Repeated application of low-frequency electroacupuncture improves high-fructose diet-induced insulin resistance in rats

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Abstract

Background Insulin resistance is frequently present in obesity and during the development of type 2 diabetes mellitus.

Objective The purpose of the present study was to investigate the effect of electroacupuncture (EA) on high-fructose diet (HFD)-induced insulin resistance.

Methods Male Wistar rats were fed HFD for 4 weeks and developed insulin resistance. Insulin sensitivity was assessed by clamp. The number of animals was seven, eight and seven in the control, HFD and HFD + EA groups, respectively. AMP-activated protein kinase (AMPK) and glucose transporter 4 (GLUT4) in skeletal muscle were measured by Western blotting analysis (n=7 in each group). EA stimulation was carried out 12 times over 4 weeks at an intensity of 1–3 mA and a frequency of 2/15 Hz in a conscious state without restraint.

Results There was no significant difference in mean body weight and fasting blood glucose concentration between groups at the end of the experiment. The mean glucose infusion rate during the clamp was significantly lower in the HFD group than in controls (p<0.05). There was no significant difference in expression of GLUT4 in skeletal muscle in the control and each group. Phosphorylated AMPKα (Thr172) in skeletal muscle showed a significant increase immediately after the final EA stimulation when compared with the control group (p<0.05).

Conclusion Repeated application of EA is capable of improving diet-induced insulin resistance, probably through activation of AMPK signalling pathways in skeletal muscle. These results suggest that repeated application of EA may have beneficial effects on diet-induced insulin resistance.

INTRODUCTION

Diabetes mellitus is a metabolic disorder that can lead to a wide range of complications, including coronary artery disease, cerebrovascular disorders, renal failure, blindness and gangrene. The prevalence of diabetes among adults (aged 20–79 years) in 2010 was estimated to be 6.4%, affecting 285 million adults, and will increase to 7.7% (439 million adults) by 2030.1 Insulin resistance is frequently present in obesity and during the development of type 2 diabetes mellitus and is generally defined as a reduction in the body’s ability to clear a glucose load from circulation in response to insulin. Although there is controversy regarding the relative importance of insulin resistance and impaired β cell function in the pathogenesis of diabetes, some studies have proposed that loss of early insulin response to glucose and poor suppression of hepatic glucose output are primarily responsible for development of the disease.2 Pharmacological agents that reduce insulin resistance are clinically available, but they carry a potential risk of serious complications.3 4 In the last decade, increasing evidence has suggested that electroacupuncture (EA) may improve glucose metabolism by modifying insulin sensitivity.5 6 Studies have suggested that EA increases insulin secretion and/or enhances insulin sensitivity in diabetic and obese rodent models.5 7 However, the electrical stimulation intensity utilised in these experiments was relatively high (10–80 mA), requiring the use of a general anaesthetic.5 8 Since administration of anaesthetic agents may suppress plasma catecholamine levels, which, in turn, reduces lipolysis and increases insulin secretion through inhibition of ATP-sensitive K+ (KATP) channels, assessment of the effects of EA under conscious conditions is of interest.9–12 In addition, the intensity of electrical stimulation used clinically is lower than that utilised in previous studies. Furthermore, the mechanisms underlying the beneficial effects of EA on insulin resistance are still unclear.

Studies have demonstrated that high-fructose diet (HFD)-fed rodents exhibit a decline in insulin sensitivity in the peripheral tissues.13 These rodents are a valuable model of insulin resistance and may provide insights into the molecular mechanisms underlying insulin resistance associated with lifestyle modification. Skeletal muscle is an important mediator of glucose homeostasis because it plays a primary role in the beneficial effects of exercise on prevention and improvement of the diabetic state.
The purpose of our study was to investigate whether EA improves impaired insulin sensitivity in HFD-fed rats, as well as to evaluate the effects of EA on glucose metabolism pathways by measuring phosphorylation of AMP-activated protein kinase α (AMPKα; Thr172) and protein expression of glucose transporter 4 (GLUT4) in peripheral skeletal muscle.

METHODS

Animals

Male Wistar rats at 4 weeks of age were purchased from CLEA Japan (Osaka, Japan). Rats were housed individually in an air-conditioned room with a 12:12-h light/dark cycle (lights on at 07:30) and allowed ad libitum access to water and their respective diet until evaluation at 8 weeks of age. Implantation of catheters was carried out 1 week before the glucose clamp study to allow recovery from the surgical procedure (refer to the section on detailed surgical procedure). Hyperinsulinaemic–euglycaemic clamp study was performed at 48 h after final (12th) EA stimulation. For protein assays of the skeletal muscle, rats were killed with an overdose of pentobarbital at specific time points: immediately before, immediately after, and at 13 and 48 h after the final EA stimulation. Figure 1 shows the flowchart of study design.

EA stimulation

EA was carried out as described by Iwa et al. Hook-shaped needles (diameter: 0.45 mm) were fabricated from ophthalmic surgical needles (ELP Brand; Akiyama Medical, Tokyo, Japan) and soldered to an electrical cord. The cord was lifted up to the lid of the cage with appropriate tension that did not restrict but follow the free moving of the animal, keeping an appropriate length to the stimulating points. Needles were inserted at bilateral Zusanli (ST36), which is located at the tibialis anterior (TA) muscle below the knees. Rats were allowed to move freely in the cage.
during EA stimulation. EA was carried out 12 times over 4 weeks using a pulse generator (Han’s Healthronics, Likon, Taipei, Taiwan). The intensity of the electrical stimulation was increased from 1 to 3 mA in 1-mA increments as follows: 1st–4th treatments: 1 mA, 5th–8th treatments: 2 mA and 9th–12th treatments: 3 mA. The frequency of the square waves was 2/15 Hz, that is, 2 Hz alternating with 15 Hz, with each lasting for 3 s over 30 min of EA application. The intensity and frequency of the electrical stimulation were monitored with an oscilloscope at both ends of a resistor (20 Ω) inserted into the circuit. A hyperinsulinaemic–euglycaemic clamp was carried out 48 h after the last EA treatment. Figure 2 shows the setup for EA stimulation under conscious conditions.

Surgery
After 3 weeks on the respective diets, rats undergoing hyperinsulinaemic–euglycaemic clamp were anaesthetised with sodium pentobarbital (45 mg/kg body weight (BW) intraperitoneally) and a horizontal incision was made in the supraclavicular area. Chronic cannulas (MRE-040 cannula; Eicom, Kyoto, Japan) were then inserted into the left carotid artery and the right jugular vein, brought around the neck subcutaneously, and passed through a small incision in the skin at the base of the neck. The rats were administered penicillin G potassium (10 000 units/kg intraperitoneally; Wako Pure Chemical Industries, Osaka, Japan) and gentamicin (0.5 mg/kg intramuscularly; Wako Pure Chemical Industries). After 7 days of recovery, rats underwent the hyperinsulinaemic–euglycaemic clamp after an overnight fast.

Hyperinsulinaemic–euglycaemic clamp
Insulin sensitivity was assessed by hyperinsulinaemic–euglycaemic clamp. Blood (approximately 10 μl) was taken from the left carotid artery for glucose measurement every 5 min using an automated blood sampling system (DR-2; Eicom). Plasma glucose was measured using the glucose oxidase method (GR102; Terumo, Tokyo, Japan). Insulin solution (Humulin R; Eli Lilly Japan, Kobe, Japan) was diluted in saline containing 0.25% wt/vol bovine serum albumin (BSA RI, A7888; Sigma, St. Louis, Missouri, USA) and infused through the venous catheter using a syringe pump (Harvard Apparatus, Holliston, Massachusetts, USA) at a rate of 5 mU/kg/min. Glucose infusion (20% wt/vol; Otsuka Pharmaceutical, Tokyo, Japan) was started 10–15 min after insulin infusion using a peristaltic pump (Minipuls 3; Gilson, Middleton, Wisconsin, USA), and the glucose infusion rate (GIR) was adjusted every 5 min in response to the blood glucose concentration with the objective of maintaining blood glucose level at the mean value obtained over a 15-min period during the fasting stage. Mean GIR was calculated based on GIR from 100 to 120

Figure 2  Setup for electroacupuncture (EA) at ST36 under conscious conditions without restraint. (A) Rats were allowed to move freely in the cage during EA (note that the lid covering the cage is not shown in this figure). The intensity (1–3 mA) and frequency (2/15 Hz) of electrical stimulation were continuously monitored with an oscilloscope located at both ends of a resistor (20 Ω) inserted into the circuit. Hook-shaped needles (B) (diameter: 0.45 mm) made from ophthalmic surgical needles were inserted bilaterally at Zusanli (ST36) (C) according to the methods of Iwa et al.14
min after starting insulin infusion, at which time GIR was in a steady state.

**Muscle preparation**

Rats undergoing skeletal muscle analysis were anaesthetised with sodium pentobarbital (45 mg/kg BW) after 4 weeks on the respective diets and the TA and extensor digitorum longus (EDL) muscles were removed and further processed for measurement of GLUT4 and phosphorylation of AMPKα (Thr172). Specimens were frozen in liquid nitrogen immediately after removal and stored at −80°C until use. After pulverisation in an iron mortar (Nonaka Rikaki, Tokyo, Japan), specimens were homogenised in HEPES buffer (25 mM HEPES, pH 7.4, 2 mM EDTA, 250 mM sucrose, 1% Triton X-100, 1 mM PMSF) using a polytron homogeniser (PT-3100; KINEMATICA, Lucerne, Switzerland). The insoluble and soluble fractions were centrifuged at 6500 rpm for 30 min at 4°C, and HEPES buffer of an equal volume to that of the soluble fraction was added to the centrifuged insoluble fraction. Protein content of the supernatant was measured using a commercially available kit (#500-0006; Bio-Rad Laboratories, Hercules, California, USA).

**Western blotting analysis**

Western blotting analysis was carried out according to previously described methods. Equal volumes of the soluble and insoluble fractions from the six experimental groups were run on a 10% sodium dodecyl sulphate–polyacrylamide gel using a minigel electrophoresis apparatus (BE-240; Bio-Craft, Tokyo, Japan). The gel was then transferred to a polyvinylidene difluoride membrane (Immobilon-PSQ Membrane; Millipore Billerica, Massachusetts, USA) at 200 V for 45 min. Membranes were blocked with 3% BSA (Further purified Fraction V, essentially γ-globulin free, A3059; Sigma) for 30 min. After two washes in Tris-buffered saline–Tween (20 mM Tris–HCl (pH 7.6), 137 mM NaCl, 0.1% Tween 20; TBS-T), membranes were incubated with GLUT4 antibody (#2299; Cell Signaling Technology, Danvers, Massachusetts, USA), phospho-AMPKα antibody (#2531; Cell Signaling Technology), α-tubulin antibody (#2144; Cell Signaling Technology) or AMPKα antibody (#2552; Cell Signaling Technology) for 1 h at room temperature. After two washes in TBS-T, membranes were incubated with anti-rabbit IgG, horse-radish peroxidase–linked antibody (#7074; Cell Signaling Technology) for 1 h at room temperature. After two washes in TBS-T, membranes were incubated in lumino chemiluminescent substrate reagent (#7003; Cell Signaling Technology) and then exposed to chemiluminescence detection film (BioMax Light Film; Eastman Kodak Company, Rochester, New York, USA). After chemiluminescent detection, bands were measured by densitometry using the ImageJ program (National Institutes of Health, Bethesda, Maryland, USA). Signals were normalised to α-tubulin or total AMPKα.

**Statistical analysis**

All data are expressed as means ± SEM. Differences between BW, blood glucose and mean GIR were determined by one-way analysis of variance (ANOVA) followed by post-hoc comparison with Tukey’s method. Since significantly skewed distribution was observed in the phospho-AMPK and the GLUT4 expression, log transformation of these data was performed before the one-way ANOVA followed by Dunnet’s multiple comparison test. A p value less than 0.05 was considered statistically significant. All analyses were performed using SYSTAT (version 12 for Windows; SYSTAT Software, San Jose, California, USA).

**RESULTS**

**BW and fasting blood glucose concentration**

There was no significant difference in mean BW and blood glucose concentration among groups at the end of the intervention period in hyperinsulinaemic–euglycaemic clamp (table 1).

**Hyperinsulinaemic–euglycaemic clamp**

In this study, we evaluated the effects of EA on diet-induced insulin resistance by comparing a standard chow diet-fed group (control), an HFD-fed group and an HFD-fed group that underwent EA (HFD + EA). Figure 3 shows the GIR for the hyperinsulinaemic–euglycaemic clamp study in the three groups. The mean GIR in the HFD group was significantly lower than that in the control group (24.4±1.9 mg/kg/min in control vs 13.0±1.9 mg/kg/min in HFD; p<0.05). In contrast, mean GIR in the HFD+EA group was similar to that in the control group and was significantly higher than that in the HFD group (24.1±3.5 mg/kg/min in HFD+EA vs 13.0±1.9 mg/kg/min in HFD; p<0.05).

### Table 1 Changes in the mean body weight and fasting blood glucose

<table>
<thead>
<tr>
<th>Group</th>
<th>Control</th>
<th>HFD</th>
<th>HFD + EA</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 Weeks of age</td>
<td>89.7±21.1</td>
<td>86.3±23.5</td>
<td>86.4±22.2</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>8 Weeks of age</td>
<td>227.7±20.1</td>
<td>208.1±26.1</td>
<td>208.3±20.6</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Fasting blood glucose (mg/dl)</td>
<td>84.0±4.5</td>
<td>84.3±3.5</td>
<td>77.7±5.1</td>
<td>&gt;0.05</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± SEM. Differences between means were determined by one-way analysis of variance followed by post-hoc comparison with Tukey’s method.
After finding that insulin resistance in HFD-fed rats was significantly improved by repeated application of EA, we investigated the mechanisms underlying this effect by measuring protein expression in the TA and EDL. We focused on GLUT4, which plays a key role in glucose uptake in skeletal muscle, and on the phosphorylation of AMPKα (Thr172), which regulates lipid metabolism and expression of GLUT4 in skeletal muscle. Figure 4 shows cytoplasmic GLUT4 content in the TA and EDL in the control and HFD groups and at each time point after final EA stimulation. Immediately after the final EA stimulation, the HFD+EA group had a two- to threefold higher GLUT4 content compared with control group. Although the GLUT4 content showed a tendency to increase after EA stimulation, the difference did not reach statistical significance. Figure 5 shows changes in the phospho-AMPKα (Thr172) in the TA and EDL. Immediately after the final EA stimulation, phospho-AMPKα (Thr172) was three- to fourfold higher in animals receiving EA than in the control group with a statistical significance (p<0.05).

DISCUSSION
This study demonstrated that repeated EA treatment improved insulin resistance in HFD-fed rats. Insulin sensitivity as evaluated by euglycaemic clamp was worsened in HFD-fed rats after 4 weeks of HFD.18 This was consistent with previous reports showing that HFD-fed rats develop insulin resistance and increased plasma free fatty acids (FFAs) and triglycerides, although fasting blood glucose...
concentration is not affected by short-term HFD. In the HFD model, decreased tyrosine phosphorylation of the insulin receptor (IR) and IR substrate (IRS)-1 has been observed in the skeletal muscle. In addition, phosphorylation of Akt (protein kinase B) and atypical protein kinase C, both of which are downstream mediators of IRS-1 in insulin signalling pathways, is also decreased in the HFD model. In this study, repeated application of low-frequency EA stimulation improved GIR in the HFD model. Since this model resembles diet-induced insulin resistance in humans, EA may have beneficial effects on prediabetic patients such as those with metabolic syndrome.

Several reports have demonstrated an improvement in insulin sensitivity or modification of plasma FFA concentration in response to EA stimulation. However, most of these studies have utilised relatively higher intensity electric current that requires the use of general anaesthesia. Since administration of anaesthesia can affect glucose metabolism, the use of these agents in conjunction with EA is not ideal for demonstrating the effects of EA on glucose homeostasis. In the present study, we were able to carry out EA under conscious and free-moving conditions, which is ideal for evaluation of the effects of EA on glucose metabolism. Another advantage of this method is that this level of EA stimulation is appropriate for clinical application. Although appropriate intensity of EA stimulation under conscious rats is unclear, several studies indicate that 1–3 mA might be applicable to the hindlimb of the rats without causing stressful reactions. We considered 1 mA was appropriate at the commencement of the stimulation to adopt the animals to the procedure, which was also found to be enough to cause muscle contraction. We set the final intensity of the stimulation as 3 mA with expectation of the effect of contractile muscle to the glucose metabolism, which caused no stressed behaviour of the animal.

Skeletal muscle is of critical importance in insulin sensitivity, as has been demonstrated through the beneficial effects of exercise and in a study that investigated alteration of glucose metabolism during and after direct electrical stimulation of skeletal muscle in vitro. It is therefore presumable that muscle contractions evoked during EA may exert beneficial effects on insulin sensitivity through activation of AMPK in the HFD model, which may, in turn, increase translocation of GLUT4 and consumption of circulating glucose. To test the hypothesis that EA affects signalling in skeletal muscle, we measured cytoplasmic GLUT4 and phosphorylation of AMPK in the EDL. It is therefore presumable that expression of GLUT4 in the plasma membrane might be increased as GIR was increased in the hyperinsulinaemic clamp study and as AMPK was significantly activated by EA stimulation. AMPK activation may be decreased in the HFD model as a result of inhibition of insulin signalling.
However, in the present study, because GLUT4 concentration in the soluble fraction was much higher than that in the insoluble fraction, we were not able to compare the contents between the fractions at the same time on the same assay. We therefore consider it necessary to investigate translocation of GLUT4 to the plasma membrane in the next investigation. In contrast, phosphorylation of AMPK was significantly increased immediately after the final EA stimulation. We believe that muscle contractions induced by EA stimulation decrease cellular ATP and elevate the ratio of AMP to ATP, which may, in turn, activate AMPK in the skeletal muscle. Activation of AMPK pathways may result in the inhibition of acetyl-CoA carboxylase 2 (ACC2) and increase glucose uptake in skeletal muscle via increased GLUT4 translocation to the plasma membrane. ACC2 may play a role in the development of insulin resistance in the HFD model through carboxylation of acetyl-CoA, producing malonyl-CoA, which inhibits carnitine palmitoyltransferase-1, which is important for mitochondrial long-chain fatty acid oxidation.

Improved lipid metabolism, which may result from EA stimulation, may help prevent insulin resistance in the HFD model. Decreased plasma FFA in response to EA was reported in a previous study. This finding supports our hypothesis that EA stimulation modifies AMPK pathways and consequently alters lipid metabolism. Many studies have suggested that similar beneficial effects can be obtained with exercise. We presume that EA is even more beneficial than exercise, as it has been reported to increase insulin secretion as well as improve AMPK pathway function, and these mechanisms may act synergistically. Although there are several reports that electrical muscle stimulation increases glucose utilisation in human subjects, it is presumably that EA at ST36 might have beneficial effects on glucose metabolism through the autonomic nervous system, such as suppression of hyperactivity in the sympathetic nervous system and/or activation of parasympathetic nervous system as it is confirmed both in animals and human subjects.

However, the mechanisms of the effects of EA on insulin secretion and lipid metabolism have not been thoroughly investigated, although some reports have suggested the involvement of opioid peptides or serotonin.

Our group has previously studied the beneficial effects of EA on glucose metabolism with respect to both insulin secretion in the pancreas and insulin sensitivity in peripheral tissues. The detailed molecular mechanisms underlying these effects are worthy of further investigation. Thus, we conclude that EA improves insulin resistance in the HFD-fed rats through activation of AMPK in the skeletal muscle.

In the present study, we investigated the effect of EA on HFD-induced insulin resistance in rats. Application of EA during HFD feeding for 4 weeks markedly improved insulin resistance. AMPK in the skeletal muscle was significantly activated after EA stimulation. These results suggest that repeated application of EA may have beneficial effects on diet-induced insulin resistance, probably through repeated activation of AMPK signalling pathways in the skeletal muscles.

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Competing interests None.

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Contributors AT and YN carried out various aspects of the experiments summarised in this manuscript. NI, YY and HK participated in the design and coordinated the performance of the experiments. NI and YN provided technical advice on this project. AT and NI drafted the manuscript.

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