The frequency of daily ethanol consumption influences the effect of ethanol on insulin sensitivity in rats fed a high-fat diet

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(Received 7 October 2010 – Revised 28 February 2011 – Accepted 23 May 2011 – First published online 6 September 2011)

Abstract
The different effects of ethanol on insulin sensitivity may be due to complex reasons. Here, we focus on the various daily ethanol consumption frequencies in rats fed a high-fat (HF) diet and explore the possible mechanism mediated by adiponectin and AMP-activated protein kinase (AMPK). A total of thirty-six male Wistar rats were fed a HF diet and were randomly divided into three groups: those that received tap water (C); those that received ethanol via a gastric tube twice per d (E1); those that received free access to ethanol for drinking (E2). The total daily ethanol dosage in groups E1 and E2 were the same (5 g/kg per d). At the end of 18 weeks, insulin sensitivity was evaluated. Adiponectin AMPK and GLUT4 levels were determined. We found that the different administration frequencies led to markedly different plasma ethanol concentrations and there were intimate relationships between plasma ethanol concentration and insulin sensitivity. Insulin resistance was markedly improved in group E1, whereas only a slight improvement was observed in group E2. Accordingly, adiponectin, phosphorylated AMPK and GLUT4 levels were significantly increased in group E1. Based on these findings, we propose that ethanol concentration might be the major influencing factor mediating the effect of ethanol on insulin sensitivity. At a total daily dosage of 5 g/kg per d, twice daily administration of ethanol was more beneficial than continuous drinking. The protective effect of ethanol might be mediated by increased adiponectin levels, which subsequently improve the activation of AMPKα and GLUT4 expression in adipose tissue.

Key words: Ethanol consumption frequency: Insulin sensitivity: High-fat diet: Rats

Both protective and detrimental effects of ethanol on insulin sensitivity have been widely reported (1–6). The majority of researchers have suggested that the differential effects of ethanol on insulin sensitivity are primarily due to the dosage of ethanol consumed (7–11). In addition, a few researchers have proposed that drinking pattern also plays an important role in mediating the effects of ethanol (12–18). However, the ethanol consumption pattern is quite complex and includes the frequency of ethanol consumption in addition to the food setting. The present study was designed to investigate the influence of daily ethanol consumption frequency on insulin sensitivity in a high-fat (HF) diet setting. To the best of our knowledge, this is the first study that focused on daily ethanol consumption frequencies, but not in a long time period.

Over the past few years, the number of studies that have investigated the effects of ethanol in combination with a HF diet has increased. The underlying reason for this increase is that alcohol consumption is often accompanied by intake of a HF diet. To date, reports on the effects of ethanol plus a HF diet on insulin sensitivity remain controversial. Some studies have shown that a combination of alcohol consumption and HF diet resulted in decreased glucose uptake.
in skeletal muscle and in adipose tissue, leading to a high incidence of diabetes\textsuperscript{19,20}. In contrast, we and others have found that ethanol consumption improved insulin resistance induced by a HF diet\textsuperscript{3,21–23}. Similar to the present and Hong’s results, Fueki et al.\textsuperscript{24} found that regular alcohol consumption improved insulin resistance in healthy Japanese men, independent of obesity. In addition to the unclear effect of ethanol and HF diet on insulin sensitivity, the underlying mechanisms of this effect are also obscure. Here, we evaluated the insulin sensitivity of HF diet-fed rats after ethanol treatments with different frequencies and explored potential mediating mechanisms by determining the expression levels of AMP-activated protein kinase (AMPK), PPARγ and GLUT4. In addition, we also measured adiponectin, a known insulin sensitizer and upstream activator of AMPK, in both adipose tissue and sera.

**Experimental methods**

**Animal feeding**

Initially, forty-eight male Wistar rats (weight, 160–180 g; age, 4–6 weeks) were acclimatized to a HF diet for 1 week. Based on energy content, the HF diet consisted of 59% fat from lard, 24% carbohydrate and 17% protein. The acclimatized rats were randomly divided into four groups according to weight, and they received ethanol with varying administration patterns: ad libitum consumption of tap water without ethanol (controls, C); twice daily administration of ethanol (E1, 5 g/kg per d); continuous drinking of ethanol (E2, 5 g/kg per d); once daily administration of ethanol (E3, 5 g/kg per d). The animals in groups E1 and E3 received ethanol via a gastric tube. Body weights were monitored and ethanol volumes were adjusted weekly. Unfortunately, a portion of the animals in group E3 died within the first 2 months, and we could only provide the complete data for groups C, E1 and E2 in the present study.

All rats were purchased from the Laboratory Animal Center of Shandong University (Jinan, China). 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 dark cycle. Water was available ad libitum. The animal study was approved by the Shandong University Institutional Animal Care and Use Committee.

**Oral glucose tolerance test**

An oral glucose tolerance test was carried out at the end of 8 and 18 weeks. After overnight fasting, rats received a glucose solution (2 g/kg body weight) via a gastric tube. Blood glucose levels were measured from whole blood samples obtained by tail bleeding at 0, 30, 60 and 120 min after the glucose load was administered. Blood glucose (BG) concentrations were determined using a OneTouch SureStep Meter (Life Scan, Milpitas, CA, USA). The area under the curve (AUC) was calculated as described previously\textsuperscript{25}:

$$\text{AUC} = \frac{1}{2} \left(\text{BG (0 min)} + \frac{1}{2} \text{BG (30 min)} + \frac{3}{4} \text{BG (60 min)} + \frac{1}{2} \text{BG (120 min)}\right)$$

**Determination of plasma ethanol concentration**

Blood samples were obtained from the inferior vena cava 40 min after gastric tube administration of ethanol in groups E1 and E3 and after the removal of ethanol in group E2. Plasma ethanol concentrations were determined with a dry chemical method (Johnson & Johnson, New Brunswick, NJ, USA).

**Tissue collection**

Most rats in group E3 died within the first 2 months of the experiment. The remaining five rats in group E3 were killed after 8 weeks of feeding; the rats in groups E1 and E2 were killed at the end of week 18. After a 10 h fast, animals were anaesthetised with an intraperitoneal injection of sodium pentobarbital (0.1 ml/100 g body weight), and blood samples were obtained from the inferior vena cava for chemical analyses, including determination of glucose, insulin and adiponectin levels. The epidymal and perirenal fat pads were rapidly removed and weighed for the calculation of the relative adipose tissue weight compared with body weight. The epidymal adipose tissues were frozen in liquid N\textsubscript{2} for mRNA and protein analyses.

**Biochemical analysis and evaluation of insulin sensitivity**

Blood glucose levels and insulin concentrations were measured using the glucose oxidase method and RIA (Northern Bioengineering Institute, Beijing, China), respectively. Adiponectin concentrations in both adipose tissue and sera were, respectively, measured using an ELISA kit (adiponectin; Bionewtrans Pharmaceutical Biotechnology Company Limited, Franklin, MA, USA), and then total adiponectin contents in adipose tissue of each rat were calculated according to adipose tissue weight. Homeostasis model assessment of insulin resistance (HOMA-IR) was calculated using the following formula\textsuperscript{25}:

$$\text{HOMA-IR} = \frac{\text{fasting plasma glucose (mmol/l)}}{\text{fasting insulin (microunits/ml)}} / 22.5.$$
Table 1. Characterisation of the rats* (8 weeks) (Mean values and standard deviations for five animals per group)

<table>
<thead>
<tr>
<th>C</th>
<th>E1</th>
<th>E2</th>
<th>E3</th>
</tr>
</thead>
<tbody>
<tr>
<td>HOMA-IR</td>
<td>4.5 ± 1.4</td>
<td>4.1 ± 1.1</td>
<td>4.4 ± 2.0</td>
</tr>
<tr>
<td>Plasma ethanol concentration (mg/l)</td>
<td>0 ± 183</td>
<td>47 ± 43</td>
<td>10 ± 970</td>
</tr>
</tbody>
</table>

C, control; E1, rats that received ethanol twice per d; E2, rats that received ethanol continuously; E3, rats that received ethanol once per d; HOMA-IR, homeostasis model assessment of insulin resistance.

* Rats received a high-fat diet only (group C) supplemented with ethanol once (group E3) or twice (group E1) daily (total 5 g/kg) via a gastric tube, or with ethanol by drinking (group E2, total 5 g/kg daily) for 8 weeks.

(antisense), accession no. NM_023991; GLUT4, 5'-ggg ctc gct gta gtt gat gtg ttc-3' (sense) and 5'-cag cga ggc aag gct aga-3' (antisense), accession no. NM_012751; PPARγ, 5'-tgt gga cct ctc tgt gat g-3' (sense) and 5'-cag cca ctc agg gcc tct ct-3' (antisense), accession no. XM_344448.

Western blotting

Total proteins were extracted from adipose tissues by using radio-immunoprecipitation assay lysis buffer supplemented with 1 mM-phenylmethylsulfonyl fluoride and Western blotting was carried out as described previously (21,26). The primary antibodies were bought from Cell Signaling Company, Danvers, MA, USA (total AMPKα and phosphorylated AMPKα) and Abcam Limited, Cambridge, UK (PPARγ and GLUT4), respectively.

Data analysis

The data shown represent a minimum of three independent experiments. All values are presented as means and standard deviations. Data were analysed with SPSS 11.5 software (SPSS, Inc., Chicago, IL, USA). After comparison by ANOVA, a least significant difference statistical test was performed for post hoc comparisons, with P<0.05 considered to be statistically significant.

Results

Fasting glucose levels, fasting insulin concentrations and homeostasis model assessment of insulin resistance

Statistics were not performed on the data obtained at the end of the 8th week (Table 1) because of the small number of rats. However, despite equal total daily ethanol dosages, HOMA-IR was obviously reduced in group E1 and was slightly decreased in group E2 compared with group C. In contrast, HOMA-IR in group E3 was obviously increased. Despite administration of the same ethanol dosage each day, the different administration frequencies led to markedly different blood ethanol concentrations, which were 183 mg/l in group E1, only 43 mg/l in group E2 and 970 mg/l in group E3. As shown in Fig. 1, there was a U-shaped relationship between HOMA-IR and ethanol concentrations.

After the rats were fed the HF diet for 18 weeks (Table 2), elevated levels of fasting glucose and fasting insulin were observed in group C, but these had been decreased by 7.5 and 23.4%, respectively, in group E1. However, the fasting plasma glucose and fasting insulin levels of group E2 were not statistically significant compared with those of group C. Accordingly, insulin resistance, which was evaluated by HOMA-IR, was observed in HF diet-fed rats, but was ameliorated with ethanol administration; the value of HOMA-IR was reduced by 30.8% in group E1 and 9.2% in group E2 (Fig. 2(a)).

Body weights and fat masses

The body weight and fat masses of rats at 18 weeks are shown in Table 2. Ethanol administration lessened the weight gain from the HF diet by 8.5% in group E1 and by 3% in group E2. Coincident with the reductions in body-weight gain, the epididymal and perirenal fat masses were reduced by 12.1 and 11.4% in group E1, respectively, but no significant...
change was observed in the fat pad masses of group E2 relative to group C.

**The area under the curve of the oral glucose tolerance test**

An oral glucose tolerance test was carried out on rats after feeding a HF diet for 18 weeks, and the AUC was calculated. As shown in Fig. 2(b), the AUC in group E1 was reduced by 10.2% relative to that in group C (P<0.05 v. C), and it was not significantly different between groups E2 and C.

**Adiponectin levels**

The concentrations of adiponectin in sera and adipose tissue were recovered towards normal by 35.3% (P<0.01 v. C) and 24.5% (P<0.01 v. C), respectively, in group E1, but were only recovered by 15.7% (P<0.05 v. C) and 10.9% (P<0.05 v. C), respectively, in group E2 (Table 2). Correlation analysis results showed an intimate correlation between the tissue and serum levels of adiponectin (r=0.572, P<0.01).

**AMP-activated protein kinase activity**

It is known that adiponectin is an activator for AMPKα. In parallel with the changes in adiponectin levels, the ratio of AMPKα (phosphorylated AMPKα)/total AMPKα in rats that received ethanol administration twice per d and in those that drank ethanol continuously increased by 97.6% (P<0.01 v. C) and 17% (P>0.05 v. C), respectively, compared with that of group C (Fig. 3(b)). Ethanol consumption had no influence on either AMPKα1 or α2 mRNA expression (Fig. 3a).

**PPARγ expression**

Relative to group C, PPARγ mRNA levels were increased by 62.9% (P<0.01 v. C) in group E1, but only by 20% (P>0.05 v. C) in group E2. In accordance with increased PPARγ gene transcript abundance, PPARγ protein expression was increased by 43.8% (P<0.01 v. C) in group E1 and by 11.3% (P>0.05 v. C) in group E2 (Fig. 4).

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**Table 2. Characterisation of the rats (18 weeks)†**

(Mean values and standard deviations)

<table>
<thead>
<tr>
<th></th>
<th>C</th>
<th></th>
<th>E1</th>
<th></th>
<th>E2</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>Mean</td>
<td>SD</td>
<td>Mean</td>
<td>SD</td>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
<td>Initial</td>
<td>219.5</td>
<td>15</td>
<td>224.6</td>
<td>19.5</td>
<td>221</td>
<td>13.9</td>
</tr>
<tr>
<td>Final</td>
<td>500.7</td>
<td>51.4</td>
<td>458.3*</td>
<td>43</td>
<td>485.8†</td>
<td>45.2</td>
</tr>
<tr>
<td>Epididymal fat mass (% body weight)</td>
<td>1.0</td>
<td>0.3</td>
<td>0.9</td>
<td>0.5</td>
<td>1.0</td>
<td>0.2</td>
</tr>
<tr>
<td>Perirenal fat mass (% body weight)</td>
<td>2.2</td>
<td>0.6</td>
<td>2.0*</td>
<td>0.7</td>
<td>2.0</td>
<td>0.4</td>
</tr>
<tr>
<td>FBG (mmol/l)</td>
<td>5.3</td>
<td>1.1</td>
<td>4.9</td>
<td>1.2</td>
<td>5.2</td>
<td>1.3</td>
</tr>
<tr>
<td>FINS (μU/ml)</td>
<td>27.4</td>
<td>4.5</td>
<td>21.0*</td>
<td>3.0</td>
<td>25.1†</td>
<td>4.3</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>6.5</td>
<td>2.3</td>
<td>4.5*</td>
<td>1.9</td>
<td>5.9†</td>
<td>2.1</td>
</tr>
<tr>
<td>Serum adiponectin (μg/ml)</td>
<td>15.3</td>
<td>3.7</td>
<td>20.7**</td>
<td>6.0</td>
<td>17.7†</td>
<td>5.2</td>
</tr>
<tr>
<td>Total adiponectin contents in epididymal adipose tissue (µg)</td>
<td>47.3</td>
<td>18.9</td>
<td>82.6**</td>
<td>19</td>
<td>55.8†</td>
<td>17.1</td>
</tr>
<tr>
<td>Serum ethanol concentration (mg/l)</td>
<td>0</td>
<td>108</td>
<td>44</td>
<td>40</td>
<td>11</td>
<td></td>
</tr>
</tbody>
</table>

C, control; E1, rats that received ethanol twice per d; E2, rats that received ethanol continuously; E3, rats that received ethanol once per d; FBG, fasting blood glucose; FINS, fasting insulin; HOMA-IR, homeostasis model assessment of insulin resistance. Mean values were significantly different from those of group C: * P<0.05, ** P<0.01. † Mean values were significantly different from those of group E1 (P<0.05). ‡ Rats received a high-fat diet only (group C) supplemented with ethanol twice daily (total 5 g/kg) via a gastric tube (group E1) or with ethanol by drinking (group E2; total 5 g/kg daily) for 18 weeks.

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**Fig. 2.** Different daily ethanol consumption frequencies restore insulin resistance induced by a high-fat diet differently. A total of thirty-six male Wistar rats fed with a high-fat diet were divided into three groups: ad libitum consumption of tap water without ethanol (controls, C); twice daily administration of ethanol (E1, 5 g/kg per d); continuously drinking of ethanol (E2, 5 g/kg per d). The oral glucose tolerance test (OGTT) was carried out after an 18-week feeding period. Blood glucose levels were measured from samples obtained by tail bleeding at 0, 30, 60 and 120 min after the glucose load (2 g/kg body weight). The area under the curve (AUC = 1/4 BG (0 min) + 1/2 BG (30 min) + 3/4 BG (60 min) + 1/2 BG (120 min)) was calculated to assess glucose tolerance (b). At 4 d after the OGTT, all rats were anaesthetised and blood samples were obtained from the inferior vena cava for the determination of glucose and insulin concentrations. Homeostasis model assessment of insulin resistance (HOMA-IR) was calculated using the following formula: fasting plasma glucose (mmol/l) x fasting insulin (microunits/ml)/22.5 (a). Values are means, with standard deviations represented by vertical bars (n=12).
frequency of twice per d was more beneficial than the once-daily or continuous drinking pattern. In the present study, rats received ethanol continuously; E3, rats that received ethanol once per d.

Based on the present data, HOMA-IR was not positively related to the frequencies of ethanol consumption. Therefore, we determined the plasma ethanol concentration because this could be a mediating factor that influences the effect of ethanol on insulin sensitivity. Whereas there must be a fluctuation in plasma ethanol concentrations, which could subsequently result in differential effects on insulin sensitivity.

Discourse

Previously, studies have shown that varying dosages, categories and drinking patterns of ethanol consumption resulted in different effects on insulin sensitivity (1–6). We presumed that all of these factors could lead to different plasma ethanol concentrations, which could subsequently result in differential effects on insulin sensitivity. Therefore, plasma ethanol concentration could be the underlying factor that determines ethanol action. In the present study, rats received ethanol at the same dosage of 5 g/kg per d, which is equivalent to an ethanol consumption of 48 g/d for a person whose body weight is 60 kg. At this same total daily ethanol dosage, variation of the daily ethanol consumption frequency led to markedly different effects. A daily ethanol administration frequency of twice per d was more beneficial than the continuous drinking pattern in the improvement of the adverse effect of a HF diet on insulin sensitivity. However, we did not verify whether this conclusion would also apply to daily ethanol dosages other than 5 g/kg per d. In fact, according to the initial design of the study, a group in which rats received ethanol once daily (group E3) at the dosage of 5 g/kg per d was also included. Unfortunately, a portion of the rats in group E3 died within the first 2 months of the study and only five rats survived to 8 weeks. Thus, we could not provide complete data for this group. However, after only 8 weeks of feeding a HF diet, we found that HOMA-IR was ameliorated in the groups that were administered ethanol via a gastric tube twice per d and by continuous drinking. In contrast, HOMA-IR was worsened in the group that was administered ethanol administration once per d. Thus, the twice-daily administration pattern showed a more beneficial effect on HF diet-induced insulin resistance than the once-daily or continuous drinking patterns.

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Different daily ethanol consumption frequencies improve GLUT4 mRNA and protein expression differently. After feeding the rats for 18 weeks, we determined GLUT4 (a) mRNA levels by RT-PCR, (b) protein levels by Western blotting and (c) immunofluorescence (×200). GLUT4 mRNA levels were normalised by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and protein levels were normalised by β-actin. Values are means, with standard deviations represented by vertical bars (n=12). E1, rats that received ethanol twice per d; E2, rats that received ethanol once per d.

Fig. 5. Different daily ethanol consumption frequencies improve GLUT4 mRNA and protein expression differently.
that the effect of ethanol was directly associated with plasma ethanol concentration, but not with ethanol dosage or drinking frequency. Thus, it might be more reasonable to define light, moderate and heavy drinkers according to plasma ethanol concentrations because, by doing so, many of the confounding factors, such as frequency and liver metabolic ability, can be eliminated.

Taken together, the present data show that a particular ethanol consumption pattern can improve insulin resistance induced by a HF diet, and this improvement is associated with a mechanism involving adiponectin and AMPK. Moreover, we found that a twice daily administration of ethanol was more beneficial than a continuous intake of ethanol at the total dosage of 5 g/kg per d. The differential plasma ethanol concentrations resulting from these administration patterns might be the key factor influencing the effect of ethanol on insulin sensitivity.

Acknowledgements

This study was supported by the grant from the National Natural Science Foundation of China (30940038 and 81000323) and the Natural Science Foundation of Shandong Province, China (Y2001C12, ZR2009CM008 and 2009ZRB14022). The authors acknowledge the expert technical assistance by teachers in the Central Laboratory and Experimental Animal Center of Provincial Hospital affiliated to the Shandong University. Partial data of the present study were presented at 48th EASD Annual Meeting (A-08-1890-EASD) and 45th EASD Annual Meeting (A-09-2096-EASD). This study was carried out in the Central Laboratory of Shandong Provincial Hospital, Shandong Province, China. L. F. and B. H. performed most of the work, such as feeding animals, performing experiments and writing the manuscript. R. W. provided help for the experiment designing. Q. L. and D. B. helped the first author determine plasma ethanol concentration. C. M. and G. S. helped L. F. prepare the animals. J. Z. and L. G. designed the experiment and supplied essential support of technology and fund. The authors declare that there is no conflict of interest.

References