target, at a certain degree, of ethanol. Based on these findings, we hypothesize that PRKAA2 activation might be a mechanism by which ethanol partially restores insulin sensitivity impaired by HF-diet consumption.

Taken together, we found that long-term moderate ethanol reversed the adverse effect of HF diet on SLC2A4 expression in adipose tissue, then whole body insulin sensitivity. Although these findings need to be further studied, at least, it provided us a new lifestyle modulation of treating HF-diet-induced insulin resistance besides the pharmacological therapy. The result also suggests that we can consume moderate but not an overdose of ethanol when we have to intake HF diet. By doing so, it may be benefit to insulin sensitivity.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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References


Ethanol restores insulin sensitivity via PRKAA2


