

Exercise-Induced Muscle Damage Impairs Insulin Signaling Pathway Associated with IRS-1 Oxidative Modification

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Short title:

Muscle Damage and Glucose Metabolism

Summary

Strenuous exercise induces delayed-onset muscle damage including oxidative damage of cellular components. Oxidative stress to muscle cells impairs glucose uptake via disturbance of insulin signaling pathway. We investigated glucose uptake and insulin signaling in relation to oxidative protein modification in muscle after acute strenuous exercise. ICR mice were divided into sedentary and exercise groups. Mice in the exercise group performed downhill running exercise at 30 m/min for 30 min. At 24 hr after exercise, metabolic performance and insulin-signaling proteins in muscle tissues were examined. In whole body indirect calorimetry, carbohydrate utilization was decreased in the exercised mice along with reduction of the respiratory exchange ratio compared to the rested control mice. Insulin-stimulated uptake of 2-deoxy-[³H]glucose in damaged muscle was decreased after acute exercise. Tyrosine phosphorylation of insulin receptor substrate (IRS)-1 and phosphatidyl-3-kinase/Akt signaling were impaired by exercise, leading to inhibition of the membrane translocation of glucose transporter 4. We also found that acute exercise caused 4-hydroxy-nonenal modification of IRS-1 along with elevation of oxidative stress in muscle tissue. Impairment of insulin-induced glucose uptake into damaged muscle after strenuous exercise would be related to disturbance of insulin signal transduction by

oxidative modification of IRS-1.

Key words Muscle damage • Glucose uptake • Oxidative stress • 4-HNE • IRS-1

Introduction

Transport of glucose across the cell membrane is the rate-limiting step in its utilization by skeletal muscle. It is well-known that a single bout exercise improves glucose uptake into skeletal muscle via insulin dependent- and independent- signal transduction mechanisms (Catee *et al.* 1989, Goodyear *et al.* 1998, Hamada *et al.* 2006, Hayashi *et al.* 1997, Perseghin *et al.* 1996). This effect is observed for several hours after exercise and it often persists until the next day. Elevation of glucose uptake is caused by the translocation of glucose transporter 4 (GLUT4) to the plasma membrane after activation of the insulin receptor (IR) and phosphatidylinositol 3-kinase (PI3K)/Akt signaling (Bryant *et al.* 2002, Wang *et al.* 1999). AMP-activated protein kinase (AMPK) and intracellular calcium can also induce GLUT4 translocation independently of the insulin signaling pathway (Fisher *et al.* 2002, Wright *et al.* 2004, 2005).

Unaccustomed and strenuous exercise causes muscle damage that presents clinically as muscular pain and involves protein degradation and ultrastructural changes, it is called delayed-onset muscle damage. Infiltration of phagocytes into the damaged muscle is seen after strenuous exercise and an inflammatory response is involved in the occurrence of delayed-onset muscle damage (Aoi *et al.* 2004, Tidball 1995). Elevation

of the levels of oxidative damage in cellular components such as lipids, proteins, and DNAs is also found in damaged muscle (Aoi *et al.* 2003, 2004). Previous studies (Bloch-Damti and Bashan 2005, Paolisso *et al.* 1994, Singh *et al.* 2008) have indicated that oxidative stress can provoke the development of insulin resistance in skeletal muscle. Stimulation of oxidants such H₂O₂ to muscle cells blocks insulin-induced glucose uptake and GLUT4 translocation by impairment of IR activation and PI3K/Akt signaling (Maddux *et al.* 2001, Wei *et al.* 2008). Interestingly, oxidative products are elevated in the muscles of patients with type 2 diabetes (Singh *et al.* 2008, Russell *et al.* Scheede-Bergdahl *et al.* 2005) and diabetic mice (Bonnard C *et al.* 2008), supporting a role for oxidative stress in the development of glucose resistance. In contrast, several antioxidants improve insulin-sensitive glucose uptake by muscle cells along with a reduction of oxidative products (Singh *et al.* 2008, Maddux *et al.* 2001, Henriksen *et al.* 2006). It has been reported about glucose metabolism after muscle-damaging exercise (del Aguila LF *et al.* 2008), unlike after non-muscle-damaging exercise, although there is little information available. In the damaged muscle, elevation of oxidative stress in muscle tissue may diminish glucose transport via inhibition of insulin signal transduction.

Previously, we found that proteins modified by 4-hydroxy-nonenal (4-HNE), a

major lipid peroxidation end product (Aoi *et al.* 2003), accumulated in muscle damaged by acute exercise. Growing evidence has shown that oxidative stress functions as a modulator of signal transduction by modifying proteins posttranslationally as well as oxidation of cellular components (Cloos and Christgau 2004). Several lipid peroxide products including 4-HNE non-enzymatically modify amino acid residues of target protein by an irreversible manner, which can result in inhibition of the protein activity (Cloos and Christgau 2004, Selley 1998). In this case, some proteins in insulin signaling pathway may be a target of 4-HNE. Therefore, suppose insulin resistance develops in the damaged muscle, the oxidative modification of protein is likely to affect impairment of insulin signaling pathway. In the present study, we investigated the changes of glucose metabolism in delayed-onset muscle damage after exercise along with 4-HNE modification of insulin-signaling protein.

Material and Methods

Animals and experimental design

The present study complied with the principals and guidelines of the Japanese Council on Animal Care and it was also approved by the Committee for Animal Research of Kyoto Prefectural University of Medicine (permission No. M13-91). ICR

mice (10 weeks old) (Shimizu Laboratory Supplies Co., Ltd., Kyoto, Japan) were acclimatized for 2 weeks in an air-conditioned ($22 \pm 2^\circ\text{C}$) room with a 12-h light/dark cycle (lights on from 07:30 to 19:30 h). The mice were divided into two groups consisting of a sedentary group and a running exercise group. Mice in the exercise group performed downhill treadmill exercise at 5° of decline grade at 30 m/min for 30 min after gradually increasing the speed over the initial 10 min, which is the condition of muscle-damaging exercise as referred to in previous studies (Aoi *et al.* 2003, 2004, Armstrong *et al.* 1983). Eight animals of each group were used for measurement of respiratory metabolic performance. At 24 hours after exercise, indirect calorimetry analysis was performed for assessment of metabolic performance in both exercise and sedentary groups. Oxygen consumption and carbon dioxide production were measured every 3 minutes with a metabolic measurement system for small animals (MK-5000R, Muromachi Kikai, Tokyo, Japan) for 60 min. The respiratory exchange ratio (RER) and substrate utilization were calculated from the level of oxygen consumption (VO_2) and carbon dioxide production (VCO_2) as described previously (Aoi *et al.* 2008).

Additional animals were used for measurement of biochemical parameters. The mice were euthanized at 24 hours after exercise following fasting for 15 hours, and soleus muscle and blood were collected for the measurements. Muscle samples

obtained from 8 animals each were used for glycogen and protein assay, and samples from remaining 8 animals were used for glucose transport measurement.

Blood analysis

The blood glucose was determined with GluTest (Sanwa Kagaku Co., Ltd., Nagoya, Japan). Each blood sample obtained from mice was centrifuged at 3,500 rpm for 15 min at 4°C immediately after collection. Then enzyme linked sorbent assay (ELISA) for insulin was performed with a commercially available kit developed by Merckodia AB (Uppsala, Sweden) according to the manufacturer's instructions. The absorbance was measured with a microplate reader (MPR-A4I; Tosoh, Tokyo, Japan) and the concentration of insulin was calculated by comparison with a calibration curve. Total creatine kinase (CK) activity in plasma was measured by using a kit (CPK 45-5; Sigma, St. Louise, MO) according to the manufacturer's instructions.

Muscle glycogen

Muscle samples were weighed and boiled in 2 M HCl for 3 h, and then neutralized with 0.5 ml of 2 N NaOH. The supernatant was separated from the mixture and was assayed by a standard enzymatic technique with fluorometric detection (Aoi *et al.*

2008).

Glucose transport in muscle tissue

Soleus muscles were dissected out and incubated with 100 μ U/ml insulin (Sigma) for 1 hr at 37°C in oxygenated (95% O₂-5% CO₂) Krebs-Henseleit buffer (KHB) supplemented with 0.1% BSA, 2 mM sodium pyruvate, and 6 mM mannitol. Thereafter, the muscles were transferred to KHB containing 0.1% BSA and 100 μ U/ml insulin, and incubated at 37°C for 15 min. Then the muscles were incubated KHB containing 0.1% BSA, 1 mM 2-deoxy-[1,2-³H]glucose (2-DG, 222 MBq/mmol; PerkinElmer, Boston, MA), 9 mM [U-¹⁴C]mannitol (1.96 MBq/mmol; PerkinElmer), and insulin for 20 min. On the other hand, muscles obtained from the other limb were incubated in the same medium in the absence of insulin for measurement of insulin-independent manner. After the incubation, the muscles were removed, rapidly frozen, weighed, and dissolved in 0.5 ml of 0.5 N NaOH. The homogenate was centrifuged at 15,000g for 15 min, and 5 ml of scintillation cocktail were added to aliquots of supernatant, and intracellular accumulation of 2-DG were measured with a scintillation counter (Packard 2700TR; Perkin Elmer).

Preparation of protein sample

The muscles of each mouse were mechanically homogenated in liquid nitrogen after insulin stimulation as described above. The one portion of tissue homogenates were incubated in lysis buffer (CellLytic-MT Lysis/Extraction Reagent; Sigma) with rotation for 60 min at 4°C. Then the resulting homogenate was centrifuged at 12,000 rpm for 20 min at 4°C, and total protein concentration of the supernatant was determined (BCA protein assay; Pierce, Rockford, IL), after which aliquots were stored at -80°C. Samples were immunoprecipitated with an antibody specific for insulin receptor substrate-1 (IRS-1). In brief, the protein sample was rotated overnight at 4°C with 4 µg of a IRS-1 antibody (Upstate, Charlottesville, VA) and protein A-agarose beads (GE Healthcare Bio-Sciences, Buckinghamshire, UK). On the next day, the agarose beads were washed three times in lysis buffer and were then washed in a solution of 0.1 mM Na₃VO₄ in PBS. Antigens were eluted from the beads with 2x Laemmli SDS buffer and were boiled for 5 min before separation by SDS-polyacrylamide gel electrophoresis (PAGE).

The other portion of tissue homogenates were used for extraction of membrane protein. Extraction of membrane protein was performed with a membrane extraction kit (Mem-PER Eukaryotic Membrane Extraction Reagent; Pierce) according to the

manufacturer's instructions.

Protein measurement

The immunoprecipitates, membrane protein lysates, and total protein lysates were separated by SDS-PAGE and then proteins were transferred onto polyvinylidene difluoride membranes. The blots were incubated with primary antibodies for phospho-tyrosin, IRS-1 (Upstate), p85, phospho-Akt, Akt (Cell Signaling Technology, Beverly, MA), GLUT4 (Chemicon International Inc., Temecula, CA), and 4-HNE (Japan Institute for the Control of Ageing, Fukuroi City, Shizuoka, Japan) which were visualized using horseradish peroxidase (HRP)-conjugated secondary antibodies (GE Healthcare Bio-Sciences) and enhanced chemiluminescence (Western pico; Thermo Scientific, Rockford, IL). Band densities were measured with Scion image software (NIH, Research Service Branch).

Thiobarbituric-acid-reactive substances (TBARS) in the muscle homogenate was measured as reported previously (Aoi *et al.* 2004). The level of TBARS was expressed as nanomoles of malondialdehyde per milligram of protein using 1,1,3,3-tetramethoxypropane as standard.

Statistical analysis

All data are reported as the mean \pm SE. Differences between groups were evaluated by using student t-test. Significant levels between means were set at $P < 0.05$.

Results

Metabolic and damaging parameters in blood and muscle

Previously, we found a marked elevation of inflammation and oxidative damage in skeletal muscle at 24 hour after acute exercise (Aoi *et al.* 2003, 2004, 2007). Thus, Metabolic and damaging parameters were examined at 24 hour after exercise. Indirect calorimetry analysis was performed for assessment of whole body metabolic performance. Mean values obtained from 60 min measurement were compared between exercise and sedentary groups on each parameter. RER was significantly lower at rest in the exercise group more than the control group ($P < 0.05$, Table 1). Corresponding to the RER, carbohydrate utilization was significantly lower in the exercise group compared with the control group ($P < 0.05$, Table 1), while fat utilization was higher in the exercise group (data not shown).

Biochemical parameters in blood and muscle were measured after fasting period.

Blood glucose showed a tendency to an elevation in the exercise mice more than sedentary mice (Table 1). Plasma insulin was not different between control and exercise groups. Plasma CK was remarkably elevated by exercise compared with sedentary ($P < 0.01$, Table 1). Glycogen content of the muscle tended to be lower in exercised group compared with control group whereas the difference was not statistical significance.

Insulin-dependent and -independent glucose transport into muscle

Insulin-dependent and -independent glucose uptake in muscle was measured in the presence or absence of insulin. 2-DG uptake by the soleus muscle after insulin stimulation was significantly decreased in the exercise group compared with the sedentary group ($P < 0.05$, Fig. 1). On the other hand, there was no significant difference between control and exercise groups in the absence of insulin.

Insulin signaling proteins

Insulin signaling proteins in soleus muscle were measured after insulin stimulation. The GLUT4 content of the plasma membrane was significantly decreased after exercise compared with that of the sedentary group ($P < 0.05$, Table 2). Tyrosine

phosphorylation of IRS-1 was decreased in exercised muscle ($P < 0.05$, Table 2), while the total content of IRS-1 did not differ between the groups. Coimmunoprecipitation of p85 with IRS-1 showed a significant decrease after exercise ($P < 0.05$, Table 2). Also, phosphorylation of Akt was decreased in the exercise group compared with the rested control group ($P < 0.05$, Table 2), but total Akt was not changed between groups.

Oxidative stress parameters

To examine an elevation of oxidative stress in muscle tissue, the level of TBARS, a major marker of lipid peroxidation, was measured. TBARS was increased in exercised muscle compared with rested muscle ($P < 0.05$, Table 1). We next measured 4-HNE modification of IRS-1, which lies in upstream in insulin signaling pathway was measured. Consequently, 4-HNE-modified IRS-1 was elevated in the muscle tissue of mice from the exercise group compared with mice from the sedentary group ($P < 0.05$, Fig. 2).

Discussion

The present study revealed the following main findings: 1) insulin-stimulated glucose uptake was decreased in skeletal muscle after strenuous exercise, 2) insulin

signal transduction was reduced along with elevation of oxidative stress in the damaged muscle, and 3) 4-HNE modification of IRS-1 was elevated in the muscle from exercised mice. There is little information regarding glucose metabolism after muscle-damaging exercise, unlike after non-muscle-damaging exercise. These observations provide that insulin-sensitive glucose transport into muscle can be diminished in eccentric exercise-induced damaging muscle after exercise due to oxidative modification of insulin signaling protein.

It is well known that a single bout of exercise elevates glucose uptake for some time afterwards (Kubo and Foley 1986, Catee *et al.* 1989, Goodyear *et al.* 1998; Hayashi *et al.* 1997). However, the present study showed that glucose uptake in response to insulin stimulation was decreased after acute eccentric exercise along with a reduction of the membrane GLUT4 content in skeletal muscle. Additionally, indirect calorimetry analysis demonstrated that carbohydrate utilization as an energy substrate at rest was decreased along with a reduction of the RER in the exercise group, which is consistent with a previous study (del Aguila *et al.* 2000). This decrease of carbohydrate utilization was presumably due to reduction of glucose uptake via GLUT4 because glucose transport into myocytes is the rate-limiting step in glucose metabolism. Therefore, the present study demonstrates that strenuous eccentric exercise causes

transient decrease of insulin sensitivity in the presence of delayed-onset muscle damage. In contrast, fat utilization as an energy substrate was increased by exercise, which would occur in compensation for decrease of glucose metabolism due to insulin resistance. It has been reported that reduction in blood lipids such triacylglycerols 1 to 4 day after exercise is accelerated by muscle-damaging exercise more than non-muscle damaging exercise (Nikolaidis *et al.* 2008, Shahbazzpour *et al.* 2004), also suggesting the compensatory lipid utilization in damaging muscle. On the other hand, muscle glycogen content, one of factors affecting glucose transport, was not changed between groups in the present study, thus the effect of muscle glycogen on the reduction of glucose uptake would be difficult to consider.

Exercise reduced insulin-dependent glucose transport into muscle but not insulin-independent glucose uptake, indicating insulin signal transduction in the muscle was impaired. GLUT4 translocation to the cell membrane after stimulation by insulin is induced in a PI3K/Akt-dependent manner along with the role of IR and IRS (Bryant *et al.* 2002, Wang *et al.* 1999). Tyrosine phosphorylation activates IRS-1, and the level phosphorylated IRS-1 was lower after insulin stimulation in the exercise group. Additionally, the interaction of the p85 regulatory subunit of PI3K with IRS-1 was decreased after exercise along with a reduction in the phosphorylation of Akt. These

results showed that blocking of the IRS-1/PI3K/Akt signaling pathway would limit GLUT4 translocation and glucose uptake in the damaged muscle.

Oxidative stress has been reported to impair insulin sensitivity in skeletal muscle (Paolisso *et al.* 1994, Singh *et al.* 2008). Our results showed that elevation of TBARS was observed in the muscles of mice which had performed exercise, showing an elevation of oxidative stress, consisting with previous studies (Aoi *et al.* 2004). Previously, we reported that proteins modified by 4-HNE accumulated in the damaged muscle (Aoi *et al.* 2003). 4-HNE is a lipid peroxidation product that covalently modifies proteins on cystine, histidine, and lysine residues (Esterbauer *et al.* 1991, Uchida and Stadman 1992) and the modified proteins accumulated in several diseases (Selley 1998, Manabe *et al.* 2008, Reed *et al.* 2008). In this study, we found an increase of 4-HNE-modified IRS-1 in the damaged muscle. IRS-1 is an upstream regulator of insulin signaling transduction and can be a target of 4-HNE. Demozay *et al.* (2008) showed that 4-HNE easily binds to IRS and decreases the tyrosine phosphorylation in adipocytes. Thus, change of the protein structure by 4-HNE modification may lead to the decrease of tyrosine phosphorylation and interaction with PI3K.

There is argument about the role of muscle damage in adaptation to exercise

training. It has been believed that muscle damage must precede restoration after exercise that leads to an increase in strength and muscle hypertrophy (Higbie *et al.* 1996). In fact, resistance training using an eccentric load causes more severe muscle damage compared with concentric contraction, and can often induce an early increase of muscle mass. On the other hand, it has been reported that there is no correlation between inflammation and muscle strength adaptation (Behm *et al.* 2001). It has been shown that recurrent inflammation caused by exercise leads to muscle fibrosis with an increase of collagen deposition (Machey *et al.* 2004, Shu *et al.* 2007). Such muscle cannot exert strength corresponding to its mass. The present study showed that muscle damage caused by strenuous exercise also induces transient insulin resistance. These observations suggest that there are negative effects of muscle damage induced by exercise, so it may be useful to prevent muscle damage during physical activity for health promotion or exercise for persons especially with diseases such as diabetes. In addition, the transient reduction of glucose metabolism may be disadvantage for conditioning before game in athletes. However, further studies are needed to elucidate the effect of muscle damage-induced metabolic disturbance on physiological and pathological significance.

In conclusion, we found that insulin-stimulated glucose uptake into muscle tissue

was decreased by acute strenuous exercise along with blocking of GLUT4 translocation via IRS-1/PI3K/Akt signaling. Exercise also induced 4-HNE modification of IRS-1 along with an elevation of oxidative damage in muscle tissue. These observations suggest that impairment of insulin-induced glucose uptake into damaged muscle after strenuous exercise is related to disturbance of insulin signal transduction by IRS-1 modification.

Conflict of Interest

No conflicts of interest are declared by the authors.

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Abbreviations

GLUT4—glucose transporter 4; IR—insulin receptor; PI3K — phosphatidylinositol 3-kinase; AMPK — AMP-activated protein kinase; 4-HNE — 4-hydroxy-nonenal; RER — respiratory exchange ratio; VO₂ — oxygen consumption; VCO₂ — carbon dioxide production; ELISA — enzyme linked sorbent assay; KHB — Krebs-Henseleit buffer; IRS-1 — insulin receptor substrate-1; TBARS — thiobarbituric-acid-reactive substances.

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Figure legends

Fig. 1. Insulin-dependent and -independent glucose uptake in soleus muscle at 24 hr after acute exercise. After incubation in the buffer with the absence or presence of insulin (100 μ U/ml), 2-deoxy-[3 H]glucose uptake by isolated muscle tissue was measured. Values are the mean \pm SE obtained from 8 mice. *, significant difference from the control in the presence of insulin at the level of $P < 0.05$.

Fig. 2. 4-HNE modification of IRS-1 in soleus muscle at 24 hr after acute exercise. Lysate protein from the muscle was immunoprecipitated with IRS-1 antibody. Immunoprecipitates were separated by SDS-PAGE and membranes probed for 4-HNE. Values are the mean \pm SE obtained from 6-7 mice. *, significant difference at the level of $P < 0.05$.

Table 1 Metabolic and damaging parameters in blood and muscle

	Control	Exercise
Blood glucose (mg/dl)	58.8 ± 2.5	66.1 ± 2.7
Plasma insulin (µg/L)	0.42 ± 0.04	0.47 ± 0.06
Plasma CK (U/L)	938 ± 53	3812 ± 285 ^a
Muscle glycogen (mg/g)	1.12 ± 0.21	1.00 ± 0.19
RER	0.895 ± 0.01	0.869 ± 0.01 ^a
Carbohydrate utilization (mg/kg/min)	43.1 ± 2.5	36.1 ± 1.7 ^a
Muscle TBARS (nmol/mg prot.)	0.52 ± 0.03	0.88 ± 0.06 ^a

Values are mean ± SE for 8 mice. ^a Statistically significant differences from the control, $P < 0.05$.

Table 2 Insulin signaling pathway in muscle after acute exercise

	Control	Exercise
GLUT 4 (%)	100 ± 17	62 ± 9 ^a
Total IRS-1 (%)	100 ± 10	108 ± 8
Phospho-Tyr-IRS-1 (%)	100 ± 9	73 ± 6 ^a
IRS-1-associated p85 (%)	100 ± 11	61 ± 13 ^a
Total Akt (%)	100 ± 12	111 ± 8
Phospho-Akt (%)	100 ± 18	71 ± 6 ^a

After insulin stimulation, membrane protein was separated from the soleus muscle homogenate and then GLUT4 in plasma membrane was quantified by immunoblotting. IRS-1, IRS-1-associated p85, and Akt in the muscle tissues were also measured. Values are the mean ± SE obtained from 6-7 mice. ^a, significant difference at the level of $P < 0.05$.

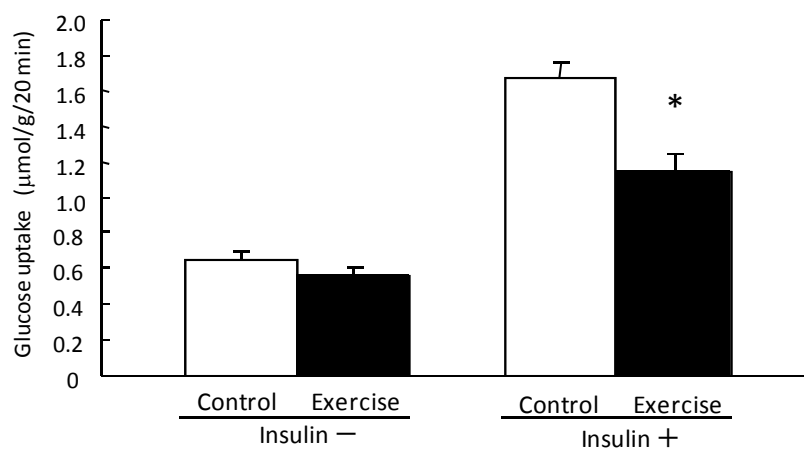


Fig. 1

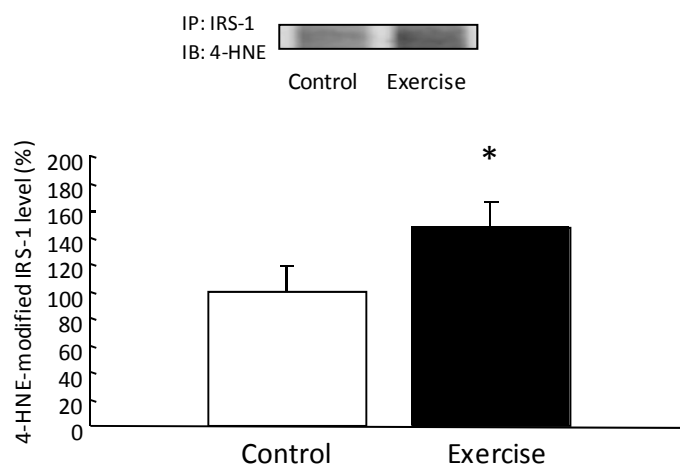


Fig. 2