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A DIGE proteomic analysis for high-intensity exercise-trained rat skeletal muscle

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Exercise training induces various adaptations in skeletal muscles. However, the mechanisms remain unclear. In this study, we conducted 2D-DIGE proteomic analysis, which has not yet been used for elucidating adaptations of skeletal muscle after high-intensity exercise training (HIT). For 5 days, rats performed HIT, which consisted of 14 20-s swimming exercise bouts carrying a weight (14% of the body weight), and 10-s pause between bouts. The 2D-DIGE analysis was conducted on epitrochlearis muscles excised 18 h after the final training exercise. Proteomic profiling revealed that out of 800 detected and matched spots, 13 proteins exhibited changed expression by HIT compared with sedentary rats. All proteins were identified by MALDI-TOF/MS. Furthermore, using western immunoblot analyses, significantly changed expressions of NDUFS1 and parvalbumin (PV) were validated in relation to HIT. In conclusion, the proteomic 2D-DIGE analysis following HIT-identified expressions of NDUFS1 and PV, previously unknown to have functions related to exercise-training adaptations.

Keywords: 2D-DIGE/exercise training/NDUFS1/parvalbumin.

Abbreviations: 2DE, two-dimensional gel electrophoresis; 2D-DIGE, two-dimensional differential gel electrophoresis; HIT, high-intensity exercise training; MS, mass spectrometry; PV, parvalbumin.

It is well known that physical training alters protein expressions in skeletal muscle recruited during training (*1–3*), while inactivity induces inverse effects (*4*). For example, exercise training elevates contractile, mitochondrial and transporter proteins (*5*, *6*). However, the molecular mechanisms of these changes remain unknown. 5′-AMP-activated protein kinase (AMPK) activation and elevation of calcium in skeletal muscle during muscle contraction have been postulated as two mechanisms that induce GLUT-4 expression after exercise training (*7*, *8*). Since both signals are dependent on exercise intensity (*9*, *10*), the glucose transporter 4 (GLUT-4) content after high-intensity intermittent exercise training of short duration (net exercise time: 280 s) is comparable with that induced by low-intensity prolonged (360 min) exercise training, which is regarded as the maximal stimulus related to exercise training (*11*). Therefore, high-intensity intermittent exercise has received attention from both scientific and practical points of view. From a practical point of view, the shorter duration high-intensity exercise training is easier to fit into a modern person's busy schedule than conventional moderate-intensity aerobic training. High intensity exercise training can also be a tool to elucidating signal intensity-related mechanisms evoking changes of protein expression in recruited skeletal muscle during exercise training. Therefore, it is of interest to clarify differences in protein expression between low- to moderate intensity and high intensity exercise training.

Proteomics is frequently used to detect pathological changes in protein expression in specific organs; we used it here for the purpose of finding changes in protein expression after exercise training. This technique of high-resolution two-dimensional gel electrophoresis (2 DE) followed by protein identification using mass spectrometry (MS) and database searching offers the possibility of detecting changed expression of a large number of proteins, and has been used previously to investigate post-translational modifications after exposure to an altered environment including exercise training (*12*). Previously, effects of low-intensity endurance training (*13*) and a session of relatively high-intensity exercise (*14*) on skeletal muscle by proteomic analysis were reported. However, these previous studies used CBB or colloidal Coomassie blue as conventional protein dyes (*13*). Therefore, only proteins of disproportionately high expression after the training were detected. Furthermore, the lack of reproducibility between gels in the traditional 2DE system leads to significant system variability, making it difficult to distinguish between system variation and induced biological change in protein abundance (*15*).

The two-dimensional differential gel electrophoresis (2D-DIGE) system has come to be known as a reliable technique. Indeed, this system has been demonstrated to be a powerful tool for the assessment of skeletal muscle plasticity, allowing an accurate quantification of protein changes, excluding non-biological variations and increasing the dynamic range and sensitivity of traditional 2D-PAGE (*15*). However, until now, this technique has not been used for research on exercise training-induced changes in protein expression.

Furthermore, this technique has not been adopted for investigations on changes induced in skeletal muscle proteins by high intensity exercise training, which has been of increasing interest because of its effectiveness at relatively short durations. For example, we have revealed changes in several metabolic protein expressions, such as GLUT4, the peroxisome proliferator-activated receptor γ coactivator-1 α(PGC-1α) and citrate synthase, by similar high intensity exercise training (*11*, *16*).

Therefore, the purpose of this study was, using the 2D-DIGE system, to analyse globally the expression of skeletal muscle proteins induced by high intensity intermittent training.

Materials and Methods

Materials

CyDye DIGE Fluor minimal dyes Cy2, Cy3 and Cy5, electrophoresis grade chemicals, IPG strips of pH 3-10 and IPG buffer of pH 3-10 for IEF and iodoacetamide were purchased from GE Healthcare (Little Chalfont, Bucks, UK). SYPRO Ruby was obtained from Molecular Probes (Eugene, OR, USA). For peptide generation, sequencing grade-modified trypsin was purchased from Promega (Madison, WI, USA) and ZipTip μ-C18 pipette tips from Millipore (Billerica, MA, USA).

Primary antibodies to NDUFS1, F_1-ATPase and glycogen phosphorylase were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and parvalbumin (PV) antibody from Abcam (Cambridge, MA, USA).

All other chemicals were purchased from Sigma Chemical (St Louis, MO, USA), Kanto Chemical (Tokyo, Japan) and Wako Pure Chemical (Tokyo, Japan).

Animal care

Four-week-old male Sprague-Dawley rats with body weights 70–85 g were used in the present experiment. All rats used for the present investigation were purchased from CREA Japan (Tokyo).

The animals were housed in rooms lighted from 7 a.m. to 7 p.m. and maintained on an *ad libitum* diet of standard chow and water. Room temperature was maintained at 20–22°C. Prior to the swimming exercise training experiment, all rats were acclimated to swimming exercise for 10 min/day for 2 days. All animal experiments were conducted with the approval of the National Institute of Health and Nutrition Ethics Committee on Animal Research.

Training protocol

Rats were randomly assigned to an extremely high-intensity intermittent training group (HIT; $n=4$) or an age-matched sedentary group (CON; $n=4$). During the 5-day HIT period, the rats daily performed 14 20-s swimming bouts while bearing a weight equivalent to 14 and 15% of their body weight for the first 3 and the last 2 days, respectively (*17*). A 10-s pause was allowed between bouts. Each rat performed the swimming exercise alone in a barrel filled to a depth of 25 cm. This training protocol has been shown to induce substantial increases in some proteins, such as citrate synthase and GLUT-4 (*17*), in recruited skeletal muscles during the exercise.

On the last training day, all exercises were finished before 6:00 p.m. Between 10:00 a.m. and 12:00 p.m. the next day (~18 h after the last exercise), the rats were anaesthetized with an intraperitoneal injection of pentobarbital sodium (5 mg/100 g body weight), and the epitrochlearis muscle was excised. The glycogen in this muscle is markedly depleted after high-intensity exercise; hence, it was selected for investigation as a muscle assumed to be vigorously recruited during the swimming exercise adopted for the training in the present investigation (*18*). The epitrochlearis muscle was clamp-frozen in liquid nitrogen and stored at −80°C until analysis.

Sample preparation

Epitrochlearis muscles were homogenized in 29 ice-cold volumes of 50 mM Tris–HCl pH 7.4, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM NaF, 1 mM sodium orthovanadate (Na_3VO_4) and 2 μl/ml protease inhibitor cocktail. The homogenates were frozen and thawed three times and centrifuged for 15 min at 700*g* at 4°C.

Protein extracts were then prepared following general guidelines recommended for posterior DIGE labelling. Briefly, proteins were precipitated using the 2D clean-up kit (GE Healthcare), resuspended in a buffer containing 7 M urea, 2 M thiourea, 4% w/v CHAPS, 30 mM Tris, pH 8.5 and finally, the protein content was quantified using a 2D Quant kit (GE Healthcare).

DIGE labelling

For DIGE analysis, four HIT and four CON samples were used ($n=4$). Samples were minimally labelled with Cy3 or Cy5 fluorescent dyes (50 μg protein/400 pmol dye) for 30 min at 4°C following the manufacturer's instructions (GE Healthcare). To minimize the system and inherent biological variation, half of the samples from each group were labelled with Cy3 and other half labelled with Cy5. An internal standard was prepared mixing equal amounts of all samples analysed and was labelled with Cy2 fluorescent dye.

2D-DIGE and image analysis

IPG strips (pH 3–10, 24 cm) were loaded with 50 μg each of Cy2-, Cy3- and Cy5-labelled samples in a buffer containing urea (7 M), thiourea (2 M), CHAPS (4 % w/v), DeStreak Reagent (2.4 % v/v, GE Healthcare) and IPG buffer (1% v/v). Isoelectric focusing was carried out in an IPGphor III Isoelectric Focusing Unit (GE Healthcare) for a total of 40–45 kVh at 0.05 mA/Strip (at 20°C).

After focusing, IPG strips were equilibrated in an SDS reducing buffer (6 M urea, 30% v/v glycerol, 2% w/v SDS, 50 mM Tris–HCl pH 8.8, 1% DTT) for 15 min, and then alkylated for 15 min in the same buffer containing 130 mM iodoacetamide instead of DTT. Second-dimension SDS–PAGE was run by overlaying the strips on 12.5% isocratic Laemmli gels (24 × 20 cm), cast in low-fluorescence glass plates, on an Ettan DALT*six* Large Electrophoresis System (GE Healthcare). Gels were run at 30°C at a constant power of 1 W/gel for 60 min followed by 2 W/gel until the bromophenol blue tracking front had run off. Fluorescence images of the gels were acquired on a Typhoon 9400 scanner (GE Healthcare). Cy2, Cy3 and Cy5 images for each gel were scanned at 488/520 nm, 532/580 nm and 630/670 nm excitation/emission wavelengths, respectively, at a 100-μm resolution.

Image analysis was performed using DeCyder V. 5.0 software (GE Healthcare) following published and manufacturers' recommendations (*15*, *19*). A differential in gel analysis (DIA) module was used for intra-gel co-detection of samples and internal standard protein spots. Artefactual spots (dust and others) were filtered (area <100, volume <70,000) and removed. The biological variation analysis (BVA) module was used for inter-gel matching of the internal standard and samples across all gels, and for performing comparative cross-gel statistical analysis of all spots, based on spot volumes (*19*), permitting the detection of differentially expressed spots between experimental conditions (Student's *t*-test, $P<0.05$). 2D-spots that exhibited a 1.3-fold or more decrease or increase were manually checked to avoid false positives, and then identified by PMF analysis.

In-gel protein digestion

The same gels used for DIGE analysis were used as preparative gels and were SYPRO Ruby stained.

In-gel digestion was carried out according to the method of Shevchenko *et al*. (*20*) with some modifications. Protein spots were excised from gels on a transilluminator, washed by 100 mM NH_4HCO_3 twice (10 min each time) and shrunk by dehydration in ACN and then dried *in vacuo*.

The gels were rehydrated with 20 μl of trypsin solution (12.5 ng/μl sequencing grade modified trypsin, 50 mM NH_4HCO_3, 5 mM $CaCl_2$) in an ice-cold bath for 45 min. The supernatant was removed and 20 μl of 50 mM NH_4HCO_3 and 5 mM $CaCl_2$ were added to keep gels wet overnight at 37°C. Peptides were extracted by one change of 20 μl of 20 mM NH_4HCO_3, and three changes of 20 μl of 5% formic acid in 50% ACN (20 min for each) at room temperature and dried to a few microlitres *in vacuo*.

MS and protein identification

ZipTip μ-C18 pipette tips were utilized to condense the peptides and to remove inorganic compounds. After 10 repetitions of passage of the sample through the μ-C18 resin, the peptides were eluted with

1–3 μl of 50% ACN, 1% TFA solution on the plate for matrix-assisted laser desorption/ionization-time of flight MS (MALDI-TOF/MS) analysis (Voyager DE-STR, Applied Biosystems). One microlitres of the matrix solution (10 mg/ml α-CHCA dissolved in 50% ACN/0.1% TFA) was mixed with the peptides on the plate, which was left until crystallization occurred. ACTH and bradykinin were used as internal standards. Peptide mass measurements were taken by using the reflector mode in a mass range from 700 to 3600 Da.

Protein identification was performed using a peptide mass database facilitated by the MASCOT search program from Matrix Science (http://www.matrixscience.com).

Validation

Validation of selected proteins identified by the MS analysis was carried out by western blot (immunoblot) analysis. Using additional rats (4-week-old male Sprague-Dawley rats), the protein content in EPI muscle was compared between controls ($n=6$) and rats ($n=6$) trained by the identical procedure for the DIGE analyses. Aliquots of epitrochlearis muscle homogenates were solubilized in the Laemmli sample buffer, subjected to SDS–PAGE and electrophoretically transferred to a polyvinylidene fluoride membrane. The membrane was incubated with each primary antibody in 5% skimmed milk overnight at 4°C. After overnight incubation, the membrane was incubated for 1 h at room temperature with anti-rabbit (for PV, F_1-ATPase; ATPsynβ) or anti-goat (for NDUFS1, glycogen phosphorylase) IgG conjugated to horseradish peroxidase (HRP; Jacson ImmunoResearch, West Grove, PA, USA). Immunoreactive bands were detected by ECL plus (GE Healthcare).

Values are expressed as means ± SD. Differences between two groups were analysed using the unpaired Student's *t*-test. Statistical significance was defined as $P<0.05$.

Results

Gel images of muscle protein extracts are shown in Fig. 1. They illustrate the representative protein spot pattern in a 2D gel of Cy2-labelled pooled standards.

In the fluorescent DIGE analysis of exercise-trained rat epitrochlearis muscles, 800 protein spots were detected and matched in each experiment. In addition, gel-to-gel variations in the 2D protein distribution patterns were greatly reduced due to the use of two different proteomes and a pooled standard on the same gel (*15*).

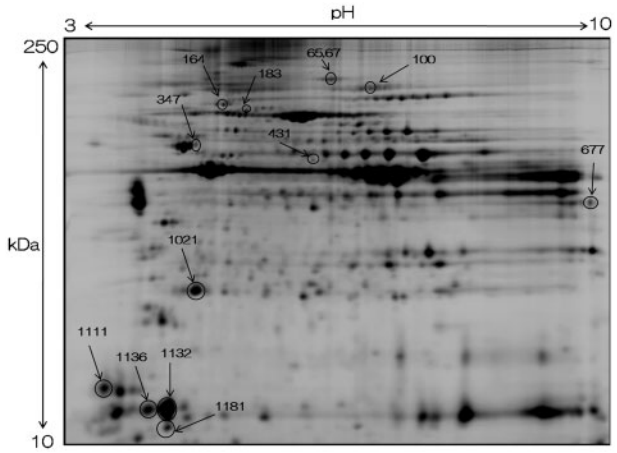

Fig. 1 Epitrochlearis muscle protein profiling by 2D-DIGE. A typical 2D-pattern (Cy2-labelled) gel image of a 50-μg protein extract separated in a pH 3–10 IPG strip in the first dimension and 12.5% polyacrylamide gel in the second. Automated image analysis by Decyder detected and matched 800 protein spots in single-gel images. Differential analysis of the epitrochlearis muscle extracts after HIT and control (CON) extracts revealed 13 differentially expressed ($P<0.05$) spots. Identified protein spots were labelled with numbers as they appear in the MS list (see Table I).

DIGE analysis found 13 differentially expressed ($P<0.05$) spots, and all of them were identified by MALDI-TOF/MS. Table I lists the DIGE-identified proteins that exhibited 1.3 or more change of expression in the rat epitrochlearis muscle following HIT. In order to correlate protein spots with an altered density to the list of MS-identified protein species (Table I), relevant 2D spots are shown in the DIGE Cy2 master gel of the HIT and control group's skeletal muscle proteome (Fig. 1). The Cy2-labelled gel with the mixed standard marked and numbered all of the 13 HIT-altered muscle protein spots. Among the differentially expressed proteins, seven proteins were up-regulated after HIT while six proteins were down-regulated.

At least seven of the HIT-altered proteins were related to metabolism. HIT up-regulated three mitochondrial electron transport chain enzymes: ATP synthase β-subunit (ATPsynβ; spot 347), NADH dehydrogenase (ubiquinone) Fe-S protein 1, 75 kDa (NDUFS1; spot 164) and NADH dehydrogenase (ubiquinone) Fe-S protein 2 (NDUFS2; spot 431). Two mitochondrial oxidative enzymes were also increased: oxoglutarate dehydrogenase (OGDH, spots 65 and 67) and mitochondrial malate dehydrogenase (m-MDH, spot 677). In contrast, muscle glycogen phosphorylase (GPh; spot 100), relevant to glycogenolysis, was decreased in HIT muscle.

Stress protein grp75 (spot 183) was increased by HIT.

Three contractile proteins found in fast-twitch muscle were down-regulated by HIT [myosin light chain, phosphorylatable, fast skeletal muscle (spots 1111 and 1136) and myosin light chain MLC1-f (spot 1021)].

PV and a related protein (spots 1132 and 1181) were decreased after HIT.

To confirm the results of the DIGE analysis, changes in some protein expressions after HIT were investigated by western analysis (Fig. 2). For PV and NDUFS1, the immunoblot analysis fully agreed with the results of DIGE analysis. However, western blot produced results differing from those of DIGE for F_1-ATPase (ATPsynβ) and GPh.

Discussion

Using proteomic 2D-DIGE analysis, the present investigation revealed, for the first time, changes in the expressions of several skeletal muscle proteins after high-intensity exercise.

The fluorescent DIGE analysis of exercise-trained rat skeletal muscle resulted in the detection and match of 800 protein spots. In contrast to staining with conventional protein dyes such as CBB or silver, fluorescent tagging of the entire muscle protein complement drastically increases the number of detectable protein spots in standard 2DE (*21*). A previous proteomic study of exercise-trained muscle in rats used colloidal coomassie as a total protein dye and resulted in

Table I. List of DIGE-identified proteins with a changed expression level following HIT in rat epitrochlearis muscle.

Protein function (classification)	Master no. (HIT)	gi no.	Protein name	Molecular mass (kDa)	Isoelectric point (pI)	Sequence coverage (%)	Average ratio V_E/V_C fold* change)	*t*-test *P*-value
Metabolic proteins	347	1374715	ATP synthase β-subunit	51.2	4.9	23	1.53	2.30E-02
	164	53850628	NADH dehydrogenase (ubiquinone) Fe-S protein 1, 75 kDa	80.3	5.6	29	1.53	2.30E-02
	431	58865384	NADH dehydrogenase (ubiquinone) Fe-S protein 2	52.9	6.5	38	1.45	4.30E-02
	65	62945278	Oxoglutarate (α-ketoglutarate) dehydrogenase	117	6.3	22	1.46	1.60E-02
	67	62945278	Oxoglutarate (α-ketoglutarate) dehydrogenase	116	6.3	12	1.42	8.00E-03
	677	42476181	Malate dehydrogenase, mitochondrial	36.1	8.9	46	1.33	3.60E-02
	100	158138498	Muscle glycogen phosphorylase	97.7	6.7	28	−1.35	3.50E-02
Stress protein	183	1000439	grp75	74	5.9	20	1.62	3.50E-02
Contractile proteins	1111	6981238	Myosin light chain, phosphorylatable, fast skeletal muscle	19.1	4.8	37	−1.30	4.60E-02
	1136	6981238	Myosin light chain, phosphorylatable, fast skeletal muscle	19.0	4.8	29	−1.34	3.10E-02
	1021	205485	myosin light chain MLC1-f	20.8	5.0	42	−1.31	5.80E-03
Other	1132	11968064	PV	11.9	5.0	79	−1.45	1.80E-02
	1181	494573	Chain 1, refined X-ray structure of rat PV	11.8	5.0	53	−1.48	1.70E-02

[a] V_E/V_C indicates the value ratio derived from the normalized spot volume standardized against the intra-gel standard provided by DeCyder software analysis.

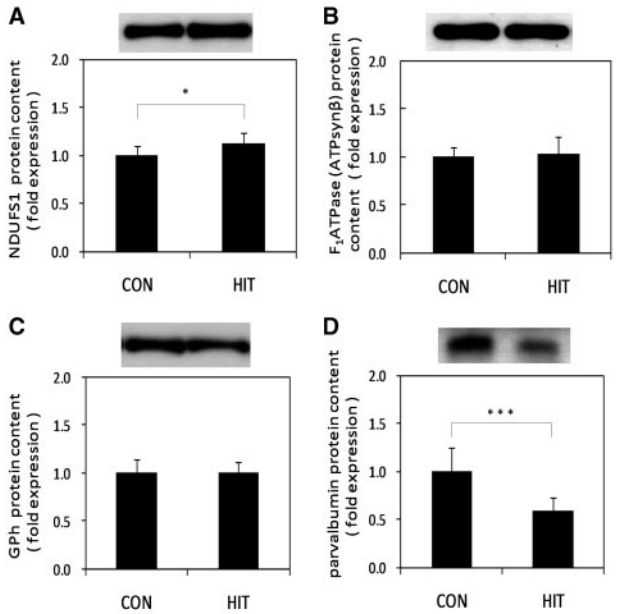

Fig. 2 Western blot analysis of selected muscle proteins following HIT. Identical immunoblots labelled with antibodies to NDUFS1 (A), F_1-ATPase (ATPsynβ) (B), GPh (C) and PV (D). '*' and '***' indicate significant differences from results in the control group (CON) at levels of $P<0.05$ and $P<0.001$. Values are means ± SD for six muscles.

the detection of only 187 individual 2D spots matched across gels (*13*). Hence, in terms of protein-profiling ability for exercise-trained muscles, the DIGE-based proteomic method adopted in the present investigation is ~4.3-fold more sensitive than conventional protein-labelling techniques.

As shown in Table I, we identified five mitochondrial enzyme proteins with altered expression after HIT. These proteins included the ATP synthase β-subunit (ATPsynβ), NADH dehydrogenase (ubiquinone) Fe-S protein 1, 75 kDa (NDUFS1), NADH dehydrogenase (ubiquinone) Fe-S protein 2 (NDUFS2), oxoglutarate dehydrogenase (OGDH) and mitochondrial malate dehydrogenase (m-MDH) (~1.3- to 1.5-fold change of expression). Using immunoblot analysis, we confirmed the results for NDUFS1 (Fig. 2A). However, for ATPsynβ and GPh, different results were obtained (Fig. 2B and C).

NDUFS1 and NDUFS2 are subunits that compose mitochondrial electron transport enzyme complex, which catalyses NADH oxidation with concomitant ubiquinone reduction and proton ejection out of mitochondria. To our knowledge, this is the first report that exercise training induces NDUFS protein expression. In addition, we confirmed that NDUFS1 was also up-regulated by low-intensity exercise training (data not shown). Since a high-fat diet was shown to down-regulate NDUFS1 mRNA in human skeletal muscle (*22*), this protein may be related to high-fat diet-induced deterioration of muscle metabolism. These results suggested that increased NDUFS protein content by exercise training might be important in mitochondrial functions.

The previous study reported that the activity of NADH-oxidase, which reflects the activity of the electron transport enzyme complex I–IV was elevated by moderate-intensity (60–70% HR_{max}) exercise training in human skeletal muscle (*23*). In addition, another previous study showed that male athletes who regularly train at a high intensity (involving supramaximal intensity exercise) have higher mitochondrial complex I activity than sedentary men (*24*). The present investigation extended the evidence that this elevated

activity of the mitochondrial electron transport enzyme after moderate- to high intensity exercise training is a reflection of the increased expression of NDUFS protein by exercise training.

In this investigation, PV was decreased in the epitrochlearis muscle after HIT. While long-term endurance training reduces the PV content in skeletal muscle (*25*), we found, for the first time, that short-term high-intensity intermittent training also decreased PV expression in fast-twitch skeletal muscle. Furthermore, the result was confirmed by the immunoblot analysis (Fig. 2D). PV is a soluble calcium-binding protein highly expressed in fast-twitch muscle fibres (*26*). In mammalian fast-twitch muscles, this protein facilitates rapid relaxation of the muscle by acting as a temporary Ca^{2+} buffer (*27*). After relatively long-term endurance training, PV expression in fast-twitch muscles was decreased (*25*). Recently, $PV^{-/-}$ fast-twitch muscles were found to have more mitochondrial protein expression than wild-type fast-twitch muscles (*28*, *29*). Therefore, there is a limited possibility that the reduction in PV expression is related to exercise-induced mitochondria biogenesis in skeletal muscle. Because PV is a major Ca^{2+} buffering protein in skeletal muscle, decreased expression of PV may positively affect Ca^{2+}-dependent signals possibly including calcineurin and CaMKs. It has been shown that raising cytosolic Ca^{2+} induced an increase in mitochondria biogenesis and GLUT4 in recruited skeletal muscle (*30*, *31*). Furthermore, overexpression of PV in slow-twitch muscle resulted in decreased calcineurin activity (*32*). However, the mechanisms should be clarified in future investigations.

For contractile proteins, the differential analysis indicates that the levels of two fast-twitch fibre-type skeletal muscle proteins, MLC-p and MLC1-f, were down-regulated by HIT. A previous study reported that high-intensity training also induces a MHC type IIb to type IIa transition in human skeletal muscle (*33*). Taken together, these results including our study may suggest that the recruitment of muscle fibres during high-intensity exercise induces fast-to-slow transformation in skeletal muscle.

Grp 75 (glucose-regulated protein 75) was found to increase after HIT. Previous studies have also reported that expression of Grp75 is elevated after low-intensity prolonged exercise training (*34*, *35*). It was previously found that both high-intensity intermittent and low-intensity prolonged exercise depletes muscle glycogen (*36*) that is the store of glucose equivalents in skeletal muscle. Since Grp75 has been shown to be induced by glucose deprivation in cells (*37*), it is reasonable to speculate that exercise-induced increase in Grp75 is due to glycogen depletion.

Up-regulation of ATPsynβ and down-regulation of glycogen phosphorylase (GPh) after HIT were detected by DIGE analysis, whereas no significantly increased expression of the enzymes were detected by the western immunoblot analysis (Fig. 2B and C). These discrepancies could be attributed to unsatisfactory specificity of the antibodies to the proteins.

The present investigation has limitations. First of all, we only focused on the 13 proteins whose expression were changed ±1.3-fold by high-intensity exercise; proteins whose expression changed less than ±1.3-fold were not reported. It is postulated that some proteins in the epitrochlearis muscle may have been increased <1.3-fold after the high-intensity swimming training. In 2D-spot testing, proteins whose expression was changed by less than ±1.3-fold could be detected. However, we used the ±1.3-fold criteria because we assumed that changes in protein expression less than ±1.3-fold may not be accurately and specifically detected, as the amount of proteins of such spots is too small to be identified by MALDI-TOF/MS, even if the DeCyder analysis provides greater sensitivity. Furthermore, proteomics does not detect all proteins existing in skeletal muscle. More than 50,000 proteins are expected to be expressed in skeletal muscle, while only 800 proteins were detected and matched in 2D electrophoresis of the present investigation, suggesting that our analysis was not comprehensive. Therefore, it is reasonable to speculate that possible proteins, including GLUT-4 (*11*) and PGC1-α (*18*) that were demonstrated by western blot analysis to increase after the same types of trainings, were not detected by the 2D electrophoresis, nor found to change their expression after HIT in the present investigation. Halloway *et al.* (*38*) also reported that, among 256 spots identified by 2D electrophoresis, neither PGC1-α nor GLUT-4 was found to increase after relatively high-intensity interval exercise training in humans.

In conclusion, we employed proteomic 2D-DIGE analysis to elucidate changed expression in skeletal muscle protein after HIT and identified several proteins, including NDUFS1 and PV, previously unknown to have functions related to exercise-training adaptations in skeletal muscle.

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Conflict of interest

None declared.

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