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THE EFFECT OF RESISTANCE AND ENDURANCE EXERCISE TRAINING ON MUSCLE PROTEOME EXPRESSION IN HUMAN SKELETAL MUSCLE

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ABSTRACT: To investigate the effect of resistance and endurance training on muscle proteome expression, samples of vastus lateralis from 10 physically active young men were analysed by 2-dimensional electrophoresis (2-DE) and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS). Differential patterns of protein expression were determined after 4 weeks of endurance or resistance exercise training. Following endurance exercise training, carbonic anhydrase III immunoglobulin heavy chain, myosin heavy chain 1, titin, chromosome 12, and fructose-1,6-bisphosphatase 2 were up-regulated while pyruvate kinase 3 isoform, ubiquitin carboxyl-terminal hydrolase, and phosphoglucomutase were down-regulated. After the 4 weeks of resistance exercise training, five proteins, apolipoprotein A-IV precursor, microtubule-actin cross linking factor 1, myosin light chain, growth hormone inducible transmembrane protein, and an unknown protein were up-regulated and pyruvate kinase 3 isoform, human albumin, and enolase 3 were down-regulated. We conclude that endurance and resistance exercise training differently alter the expression of individual muscle proteins, and that the response of muscle protein expression may be associated with specific myofibre adaptations to exercise training. Proteomic studies represent one of the developing techniques of metabolism which may substantially contribute to new insights into muscle and exercise physiology.

KEY WORDS: proteomics, 2-dimensional electrophoresis, MALDI-TOF, skeletal muscle

INTRODUCTION

Skeletal muscle is capable of highly specific metabolic and morphological adaptations in response to acute/repeated bouts of contractile activity. Contractile activity-induced adaptations in skeletal muscle are dependent upon the type of exercise (i.e. resistance or endurance), as well to its frequency, intensity and duration [1]. In particular, exercise modulates muscle protein synthesis and degradation [16]. Thus, physical activity is an important regulator of protein synthesis and gene transcription in skeletal muscle [21]. Specific proteins determine contractility properties that are characterized by significant morphological and biochemical differences [17].

The molecular mechanisms underlying exercise-induced muscle adaptation where analysed by Sangdun et al. [17], who determined mRNA expressions in mouse skeletal muscle following a single bout of running. The findings of that study suggested that skeletal muscle undergoes a phase of active cellular remodelling after a single bout of running. Transcriptional or translational activities of a specific gene cannot give a precise index of protein synthesis and protein changes at the gene level; cells, blood or tissue information needs to be clarified. In spite of accumulation of specific proteins [12], after exercise training, little is known about the specific signalling molecules and pathways that enable exercise to modulate cellular processes in skeletal muscle. Furthermore, there have been few studies [5,9,11,22] on quantitative analysis of alterations in protein expression related to exercise.

Currently, proteomics denotes nearly any type of technology focusing on protein analysis by 2-dimensional electrophoresis (2-DE) combined with matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) providing information on a single protein to thousands of proteins in a single experiment. The use of these methods is to provide extensive information on the biochemical properties of proteins in living systems. Its major goal is to create an inventory of all human proteins and a molecular protein atlas of cells, organs and tissues highlighting protein-protein

Abbreviations:

2-DE	- 2-dimensional electrophoresis;
MALDI-TOF	- matrix-assisted laser desorption/ionization time-of flight

interactions, the development of specific informational databases and identification of specific markers of pathological processes or exercise interventions [10].

Only a few studies of the effects of exercise on protein expression have been undertaken, and then mostly using animal models [5,9,17]. Takahashi and Kubota reported on the changes in protein expression after prolonged swimming exercise and the authors suggested that there existed a close link between exercise and skeletal muscle differentiation [19]. One study reported human muscle proteomic expression response to interval exercise training [11]. However, no information was recorded on human muscle protein expression according to different types of exercise training, either qualitatively or quantitatively. The purpose of the present study was to identify proteins as bio-markers which relate to different types of exercise, using 2-DE and MS.

MATERIALS AND METHODS

Subjects. Ten healthy physically active male subjects volunteered to participate in the present study. The subjects were allocated to either an endurance training group (mean ± SD; n=5, 23.4±3.2 years, 179.2±3.5 cm, 70.1±5.4 kg) or a resistance training group (mean ± SD; n=5, 22±2.8 years, 171.3±3.6 cm, 60.5±3.1 kg). The subjects gave their free consent after having been fully informed of the experimental procedures and any risk or discomfort associated with the experiment. This investigation has been approved by the Ethical Committee of Korea National Sport University.

Overview of experimental design

After a week of adaptation, both groups performed training exercise for a period of 4 weeks using one leg, the collateral leg serving as a control. For the endurance group, training comprised bicycle exercise at a speed of 70 rpm $\cdot$ min^{-1} for 1 hour per day for 5 days a week. The exercising leg was secured to the pedal by means of a toe clip. The resting leg was placed on a stool. For the resistance group, training comprised extensions and flexions of the quadriceps of one leg, on a KinCom isokinetic dynamometer, with 10 × 10 sets at 90° $\cdot$ s^{-1} and 180° $\cdot$ s^{-1} with 1 minute rest between each set. Training was undertaken daily and maintained for 4 weeks. All sessions were supervised by the researcher's team.

Muscle biopsies

Percutaneous muscle biopsies were taken from both the trained and untrained legs before and after 4 weeks of training. Initial biopsies were taken 1 week before the initiation of exercise to allow for adequate healing. Muscle samples were obtained under local anaesthesia from the vastus lateralis muscle using the percutaneous needle biopsy technique [2]. Muscle samples were immediately frozen in liquid nitrogen for proteomic assays. All muscle samples were stored at -80°C until analysed.

Protein preparation

Muscle samples were solubilised in 600 μL sample buffer composed of 7 M urea, 2 M thiourea, 4% CHAPS, 0.5% ampholyte, 100 mM DTT, 40 mM Tris, 0.002% bromophenol blue, protease inhibitor (Roche, Penzberg, Germany), and DNase. The sample was sonicated for approximately 10 s, 10 times with 10 s intervals. The sample was then incubated at 4°C for 30 min and centrifuged for 45 min at 35,300 rpm. The supernatant was collected, and its protein concentration was determined using the Bradford assay kit (Bio-Rad, USA). For each tissue, a protein sample of 75 mg was used for 2-DE.

Two-dimensional polyacrylamide gel electrophoresis and image analysis

The protein was mixed with sample buffer to obtain a total volume of 400 μl, which was applied to 18 cm long immobilized pH 3-10 nonlinear gradient (IPG) strips. The first dimension isoelectric separation was made on IPG of 3.10 L (240 × 3 × 0.5 mm) dry strips (Amersham Pharmacia Biotech, UK) using an Ettan IPGphor Isoelectric Focusing system. The strips were left in mineral oil for 12-16 h at room temperature. Isoelectric focusing was run for 80,000 total voltage hours (Vh) using a gradually increasing voltage protocol, implemented using a programmable high voltage power supply. To solubilize the electro-focused proteins and to allow the SDS to polymerize, it is necessary to soak the IPG strips in SDS equilibration buffer (6 M urea, 2% SDS, gel buffer pH 8.8, 50% glycerol, 2.5% acrylamide monomer).

Second dimension separation was conducted with an Ettan DALTsix Electrophoresis Unit (Amersham Pharmacia Biotech, UK) on 12% SDS polyacrylamide gels. The strips were embedded in a 0.5% (w/v) agarose stacking gel, and the proteins were separated at 40 mA per gel until the bromophenol blue marker dye ran off the bottom of the gel. After 2-DE, gels were fixed for 1 h in fixing solution (40% methanol and 5% phosphoric acid) and the gel was stained in Coomassie brilliant blue G-250 (17% ammonium sulfate, 3.6% phosphoric acid, and 34% methanol) overnight. The gels were washed 5-6 times in distilled water for 5 h.

Integrated signal intensities were analysed quantitatively using Image Master Platinum 5 (GE Healthcare). Two independent observers visually confirmed the differential expression. Increased and decreased spots over 2-fold in treated muscle compared with control were manually excised and subjected to in-gel tryptic digestion using the procedure described.

In order to limit the influence of the transfer effect of exercise training in both legs, the spots which were changed in the untrained leg were excluded in the image analysis.

Trypsin digestion of proteins in-gel

Gel spots were picked with an end-cut yellow tip and the selected piece was transferred into a 1.5-mL Eppendorf tube and then washed with 100 μL of distilled water. The gel pieces were mixed with 50 μL

of 50 mM NH_4HCO_3 and acetonitrile and shaken for 10 min. The gel pieces were then dried in a Speed Vac for 10 min and 5 μL of trypsin was added; the gel pieces were left on ice for 45 min. After that they were incubated at 37°C for 12 h.

MALDI-TOF MS analysis

Peptide mass fingerprinting was performed at the Yonsei Proteome Research Center, Seoul.

Database searching and MALDI-TOF MS was performed on a 4800 MALDI-TOF/TOF™ Analyzer (Applied Biosystems) equipped with a 355-nm Nd:YAG laser. The pressure in the TOF analyser is approximately 7.6e-07 Torr. The mass spectra were obtained in the reflectron mode with an accelerating voltage of 20 kV and the sum from 500 laser pulses and calibrated using the 4700 calibration mixture (Applied Biosystems). Data Explorer 4.4 (PerSeptive Biosystems) was used for the data acquisition and extraction of the monoisotopic masses.

Data searching and identification of proteins' peptide mass fingerprints were obtained by MALDI-TOF MS through the NCBInr, SWISS-PROT and MvSDB databases, using Mascot software (http://www.matrixscience.com).

RESULTS

Identification of protein spots on two-dimensional gels after 4 weeks endurance training. Protein profiles from vastus lateralis muscle were analysed by 2-DE and 500-600 spots were observed after 4 weeks

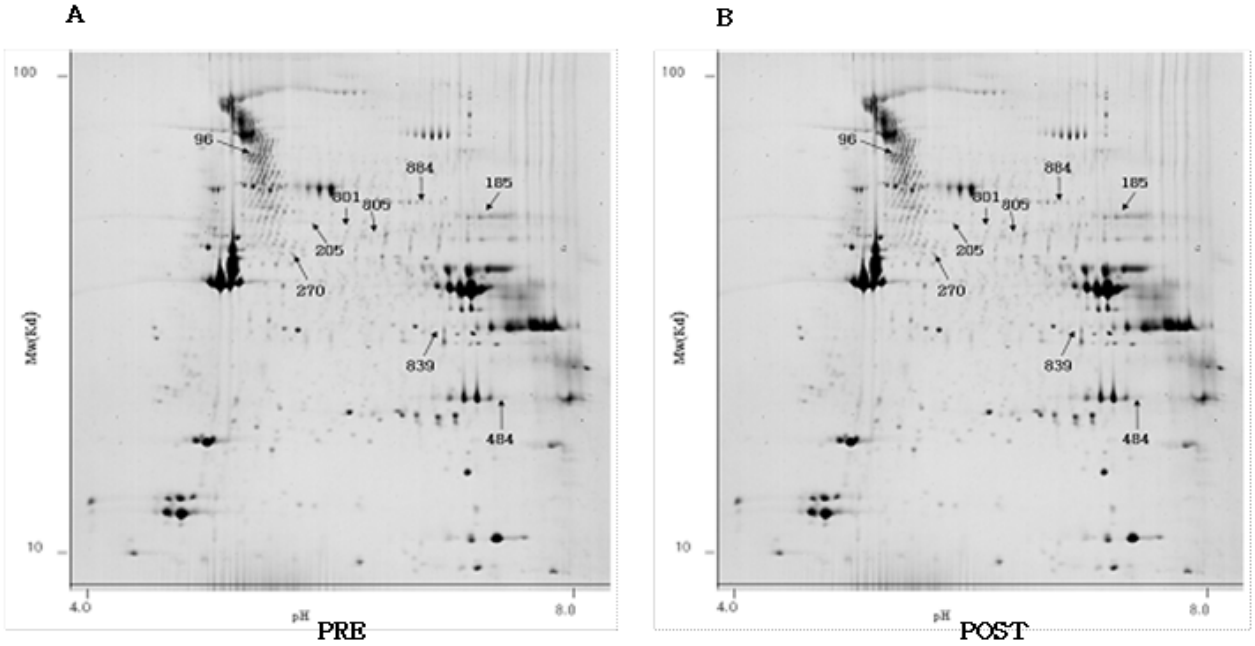

FIG. 1. REPRESENTATIVE SAMPLE OF 2-D GEL IMAGE BEFORE AND AFTER AEROBIC EXERCISE TRAINING FROM VASTUS LATERALIS MUSCLE, USING A 3-10 NL PH GRADIENT

TABLE 1. DIFFERENTIALLY EXPRESSED PROTEINS THAT WERE INCREASED AFTER AEROBIC EXERCISE TRAINING

Spot	Protein name	Accession no	Sequence of coverage (%)	Mass matched	Post/pre	Measured Mr/PI
205	Immunoglobulin heavy chain variable region	gi|112701251	68	4	2.9	10846/ 6.34
270	Myosin heavy chain 1	gi|13638390	26	22	2.8	101955/ 5.59
484	Carbonic anhydrase III	gi|4885099	65	15	7.2	29539/ 6.86
801	Titin	gi|407139	10	34	5.1	521744/ 8.06
839	Fructose-1,6-bisphosphatase 2	gi|22907028	28	14	10.4	36031/ 6.84
805	Chromosome 12	gi|13111784	29	14	2.7	73322/ 6.28

Note: Protein name and accession numbers correspond to NCBI database pI and Mr values were estimated from the relative position of each protein spot on the 2-D gel, and calculated using the ExPASY tool

TABLE 2. DIFFERENTIALLY EXPRESSED PROTEINS THAT WERE DECREASED AFTER AEROBIC EXERCISE TRAINING

Spot	Protein name	Accession no	Sequence of coverage (%)	Mass matched	Post/pre	Measured Mr/PI
96	Ubiquitin carboxyl-terminal hydrolase L5	gi|55859536	51	8	3.7	18235/ 11.23
185	Pyruvate kinase 3 isoform 2	gi|33286420	35	19	4.2	61555/ 7.6
884	Phosphoglucomutase 1	gi|21361621	41	18	6.4	61411/ 6.3

Note: Protein name and accession numbers correspond to NCBI database pI and Mr values were estimated from the relative position of each protein spot on the 2-D gel, and calculated using the ExPASY tool

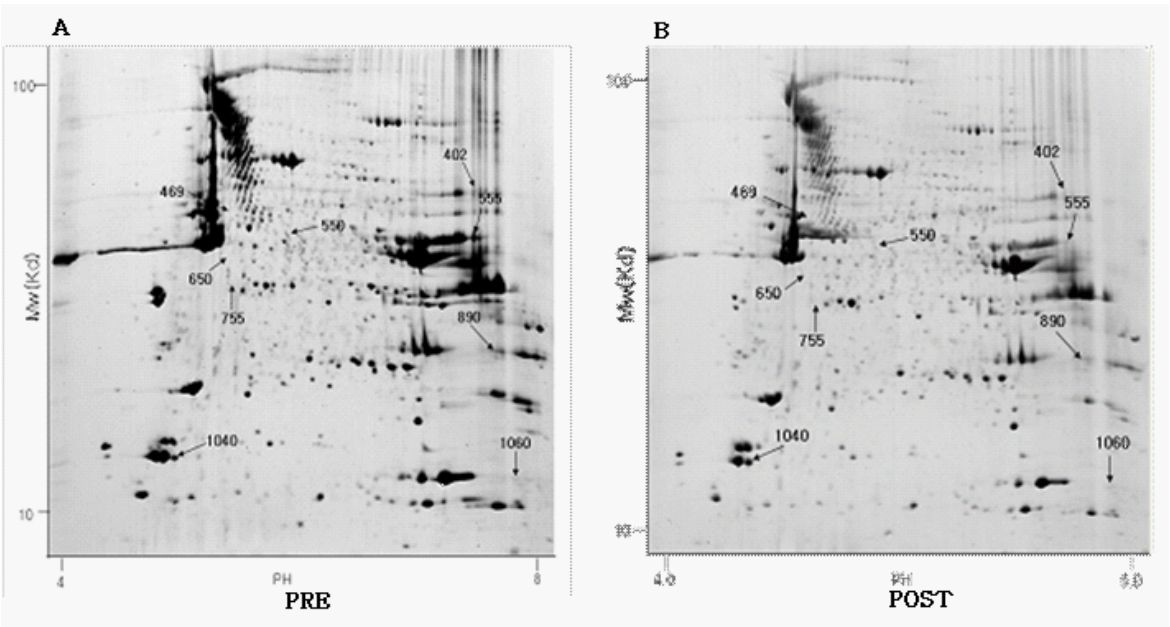

FIG. 2. REPRESENTATIVE SAMPLE OF 2-D GEL IMAGE BEFORE AND AFTER RESISTANCE EXERCISE TRAINING FROM VASTUS LATERALIS MUSCLE, USING A 3-10 NL PH GRADIENT

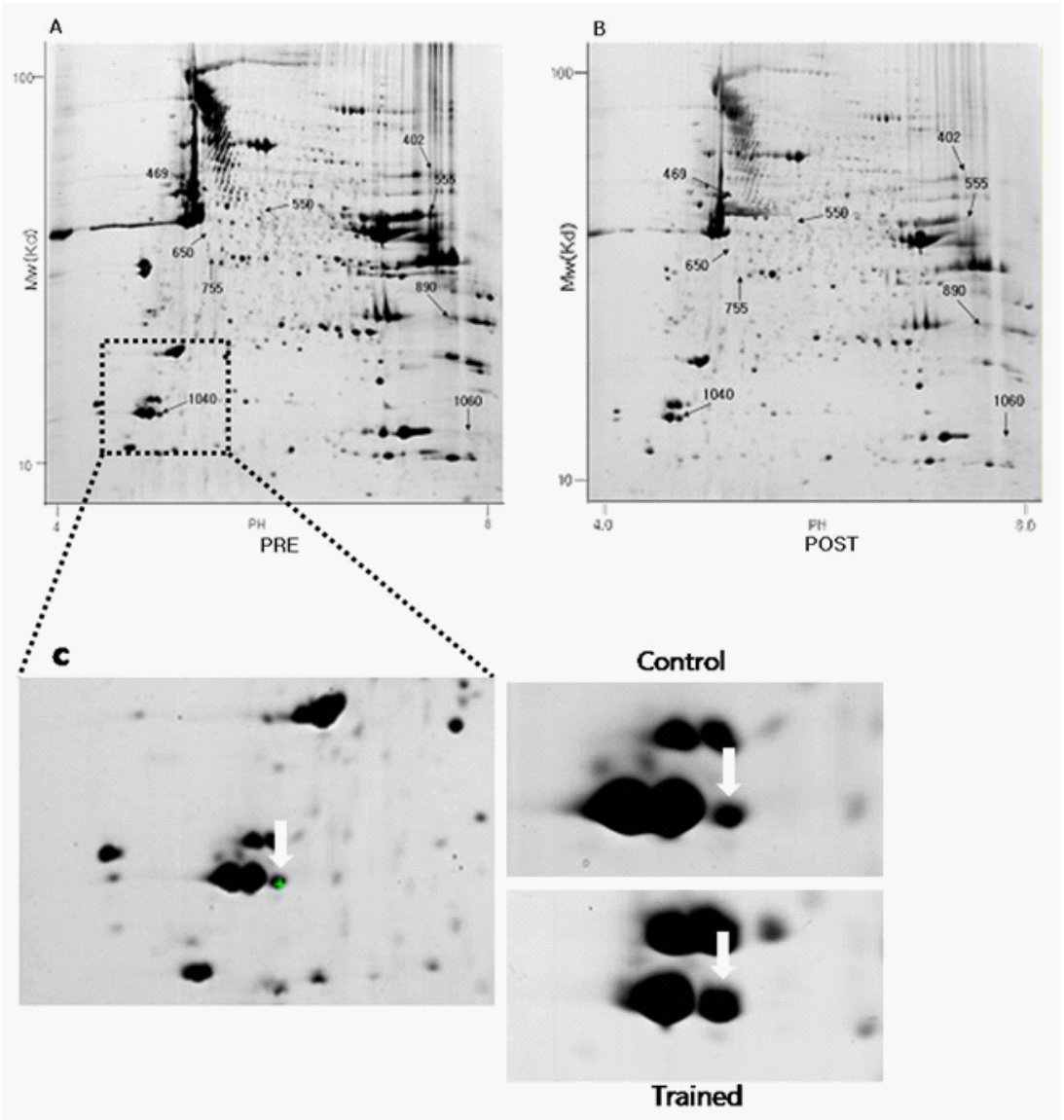

FIG. 3. REPRESENTATIVE SAMPLE OF 2D ELECTROPHORESIS ANALYSIS OF REGIONAL DIFFERENCES IN MYOSIN LIGHT CHAIN PROTEIN (1040 SPOT) EXPRESSION IN TRAINED AND CONTROL VASTUS LATERALIS MUSCLE. A AND B: REPRESENTATIVE 2D GEL AFTER RESISTANCE EXERCISE TRAINING, C: CLOSE-UP OF 1040 PROTEIN REGION OF GELS

TABLE 3. DIFFERENTIALLY EXPRESSED PROTEINS THAT WERE INCREASED AFTER RESISTANCE EXERCISE TRAINING

Spot	Protein name	Accession no	Sequence of coverage (%)	Mass matched	Post/pre	Measured Mr/PI
469	unknown protein	gi|73909110	50	10	10.1	104422/ 5.19
650	Apolipoprotein A-IV precursor	gi|178779	65	24	2.2	43358/ 5.22
890	microtubule-actin crosslinking factor 1	gi|6273778	10	28	2.1	613652/ 5.26
1040	Myosin light chain	gi|48146043	65	14	2.8	19102/ 4.89
1060	growth hormone inducible transmembrane protein	gi|55665693	42	8	33.7	35259/ 10.12

Note: Protein name and accession numbers correspond to NCBI database pI and Mr values were estimated from the relative position of each protein spot on the 2-D gel, and calculated using the ExPASY tool

TABLE 4. DIFFERENTIALLY EXPRESSED PROTEINS THAT WERE DECREASED AFTER RESISTANCE EXERCISE TRAINING

Spot	Protein name	Accession no	Sequence of coverage (%)	Mass matched	Post/pre	Measured Mr/PI
402	Pyruvate kinase 3 isoform	gi|33286422	29	15	6.7	58538/ 7.6
550	Human albumin	gi|763431	29	14	3.4	53416/ 5.69
555	Enolase 3	gi|114665857	48	13	2.2	46957/ 7.59
755	Unknown	gi|62702263	54	6	2.7	15577/ 5.2

Note: Protein name and accession numbers correspond to NCBI database pI and Mr values were estimated from the relative position of each protein spot on the 2-D gel, and calculated using the ExPASY tool

of endurance training. A representative example of muscle proteins separated on a 2-DE gel is shown in Figure 1. Most protein spots that were observed were located in the range of PI 3-10 with the molecular mass range of 10-100 kDa. Nine protein spots were significantly different between rest and endurance training. These protein spot variations were not detected in the untrained leg (n=2). Six protein spots were up-regulated whereas the other three protein spots were down-regulated. The up-regulated proteins were myosin heavy chain 1, carbonic anhydrase III, titin, chromosome 12, fructose-1,6-bisphosphatase 2, and immunoglobulin heavy chain variable region (Table 1). The down-regulated proteins were pyruvate kinase 3, ubiquitin carboxyl-terminal hydrolase L5, and phosphoglucomutase (Table 2).

Identification of protein spots on two-dimensional gels after 4 weeks of resistance training

The present study extends previous studies by identifying several proteins, such as microtubule-actin cross linking factor 1 and myosin light chain, which are associated with muscular function. After 4 weeks of resistance training, the protein profiles revealed about 800-900 spots. Most protein spots were obtained in the range of PI 3-10 and 1000 μg of muscle protein were applied for separation. Nine protein spots were significantly changed by resistance training.

Figure 3 shows a representative sample of 2D electrophoresis analysis of regional differences in myosin light chain protein (1040 spot) expression in trained and control vastus lateralis muscle.

Five proteins were up-regulated whereas the other four proteins were down-regulated. No modifications were detected in the untrained leg (n=2).

The up-regulated proteins were apolipoprotein A-IV precursor microtubule-actin cross linking factor 1, myosin light chain, and growth hormone inducible transmembrane protein (Table 3), whereas the down-regulated proteins were human albumin, enolase 3, and an unknown protein (Table 4).

DISCUSSION

Proteomic techniques such as 2D-PAGE and mass spectrometry can give extensive information on final protein expression. Most of the time, expression at the mRNA level does not reflect the expression of protein synthesis [4]. The use of proteomics offers a clearer insight into real protein expression, providing a more accurate view of the processes involved in physiological adaptations.

The major finding of the current study was that the muscle proteins are altered differently after endurance and resistance training. The identified protein isoforms are involved in contractile, regulatory and metabolic processes and enable muscle-specific protein expression patterns to be determined. We have observed that exercise training induces specific changes in protein expression of human skeletal muscle. Six proteins were up-regulated and three proteins were down-regulated in vastus lateralis muscle after 4 weeks of endurance training. The increase of these cytoskeletal proteins may enhance muscle function induced by the training. Endurance training increased the expression of myosin heavy chain and titin, both of which are located in the sarcomere. Titin is an integral component of the M-line with its N-terminal forming part of the Z-disc in each sarcomere. The increase in myosin heavy chain expression with endurance training is consistent with previous work with trained muscle [13]. Both myosin heavy chain and titin are associated with muscle building, and that endurance training induced an increase in the expression of these proteins.

Carbonic anhydrase, a sarcoplasmic protein, was up-regulated 7.2-fold after endurance training. Carbonic anhydrase catalyses the

production of HCO_3^- from CO_2 and H_2O, providing HCO_3^- substrate for transport as well as removing HCO_3^- following transport. Modulation of carbonic anhydrase activity therefore provides a means to regulate the rate of HCO_3^- transport [8]. Therefore, this prominent increase would enable a greater capacity to remove CO_2 produced by slow-twitch oxidative fibres.

Endurance training also resulted in adaptations in the glycolytic pathway. Fructose-1,6-bisphosphatase, which is an enzyme essential to a gluconeogenesis pathway, was more highly expressed in trained muscle (10.4-fold). This glycogenic enzyme catalyses a tightly regulated step of gluconeogenesis in the liver and catalyses the hydrolysis of fructose-1,6-bisphosphate to fructose 6-phosphate in the muscle.

Thus, the use of proteomic techniques for research on muscular protein expression pattern in response to endurance training gave us information on muscle construction-related proteins (titin, myosin heavy chain I), immune-related protein (immunoglobulin) and energy metabolism-related proteins (fructose-1,6, phosphate, phosphoglucomutase, pyruvate kinase) which were significantly altered by this type of exercise.

On the other hand, muscle protein spots were differentially expressed by resistance exercise training. The up-regulated spots increased by more than 200% after resistance training, namely, apolipoprotein A-IV precursor, microtubule-actin cross linking factor 1, myosin light chain, and growth hormone inducible transmembrane protein. By contrast, human albumin, enolase 3, and an unknown protein were down-regulated. All these proteins are related to HDL parameters, gluconeogenic enzyme, muscle contraction and muscle building.

Muscle building-related proteins, such as microtubule-actin cross linking factor and myosin light chain, were up-regulated (~2-fold). Microtubule actin cross-linking factor (MACF) is classified as a member of the plakin family [3]. Human MACF was also reported as trabeculin-α, which shares similar reaction mechanisms with actin-binding proteins [15]. Regulatory myosin light chain (RLC) is related to the thin filament regulatory system as well as to the Ca^{2+} sensitivity of ATPase activity [7]. At the molecular level, RLC helps cross bridges to move away from the thick filament backbone to become more accessible to actin during skeletal muscle contraction [18]. It appears that the increase of those cytoskeletal proteins may enhance muscle function during exercise adaptations.

In addition, several specific proteins related to fatty acid metabolism are implicated in resistance training adaptation. Apolipoprotein A-IV (apo A-IV) was up-regulated after resistance exercise training (2.1-fold). The lipoprotein-related variable apolipoprotein A is inversely associated with coronary artery disease [6]. Therefore resistance exercise training may generate a higher level of this HDL-related parameter.

Several proteins involved in energy metabolism are differentially expressed when comparing endurance and resistance types of training. However, we could not identify an increase in mitochondrial enzymes, which is indicative of a well-established response to exercise training. Thus, the protein molecule biomarkers for exercise responses are still unclear. The current protein separation techniques do not yet allow one to identify all types of protein, but the proteomics approach applied to striated skeletal muscle investigations still appears to be one of the most promising techniques to understand the mechanisms of adaptations in physiological states.

CONCLUSIONS

In summary, 4 weeks of endurance and resistance exercise training in young male subjects altered the expression of several skeletal muscle proteins, some being up-regulated, others down-regulated. The different responses of muscle protein expression between the two types of exercise training may be associated with differential myofibre adaptation. Proteomic studies could be applied to evaluate the functional adaptation to specific types of exercise conditions, but more developed techniques appear to be compulsory to better quantify those relative changes.

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