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 vivo* 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.* 2003). 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.

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, Sriwijitkamol *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 & Ismail-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 (Sparagna *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 acid 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: Bionewtrans 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 (Adlitteram Diagnostic Laboratories, Inc. Shanghai, China). HOMA-IR was calculated by the following formula: fasting plasma glucose (FPG) (mmol/l) $\times$ FINS (μ m/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

visible blood vessels were removed. Then the minced adipose tissues were digested in Krebs–Ringer bicarbonate HEPES (KRBH) 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 KRBH 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 1×10^7 /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 (10×), 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 AlphaImager 2200. 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-acetyl 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, Cambridgeshire, 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 1 Sequences of primers and annealing temperatures

	Primers	Annealing temperature (°C)	Product size (bp)	Accession number
Gene				
PRKAA1	Sense: 5'-ggg atc cat cag caa cta tcg-3' Antisense: 5'-ggg agg tca cgg atg agg-3'	56.4	100	NM019142
PRKAA2	Sense: 5'-cat ttg tgc aag gcc cct agt-3' Antisense: 5'-gac tgt tgg tat ctg cct gtt tcc-3'	58.5	100	NM023991
MEF2A	Sense: 5'-agt ggc tgg agg gca gtt atc-3' Antisense: 5'-tgg agg ttg tgg cgg tgg-3'	58.5	168	NM001014035
MEF2D	Sense: 5'-ggt gac atc atc cct tac gg-3' Antisense: 5'-agg ccc tgg ctg agt aaa ct-3'	58.5	447	NM030860
SLC2A4	Sense: 5'-ggg ctg tga gtg agt gct ttc-3' Antisense: 5'-cag cga ggc aag gct aga-3'	57.6	150	NM012751
GAPDH	Sense: 5'-tgg tgg acc tca tgg cct ac-3' Antisense: 5'-cag caa ctg agg gcc tct ct-3'		105	XM344448

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

	N (n=12)	HF (n=12)	HF+E (initial: n=12; final: n=9)
BW (g)			
Initial	220.25±13.1	219.5±14.97	224.62±19.54
Final	477.67±34.47	540.67±58.36 [†]	495.33±43 [‡]
Epididymal fat mass (% of BW)	0.93±0.28	1.31±0.33*	1.03±0.45
Perirenal fat mass (% of BW)	1.28±0.49	2.31±0.45 [†]	1.61±0.71 [§]
FBG (mmol/l)	4.09±1.65	5.24±1.03	4.83±1.37
FINS (mIU/l)	20.58±5.24	26.4±5.58*	21.2±3.36 [‡]
HOMA-IR	3.74±1.21	6.35±2.33 [†]	4.56±1.86 [‡]
Serum adiponectin (µg/ml)	25.8±3.15	16.5±3.68*	22.71±6.48 [§]
Total adiponectin contents in epididymal adipose tissue (µg)	88.75±20.34	47.33±18.89 [†]	2.58±18.95 [§]
FFA (µmol/l)	50.67±20.77	170.44±22.63 [†]	111.14±47.43 ^{‡,§}

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.

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 $\pm$ 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 α -subunit of PRKAA2 is essential for the PRKAA2 activity (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 (Sriwijitkamol *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 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 β-actin. (C) The immunofluorescence microscopy ($\times 200$) was adopted to measure SLC2A4 protein expression as well. Nuclei of adipocytes were stained by DAPI. Hematoxylin and eosin (HE) stain ($\times 200$) was performed to observe the appearance of adipocyte (C). Values were given as means $\pm$ 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/d¹ respectively (Koppes *et al.* 2005). Here, we fed rats with ethanol at the dosage of 5 g/kg per d¹, which was equal to a dosage of 48 g/d¹ 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 resistance 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 d¹ *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 NADs⁺, 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 $\pm$ 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.

Figure 4 Long-term ethanol consumption improved MEF2 expression in high-fat-diet-fed rat adipose tissue. After rats were fed with normal diet (N), high-fat (HF) diet, and HF diet plus ethanol (HF + E) for 22 weeks, (A) *Mef2a*, *Mef2d* mRNA levels were measured by RT-PCR and normalized by GAPDH. (B) MEF2 protein expression was determined by western blotting and 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; # $P < 0.05$, ## $P < 0.01$ 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

Figure 5 Supplement of ethanol to rat primary adipocytes restored activation of PRKAA2, expressions of MEF2 and SLC2A4 in the setting of palmitate. Rat primary adipocytes were isolated from epididymal fat pads as previously described (Materials and Methods), the 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 (20 μM). Compound C treatment was initiated 20 min before the addition of ethanol. *Slc2a4* mRNA expression was measured by RT-PCR. Protein expressions of pPRKAA2, pACACA, MEF2, and SLC2A4 were determined by western blotting. The data presented were based on the results of four separate experiments. P, E, P + E, E + C, and C represented cells treated with palmitate, ethanol, palmitate plus ethanol, ethanol plus compound C, and compound C respectively. N represented control cells. * $P < 0.05$, ** $P < 0.01$ versus controls; # $P < 0.05$, ## $P < 0.01$ versus cells treated with palmitate.

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-aminoimidazole-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 *Slc2a4* gene transcription was coincidentally declined. On the contrary, when ethanol enhanced the PRKAA2 activity in adipose tissues, the expressions of MEF2D and SLC2A4 became enlarged. The findings *in vivo* were reconfirmed by the *in vitro* 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

- Abel ED, Peroni O, Kim JK, Kim Y-B, Boss O, Hadro E, Minnemann T, Shulman GI & Kahn BB 2001 Adipose-selective targeting of the GLUT4 gene impairs insulin action in muscle and liver. *Nature* **409** 729–733.
- Belfort R, Mandarino L, Kashyap S, Wirfel K, Pratipanawatr T, Berria R, DeFronzo RA & Cusi K 2005 Dose-response effect of elevated plasma free fatty acid on insulin signaling. *Diabetes* **54** 1640–1648.
- Beulens JW, van Beers RM, Stolk RP, Schaafsma G & Hendriks HF 2006 The effect of moderate alcohol consumption on fat distribution and adipocytokines. *Obesity* **14** 60–66.
- Beulens JW, van Loon LJ, Kok FJ, Pelsers M, Bobbert T, Spranger J, Helander A & Hendriks HF 2007 The effect of moderate alcohol consumption on adiponectin oligomers and muscle oxidative capacity: a human intervention study. *Diabetologia* **50** 1388–1392.
- Boden G & Chen X 1995 Effects of fat on glucose uptake and utilization in patients with non-insulin-dependent diabetes. *Journal of Clinical Investigation* **96** 1261–1268.
- Buhl ES, Jessen N, Schmitz O, Pedersen SB, Pedersen O, Holman GD & Lund S 2001 Chronic treatment with 5-aminoimidazole-4-carboxamide-1-beta-D-ribofuranoside increases insulin-stimulated glucose uptake and GLUT4 translocation in rat skeletal muscles in a fiber type-specific manner. *Diabetes* **50** 12–17.
- Carling D 2004 The AMP-activated protein kinase cascade – a unifying system for energy control. *Trends in Biochemical Sciences* **29** 18–24.
- Cunningham CC, Coleman WB & Spach PI 1990 The effects of chronic ethanol consumption on hepatic mitochondrial energy metabolism. *Alcohol and Alcoholism* **25** 127–136.
- Furuya DT, Binsack R & Machado UF 2003 Low ethanol consumption increases insulin sensitivity in Wistar rats. *Brazilian Journal of Medical and Biological Research* **36** 125–130.
- Gaidhu MP, Fediuc S & Ceddia RB 2006 5-Aminoimidazole-4-carboxamide-1-beta-D-ribofuranoside-induced AMP-activated protein kinase phosphorylation inhibits basal and insulin-stimulated glucose uptake, lipid synthesis, and fatty acid oxidation in isolated rat adipocytes. *Journal of Biological Chemistry* **281** 25956–25964.
- Hawley SA, Davison M, Woods A, Davies SP, Beri RK, Carling D & Hardie DG 1996 Characterization of the AMP-activated protein kinase kinase from rat liver and identification of threonine 172 as the major site at which it phosphorylates AMP-activated protein kinase. *Journal of Biological Chemistry* **271** 27879–27887.
- Holmes BF, Sparling DP, Olson AL, Winder WW & Dohm GL 2005 Regulation of muscle GLUT4 enhancer factor and myocyte enhancer factor 2 by AMP-activated protein kinase. *American Journal of Physiology. Endocrinology and Metabolism* **289** E1071–E1076.
- Hong-Brown LQ, Brown CR, Huber DS & Lang CH 2007 Alcohol regulates eukaryotic elongation factor 2 phosphorylation via an AMP-activated protein kinase-dependent mechanism in C2C12 skeletal myocytes. *Journal of Biological Chemistry* **282** 3702–3712.
- Jessen N, Pold R, Buhl ES, Jensen LS, Schmitz O & Lund S 2003 Effects of AICAR and exercise on insulin-stimulated glucose uptake, signaling, and GLUT-4 content in rat muscles. *Journal of Applied Physiology* **94** 1373–1379.
- Jing M & Ismail-Beigi F 2006 Role of 5'-AMP-activated protein kinase in stimulation of glucose transport in response to inhibition of oxidative phosphorylation. *American Journal of Physiology. Cell Physiology* **290** C484–C491.
- Kao WH, Puddey IB, Bolland LL, Watson RL & Brancati FL 2001 Alcohol consumption and the risk of type 2 diabetes mellitus: atherosclerosis risk in communities study. *American Journal of Epidemiology* **154** 748–757.
- Kemp BE, Mitchellhill KI, Stapleton D, Michell BJ, Chen ZP & Witters LA 1999 Dealing with energy demand: the AMP-activated protein kinase. *Trends in Biochemical Sciences* **24** 22–25.

- Kiechl S, Willeit J, Poewe W, Egger G, Oberhollenzer F, Muggeo M & Bonora E 1996 Insulin sensitivity and regular alcohol consumption: large, prospective, cross sectional population study (Bruneck study). *BMJ* **313** 1040–1044.
- Koistinen HA, Galuska D, Chibalin AV, Yang J, Zierath JR, Holman GD & Wallberg-Henriksson H 2003 5-Amino-imidazole carboxamide riboside increases glucose transport and cell-surface GLUT4 content in skeletal muscle from subjects with type 2 diabetes. *Diabetes* **52** 1066–1072.
- Koppes LL, Dekker JM, Hendriks HF, Bouter LM & Heine RJ 2005 Moderate alcohol consumption lowers the risk of type 2 diabetes: a meta-analysis of prospective observational studies. *Diabetes Care* **28** 719–725.
- Kurth-Kraczek EJ, Hirshman MF, Goodyear LJ & Winder WW 1999 5' AMP-activated protein kinase activation causes GLUT4 translocation in skeletal muscle. *Diabetes* **48** 1667–1671.
- Lee JS, Pinnamaneni SK, Eo SJ, Cho IH, Pyo JH, Kim CK, Sinclair AJ, Febbraio MA & Watt MJ 2006 Saturated, but not N-6 polyunsaturated fatty acids, induce insulin resistance: role of intramuscular accumulation of lipid metabolites. *Journal of Applied Physiology* **100** 1467–1474.
- Liu ML, Olson AL, Edgington NP, Moye-Rowley WS & Pessin JE 1994 Myocyte enhancer factor 2 (MEF2) binding site is essential for C2C12 myotube-specific expression of the rat GLUT4/muscle-adipose facilitative glucose transporter gene. *Journal of Biological Chemistry* **269** 28514–28521.
- Liu Y, Wan Q, Guan Q, Gao L & Zhao J 2006 High-fat diet feeding impairs both the expression and activity of AMPK α in rats' skeletal muscle. *Biochemical and Biophysical Research Communications* **339** 701–707.
- Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF & Turner RC 1985 Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologia* **28** 412–419.
- Minokoshi Y, Kahn CR & Kahn BB 2003 Tissue-specific ablation of the GLUT4 glucose transporter or the insulin receptor challenges assumptions about insulin action and glucose homeostasis. *Journal of Biological Chemistry* **278** 33609–33612.
- Mora S & Pessin JE 2000 The MEF2A isoform is required for striated muscle-specific expression of the insulin-responsive GLUT4 glucose transporter. *Journal of Biological Chemistry* **275** 16323–16328.
- Olsen GS & Hansen BF 2002 AMP kinase activation ameliorates insulin resistance induced by free fatty acids in rat skeletal muscle. *American Journal of Physiology. Endocrinology and Metabolism* **283** E965–E970.
- Salt IP, Connell JM & Gould GW 2000 5-Aminoimidazole-4-carboxamide ribonucleoside (AICAR) inhibits insulin-stimulated glucose transport in 3T3-L1 adipocytes. *Diabetes* **49** 1649–1656.
- Santalucia T, Moreno H, Palacin M, Yacoub MH, Brand NJ & Zorzano A 2001 A novel functional co-operation between MyoD, MEF2 and TRalpha1 is sufficient for the induction of GLUT4 gene transcription. *Journal of Molecular Biology* **314** 195–204.
- Shepherd PR, Gnudi L, Tozzo E, Yang H, Leach F & Kahn BB 1993 Adipose cell hyperplasia and enhanced glucose disposal in transgenic mice overexpressing GLUT4 selectively in adipose tissue. *Journal of Biological Chemistry* **268** 22243–22246.
- Sierksma A, Patel H, Ouchi N, Kihara S, Funahashi T, Heine RJ, Grobbee DE, Kluij C & Hendriks HF 2004 Effect of moderate alcohol consumption on adiponectin, tumor necrosis factor- α , and insulin sensitivity. *Diabetes Care* **27** 184–189.
- Sparagna GC, Jones CE & Hickson-Bick DL 2004 Attenuation of fatty acid-induced apoptosis by low-dose alcohol in neonatal rat cardiomyocytes. *American Journal of Physiology. Heart and Circulatory Physiology* **287** H2209–H2215.
- Sriwijitkamol A, Ivy JL, Christ-Roberts C, DeFronzo RA, Mandarino LJ & Musi N 2006 LKB1-AMPK signaling in muscle from obese insulin-resistant Zucker rats and effects of training. *American Journal of Physiology. Endocrinology and Metabolism* **290** E925–E932.
- Tanaka T, Masuzaki H, Yasue S, Ebihara K, Shiuchi T, Ishii T, Arai N, Hirata M, Yamamoto H, Hayashi T *et al.* 2007 Central melanocortin signaling restores skeletal muscle AMP-activated protein kinase phosphorylation in mice fed a high-fat diet. *Cell Metabolism* **5** 395–402.
- Tanti JF, Cormont M, Gremeaux T & Le Marchand-Brustel Y 2001 Assays of glucose entry, glucose transporter amount, and translocation. *Methods in Molecular Biology* **155** 157–165.
- Thai MV, Guruswamy S, Cao KT, Pessin JE & Olson AL 1998 Myocyte enhancer factor 2 (MEF2)-binding site is required for GLUT4 gene expression in transgenic mice. Regulation of MEF2 DNA binding activity in insulin-deficient diabetes. *Journal of Biological Chemistry* **273** 14285–14292.
- Tong Y, Huang DS & Bryer-Ash M 2006 Effects of globular adiponectin, glucose and free fatty acid on AMPK and ACC phosphorylation in INS-1 beta cells. *Beijing Da Xue Xue Bao* **38** 609–613.
- Wannamethee SG, Shaper AG, Perry IJ & Alberti KG 2002 Alcohol consumption and the incidence of type II diabetes. *Journal of Epidemiology and Community Health* **56** 542–548.
- Wei M, Gibbons LW, Mitchell TL, Kampert JB & Blair SN 2000 Alcohol intake and incidence of type 2 diabetes in men. *Diabetes Care* **23** 18–22.
- Wilkes JJ, DeForrest LL & Nagy LE 1996 Chronic ethanol feeding in a high-fat diet decreases insulin-stimulated glucose transport in rat adipocytes. *American Journal of Physiology* **271** E477–E484.
- Wu X, Motoshima H, Mahadev K, Stalker TJ, Scalia R & Goldstein BJ 2003 Involvement of AMP-activated protein kinase in glucose uptake stimulated by the globular domain of adiponectin in primary rat adipocytes. *Diabetes* **52** 1355–1363.
- Wu Y, Song P, Xu J, Zhang M & Zou MH 2007 Activation of protein phosphatase 2A by palmitate inhibits AMP-activated protein kinase. *Journal of Biological Chemistry* **282** 9777–9788.
- Yamaguchi S, Katahira H, Ozawa S, Nakamichi Y, Tanaka T, Shimoyama T, Takahashi K, Yoshimoto K, Imaizumi MO, Nagamatsu S *et al.* 2005 Activators of AMP-activated protein kinase enhance GLUT4 translocation and its glucose transport activity in 3T3-L1 adipocytes. *American Journal of Physiology. Endocrinology and Metabolism* **289** E643–E649.
- Yamauchi T, Kamon J, Minokoshi Y, Ito Y, Waki H, Uchida S, Yamashita S, Noda M, Kita S, Ueki K *et al.* 2002 Adiponectin stimulates glucose utilization and fatty-acid oxidation by activating AMP-activated protein kinase. *Nature Medicine* **8** 1288–1295.
- Yang Q, Graham TE, Mody N, Preitner F, Peroni OD, Zabolotny JM, Kotani K, Quadro L & Kahn BB 2005 Serum retinol binding protein 4 contributes to insulin resistance in obesity and type 2 diabetes. *Nature* **436** 356–362.
- You M, Matsumoto M, Pacold CM, Cho WK & Crabb DW 2004 The role of AMP-activated protein kinase in the action of ethanol in the liver. *Gastroenterology* **127** 1798–1808.
- You M, Considine RV, Leone TC, Kelly DP & Crabb DW 2005 Role of adiponectin in the protective action of dietary saturated fat against alcoholic fatty liver in mice. *Hepatology* **42** 568–577.

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