

Effect of Leptin on Insulin Resistance of Muscle – Direct or Indirect?

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Summary

We examined the effect of leptin on the insulin resistance in skeletal muscles by measuring glucose transport. Male Wistar rats were fed rat chow or high-fat diets for 30 days. Before sacrifice, rats fed high-fat diet were subcutaneously injected with leptin (1 mg/kg b.w.) for 3 days. The glucose transport in epitrochlearis and soleus muscles did not differ in the experimental groups under basal conditions, however these values decreased significantly in the rats fed high-fat diet under insulin stimulation ($p < 0.01$). Leptin treatment recovered the decreased glucose transport in epitrochlearis ($p < 0.05$) and soleus muscles ($p = 0.08$). Triglyceride concentrations in soleus muscles were increased significantly in the rats fed high-fat diet as compared to rats fed chow diet ($p < 0.01$), and were decreased significantly by leptin treatment ($p < 0.01$). The glucose transport was measured under basal conditions and after 60 $\mu\text{U/ml}$ of insulin treatment with or without 50 ng/ml of leptin. Leptin had no direct stimulatory effect on glucose transport under both basal and insulin-stimulated conditions *in vitro*. These results demonstrate that leptin injection to rats fed high-fat diet recovered impaired insulin responsiveness of skeletal muscles and muscle triglyceride concentrations. However, there was no direct stimulatory effect of leptin on insulin sensitivity of skeletal muscles *in vitro*.

Key words

Leptin • Glucose transport • Insulin • Skeletal muscle

Introduction

Leptin, the protein product of the *ob* gene, affects the feeding regulatory center in the hypothalamus, where it binds to the leptin receptor in order to prevent obesity by reducing food intake through the inhibition of secretion of neuropeptide Y and by increasing lipid oxidation through the sympathetic nerve system (Schwartz *et al.* 1996). Recently, adipose tissues were

reported to be an active endocrine organs that secrete leptin, plasminogen activator inhibitor-1, angiotensinogen and cytokines, such as tumor necrosis factor- α , interleukin-6 and transforming growth factor- β . (Mohamed-Ali *et al.* 1998). It was reported that the principal target tissues of those mediators are mostly skeletal muscles (Tartaglia 1997). Therefore, the metabolic link between adipocytes and skeletal muscles has attracted a great deal of attention. Moreover, since

leptin receptors are expressed in skeletal muscles, leptin function as a mediator for delivering signals from adipose tissues to skeletal muscles is being studied. Some authors have reported that the glucose metabolism increases after leptin had been administered *in vivo* (Kamohara *et al.* 1997, Yaspelkis *et al.* 2004), whereas others observed no effect or inhibition in animals (Rossetti *et al.* 1997, Widdowson *et al.* 1998) or cells (Tajmir *et al.* 2003). When treated for a long period of time, insulin sensitivity was increased (Rouru *et al.* 1999, Yaspelkis *et al.* 1999, 2004). Glucose metabolism also increased, when leptin was injected directly into the ventromedial nucleus of the hypothalamus (Minokoshi *et al.* 1999). However, the direct effects of leptin in skeletal muscles remain controversial. It increases the glucose metabolism in C2C12 myotubes and skeletal muscles (Berti *et al.* 1997, Ceddia *et al.* 1998), but it does not affect the glucose metabolism of skeletal muscles directly (Furnsinn *et al.* 1998, Ranganathan *et al.* 1998, Zierath *et al.* 1998).

Skeletal muscles use stored glycogen and triglycerides, and glucose comes from oral ingestion as the main source of energy. Leptin stimulates the expression of uncoupling protein-2 in adipocytes (Zhou *et al.* 1997), and inhibits the incorporation of free fatty acids into triglycerides in skeletal muscles (Muoio *et al.* 1999). Such results suggest that leptin may improve the insulin sensitivity by reducing the amount of triglycerides in skeletal muscles.

In this study, the glucose transport of skeletal muscles was examined in order to elucidate the leptin effect on glucose transport in skeletal muscles. After induction of insulin resistance by high-fat diets, leptin was injected into the peritoneum for 3 days (*in vivo* effect), and leptin was also added to the incubation media of skeletal muscles *in vitro* in order to evaluate the direct effect of leptin (*in vitro* action). The amount of abdominal fat and the concentration of triglycerides in skeletal muscles were measured in order to examine the mechanism of insulin resistance of skeletal muscles and the effect of leptin on them.

Methods

Materials

2-[1,2-³H]-deoxy-D-glucose (2-DG) was obtained from American Radiolabeled Chemicals (St. Louis, MO, USA), and D-[1-¹⁴C]mannitol was from NEN Life Science Products (Boston, MA, USA). Insulin was purchased from Novo Nordisk and all other reagents were

obtained from Sigma Chemical (St. Louis, MO, USA).

Animals

Wistar male rats (~50 g) were used for studying the indirect effect of leptin in high-fat diet groups, and were given rat chow or high-fat diet for 4 weeks (Table 1). Male (~100 g) rats were used for studying the direct effect of leptin. The high-fat diet was prepared using lard, corn oil, sucrose, and casein (32, 18, 27, and 23 % of total calories, respectively), supplemented with vitamins (22 g/kg Teklad vitamin mix no. 40077), minerals (51 g/kg Teklad mineral mix no. 170915), and methionine (4.4 g/kg). The rat chow contained the following percentage of calories: 58.9 % carbohydrate, 12.4 % fat, and 28.7 % protein. The energy content of the high-fat diet was 5.1 kcal/g, whereas that of the rat chow was 3.3 kcal/g. The rats received the diets and water *ad libitum*.

Leptin administration

Rats fed the high-fat diet were divided into two groups matched for body weight after 24 days on the diet. The animals in one group were given daily subcutaneous injection of rat leptin (Research Diagnostics, NJ, USA; 1 mg/kg body weight) between 09:00 h and 10:00 h for 3 days (from the 25th to the 27th day) before the experiment). The control animals were given daily injections of the phosphate buffer.

Muscle and plasma preparation

Food was removed after 18:00 h the day before the experiment. Rats were anesthetized by an intraperitoneal injection of pentobarbital sodium (50 mg/kg body weight) and blood samples were withdrawn from the tail vein for measurement of glucose, insulin, triglycerides and leptin concentration, and then the epitrochlearis and soleus muscles were excised. Before incubation, the soleus muscle was longitudinally split into strips with an average weight of 20-25 mg.

Weights of fat pads

After the muscle dissection was completed, the abdominal cavity was opened, and the epididymal, mesenteric and retroperitoneal fat pads were excised and weighed.

Incubations of muscle (effects of insulin)

To allow recovery from the dissection and splitting procedures, muscles were incubated for 30 min

Table 1. Food intake, body weight and regional fat mass (g) in the experimental groups

	Chow	HF	HF-L
<i>Food intake (Cal/day)</i>	39.5±1.87	51.0±2.54	50.8±2.35
<i>Body weight</i>			
<i>feeding state</i>	254.5±4.71	253.3±3.50	253.5±1.74
<i>fasting state</i>	235.8±5.12	244.8±4.13	245.5±1.44*
<i>Total fat</i>	7.1±0.84	11.7±0.54**	9.8±0.44**,#
<i>Epididymal fat</i>	2.2±0.31	3.3±0.22**	2.8±0.18**,#
<i>Mesenteric fat</i>	3.1±0.24	4.1±0.23**	3.6±0.13**,#
<i>Retroperitoneal fat</i>	1.9±0.31	4.3±0.30**	3.4±0.24**,#
<i>n</i>	7	7	8

Values are mean ± S.E.M. *n* indicates the number of experimental cases. HF: high-fat diet group, HF-L: leptin-treated high-fat diet group. **p*<0.05, ***p*<0.01, vs chow. #*p*<0.05, vs HF.

Table 2. Plasma concentrations of glucose, insulin, leptin and triglyceride (TG) in experimental groups

	Chow	HF	HF-L
<i>Glucose (mg/dl)</i>	91.7±2.6	96.2±3.6	98.5±4.6
<i>TG (mg/dl)</i>	59.6±2.9	63.1±2.6	60.5±4.4
<i>Insulin (μU/ml)</i>	9.8±0.9	17.2±2.1**	16.9±3.1**
<i>Leptin (ng/ml)</i>	0.7±0.1	2.0±0.2**	1.6±0.3**
<i>n</i>	7	7	8

Values are mean ± S.E.M. *n* indicates the number of experimental cases. HF: high-fat diet group, HF-L: leptin-treated high-fat diet group. **p*<0.05, ***p*<0.01, vs chow.

at 35 °C in a shaking incubator in 2 ml of oxygenated Krebs-Henseleit buffer (KHB) supplemented with 8 mmol/l glucose, 32 mmol/l mannitol, and 0.1 % bovine serum albumin (BSA). After a 30-min recovery period, epitrochlearis muscles and soleus strips were incubated for 60 min at 35 °C in 2 ml of oxygenated KHB containing 8 mM glucose, 32 mM mannitol, and 0.1 % BSA in the presence or absence of a maximally effective concentration of insulin (2 mU/ml) before the measurement of 2-DG transport activity.

Measurement of 2-DG transport activity

Glucose transport activity was measured using 2-DG, according to Kim *et al.* (1999). After incubation with insulin, muscles were incubated for 20 min at 30 °C in 2 ml KHB containing 4 mM 2-[1,2-³H]-DG (1.5 μCi/ml), 36 mM [¹⁴C]mannitol (0.2 μCi/ml), 0.1 % BSA, and insulin if it was present in the previous incubation. Extracellular space and intracellular concentration of 2-DG (μmol/ml intracellular water/20 min)

were determined according to the method of Kim *et al.* (1999).

Analytical procedures

Plasma glucose concentrations were determined, using the glucose oxidase method by a YSI Glucose Analyzer. Plasma insulin and leptin were measured by radioimmunoassay kit obtained from Linco Research Instrument (St. Charles, MO). Serum triglyceride concentration was measured using a kit obtained from Sigma Chemical (St. Louis, MO, USA). Muscle triglyceride concentration was determined by extracting total lipids from clamp-frozen muscle samples with chloroform-methanol (2:1, vol/vol) as described by Folch *et al.* (1957), separating the chloroform and methanol-water phases, removing phospholipids and further processing the sample using Frayn and Maycock's (1980) modification of the method of Denton and Randle (1967). Triglycerides were then quantified spectrophotometrically as glycerol using a Sigma enzymatic assay

kit (Sigma Chemical, St. Louis, MO, USA).

Statistical analysis

Values are expressed as means \pm S.E.M. For multiple comparisons, the one-way analysis of variance (ANOVA) was used. When ANOVA showed significant differences, *post-hoc* analysis was performed with the Newman-Keuls multiple range test.

Results

Leptin has an appetite-suppressing effect that we wished to avoid. Since rats eat most of their food during the night, we gave the leptin injections in the morning in an attempt that the appetite suppressing effect would have worn off by the following night. This approach was reasonably successful. Body weights of the rats fed the high-fat diet were not reduced by leptin injection (Table 1), and the food intake for the 3 days of leptin injection was reduced by only 15 % (18.6 ± 1.1 g/day for the high-fat diet group vs. 15.8 ± 1.0 g/day for the high-fat diet-leptin group). However, there were no differences in calorie consumption between the high-fat diet and the high-fat diet-leptin groups for 4 weeks. In the high-fat diet group, epididymal, mesenteric, and retroperitoneal fat mass increased significantly, compared with fats in the chow-diet group ($p < 0.01$), although there was no significant difference in body weight. The increased fats in the abdominal cavity of the high-fat diet group were decreased significantly by leptin treatment ($p < 0.05$), but they were still significantly higher than those in the chow-diet group ($p < 0.01$) (Table 1). Plasma glucose and triglycerides levels did not significantly differ among the groups. Plasma insulin and leptin levels were significantly higher in both the high-fat diet group and the high-fat diet-leptin group ($p < 0.01$) compared with the chow-diet group. There was no difference in these levels between the high-fat diet group and the high-fat diet-leptin group (Table 2). Glucose transport ($\mu\text{mol}/20$ min) of the epitrochlearis muscle was not significantly different among the groups under the basal state. However, it was significantly lower in the high-fat diet group than in the chow-diet group after insulin stimulation (1.42 ± 0.109 vs 1.94 ± 0.163 , $p < 0.01$), and were significantly higher in the high-fat diet-leptin group than in the high-fat diet group (1.85 ± 0.144 vs 1.42 ± 0.109 , $p < 0.05$) (Fig. 1). Similarly, the glucose transport in the soleus muscle did not significantly differ among the groups under the basal state. It was significantly lower in the high-fat diet group than in the

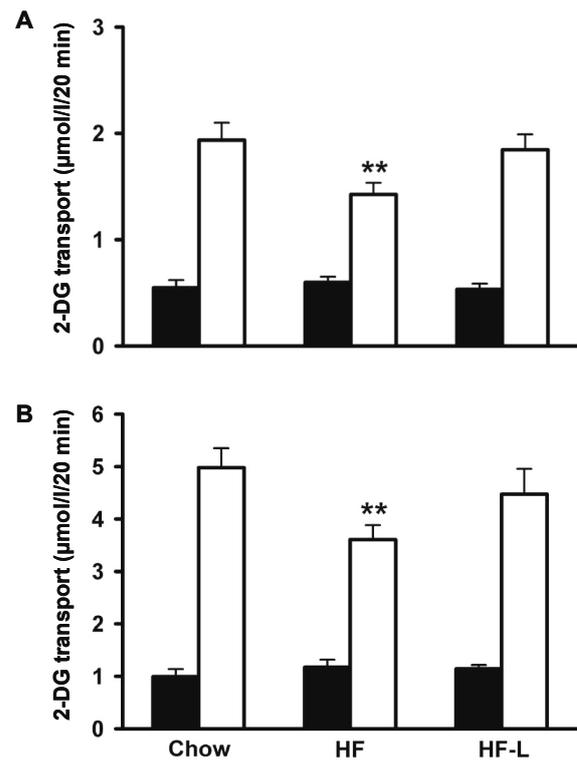


Fig. 1. 2-Deoxy-glucose (2-DG) uptake in epitrochlearis (A) and soleus (B) muscle in chow, high-fat (HF), and leptin-treated high-fat (HF-L) diet rats. Open bars indicate basal state and closed bars indicate insulin (2 mU/ml) stimulated state. ** $p < 0.01$ vs chow.

chow-diet group by insulin stimulation (3.61 ± 0.278 vs 4.98 ± 0.370 , $p < 0.01$), but tended to increase in the high-fat diet-leptin group as compared with the high-fat diet group (4.48 ± 0.484 vs 3.61 ± 0.278 , $p = 0.08$). The concentration of triglyceride in the soleus muscle was significantly higher ($p < 0.01$) in the high-fat diet group (11.5 ± 0.57 $\mu\text{mol}/\text{g}$) than in the chow-diet group (7.6 ± 0.49 $\mu\text{mol}/\text{g}$) (Fig. 2). In case of the high-fat diet-leptin group, we found about the same values as in the chow-diet group, since it was significantly lower (7.5 ± 0.55 , $p < 0.01$) than in the high-fat diet group. In order to examine the direct effect of leptin on the glucose transport of skeletal muscles, leptin was added to the incubation medium of muscle tissues, and the rate of glucose transport was measured. The result showed that leptin had no effect on the glucose transport of both epitrochlearis and soleus muscles under the basal state. There was also no stimulatory effect on the glucose transport stimulated by insulin (Fig. 3).

Discussion

Rats fed high-fat diets for four weeks had an increased amount of fat in the abdomen without any

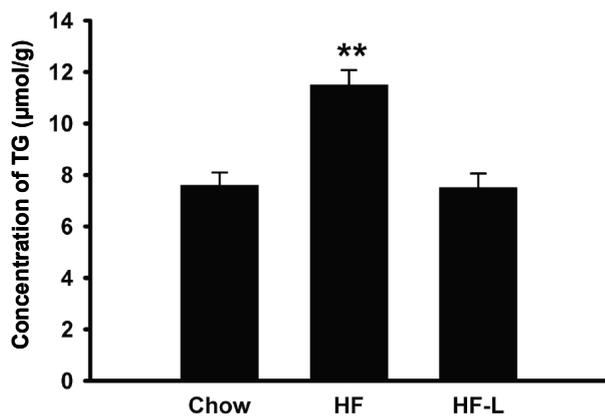


Fig. 2. Concentrations of triglyceride (TG) in soleus muscle in chow, high-fat (HF) and leptin-treated high-fat (HF-L) diet fed rats. ** $p < 0.01$, vs chow or HF-L.

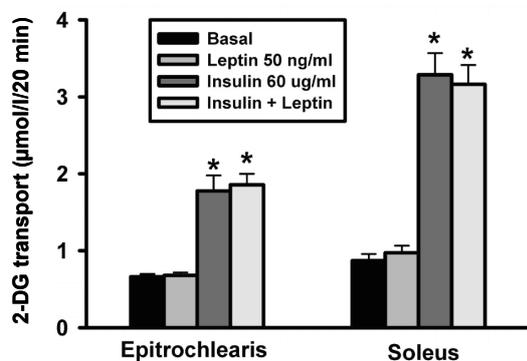


Fig. 3. 2-Deoxy-glucose (2-DG) uptake in epitrochlearis and soleus muscles after incubation with leptin *in vitro*. * $p < 0.01$ vs basal or leptin.

significant differences in body weight, compared with rats on chow diets, and had an increased plasma insulin and leptin levels. Intra-abdominal fat pads of high-fat diet group became significantly lower as a result of leptin treatment for 3 days, but these were still significantly higher, compared with those of chow-diet group. This result agrees with the report that leptin was considerably effective in reducing fats in the abdominal cavity (Barzilai *et al.* 1997).

The fasting plasma glucose of the high-fat diet group was similar to that of the chow-diet group. However, the high-fat diet group showed about 1.7 times higher plasma insulin levels than the chow-diet group. The plasma leptin level increased about 2.8 times in the high-fat diet group, compared with the chow-diet group, but there was no significant difference in the plasma glucose levels. This result apparently differs from the reports that leptin directly acts on pancreatic islets or improves the insulin sensitivity (Timothy *et al.* 1997).

The insulin responsiveness of skeletal muscles

was evaluated by the rate of glucose transport. In the high-fat diet group, glucose transport after insulin stimulation decreased significantly in both epitrochlearis muscles and soleus muscles compared with the chow-diet group. When leptin was administered to the high-fat diet group for three days, the glucose transport of epitrochlearis muscles increased significantly up to the level of the chow-diet group and also tended to increase in soleus muscles, thus verifying that insulin responsiveness of skeletal muscles was improved by leptin treatment. It means that leptin can recover insulin resistance of skeletal muscles *in vivo*. This is in agreement with other reports (Kamohara *et al.* 1997, Minokoshi *et al.* 1999).

In order to know the direct effect of leptin on the insulin responsiveness of skeletal muscles, leptin was added to the incubation medium of epitrochlearis and soleus muscles. Both muscles did not show any increase of glucose transport under the basal condition as well as after insulin stimulation. This result agrees with the reports that leptin have no direct effects on the glucose transport of skeletal muscles, although leptin receptors are expressed in skeletal muscles (Zierath *et al.* 1998). In other studies (Berti *et al.* 1997, Ceddia *et al.* 1998), leptin showed insulin-like effects to a certain degree, although leptin was not able to magnify the function of insulin.

A recent report emphasized the importance of intramuscular fat to insulin action (Greco *et al.* 2002). In this study we thus measured triglyceride concentrations of soleus muscles in order to examine whether the improved insulin resistance of skeletal muscles by leptin was related to decreased intramuscular triglycerides. The triglycerides of soleus muscles increased by 50 % in the high-fat diet group compared with the chow-diet group. After leptin treatment, however, the triglyceride accumulated in skeletal muscles following high-fat diets decreased to the level of the chow-diet group. These outcomes are most likely due to leptin's specific effect, since leptin decreases the influx of triglycerides into adipose tissues (Lopez-Soriano *et al.* 1998), and inhibits the accumulation of fatty acids as triglycerides in skeletal muscles (Muoio *et al.* 1999). It was also reported that leptin administration activates 5'-AMP-activated protein kinase (AMPK) in skeletal muscle, leading to the inhibition of acetyl coenzyme A carboxylase and subsequent stimulation of fatty acid oxidation (Minokoshi *et al.* 2002). In a recent two report, 14 days of hyperleptinemia made adipocytes shrunken, fatless and up-regulated peroxisome proliferator-activated receptor

gamma coactivator 1 α (Orci *et al.* 2004), and leptin administration in ob/ob mice resulted in improvement of insulin sensitivity concomitant with a decrease in resistin gene expression (Asensio *et al.* 2004). These might be the causes of reduction in intramuscular triglyceride by leptin treatment.

To sum up the above results, leptin is unlike to have direct effect on the glucose transport of skeletal

muscle, and leptin seems to induce depletion of the triglyceride content of tissues and therefore cause an improvement of insulin sensitivity.

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