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Differential expression of the fast skeletal muscle proteome following chronic low-frequency stimulation

Pamela Donoghue[a], Philip Doran[a], Paul Dowling[b], Kay Ohlendieck[a,*]

[a]Department of Biology, National University of Ireland, Maynooth, Co. Kildare, Ireland
[b]School of Biotechnology, Dublin City University, Dublin, Ireland

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Abstract

Physiological and biochemical responses of skeletal muscle fibres to enhanced neuromuscular activity under conditions of maximum activation can be studied experimentally by chronic low-frequency stimulation of fast muscles. Stimulation-induced changes in the expression pattern of the rabbit fast skeletal muscle proteome were evaluated by two-dimensional gel electrophoresis and compared to the altered isoform expression profile of established transformation markers such as the Ca^{2+}-ATPase, calsequestrin and the myosin heavy chain. Sixteen muscle proteins exhibited a marked change in their expression level. This included albumin with a 4-fold increase in abundance. In contrast, glycolytic enzymes, such as enolase and aldolase, showed a decreased expression. Concomitant changes were observed with marker elements of the contractile apparatus. While the fast isoforms of troponin T and myosin light chain 2 were drastically down-regulated, their slow counterparts exhibited increased expression. Interestingly, mitochondrial creatine kinase expression increased while the cytosolic isoform of this key muscle enzyme decreased. The expression of the small heat shock protein HSP-B5/αB-crystallin and the oxygen carrier protein myoglobin were both increased 2-fold following stimulation. The observed changes indicate that the conversion into fatigue-resistant red fibres depends on: (i) the optimum utilization of free fatty acids via albumin transportation, (ii) a rearrangement of the creatine kinase isozyme pattern for enhanced mitochondrial activity, (iii) an increased availability of oxygen for aerobic metabolism via myoglobin transport, (iv) the conversion of the contractile apparatus to isoforms with slower twitch characteristics and (v) the up-regulation of chaperone-like proteins for stabilising myofibrillar components during the fast-to-slow transition process.

Keywords: Skeletal muscle proteome; Chronic low-frequency stimulation; Muscle transformation; Muscle plasticity; Muscle proteomics

1. Introduction

Chronic low-frequency stimulation of fast-twitch skeletal muscles is an experimental technique that has been extensively used to enhance our basic understanding of the physiological properties of transforming muscle fibres [1,2] and to develop improved biomedical treatments that exploit muscle plasticity [3,4]. The detailed analysis of fibre transition processes on the molecular and cellular level has established skeletal muscles as a class of tissue that can quickly adapt to changed functional demands [5,6]. A decreased fibre caliber, an elevation of the aerobic-oxidative capacity and an increase in the time to peak twitch tension and half-relaxation time are hallmarks of chronically stimulated muscle fibres resulting in an improved resistance to fatigue [7]. The molecular physiological and biochemical characterization of conditioned muscles have shown that electro-stimulation has pronounced effects on elements of the contractile apparatus [8], regulatory proteins of the excitation–contraction–relaxation cycle [9], components involved in ion homeostasis [10], metabolic enzymes [11] and neuromuscular junction proteins [12]. The transformation consists of changes in the abundance and isoform expression pattern of key muscle proteins [2], as well as variations in protein–protein interactions within supramolecular protein assemblies [13]. Thus, in order to investigate responses of skeletal muscles to enhanced neuromuscular activity under condi-

* Corresponding author. Tel.: +353 1 708 3842; fax: +353 1 708 3845.
E-mail address: kay.ohlendieck@nuim.ie (K. Ohlendieck).

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tions of maximum activation, long-term low-frequency stimulation represents an ideal model system.

Representative examples of stimulation-induced changes in major muscle proteins is the stepwise replacement of myosin heavy chains from the MHCII isoforms to MHCI [14], a shift from the fast SERCA1 isoform of the sarcoplasmic reticulum Ca^{2+}-ATPases to the slower SERCA2 isoform [15], the increase in the levels of the α- and δ-subunits of the nicotinic acetylcholine receptor [12] and the gradual increase in the activity of enzymes involved in the main metabolic systems of aerobic substrate oxidation, i.e., the citric acid cycle, fatty acid oxidation and the respiratory chain [11,16,17]. The recent blot overlay analysis of the sarcoplasmic reticulum Ca^{2+}-binding protein calsequestrin, using a peroxidase conjugate probe, revealed that the fast-to-slow transition process triggers a distinct reduction in fast calsequestrin and a concomitant alteration in calsequestrin interactions with the ryanodine receptor Ca^{2+}-release channel [13]. Hence, chronic electro-stimulation has not only an effect on the activity, density and isoform expression pattern of muscle proteins, but also influences sensitive protein–protein interactions in ion-regulatory membrane complexes. Based on these findings, it would now be of interest to establish a more comprehensive picture of the overall fibre transition process. Due to the apparent complexity of stimulation-induced changes on the protein level, modern proteomics technology presents itself as a possible way forward to do this and further our understanding of the finely tuned adaptation process of skeletal muscles.

Following the emergence of proteomics technology over the last few years [18,19], two-dimensional gel electrophoresis and mass spectrometry has been successfully adapted for the analysis of the skeletal muscle proteome [20–22]. Skeletal muscle proteomics has been used in the identification of O-linked N-acetylglucosamine proteins [23] and age-dependent protein nitration [24], as well as expression profiling during chicken muscle growth [25] and bovine skeletal muscle hypertrophy [26]. Recently, the analysis of pathological skeletal muscle fibres led to the identification of novel protein factors involved in neuromuscular disorders [27–29]. In analogy, we have used in this study two-dimensional electrophoresis, mass spectrometry and immunoblotting to determine the differential expression of the fast skeletal muscle proteome following chronic low-frequency stimulation. Our findings might further our understanding of the molecular and cellular effects of fibre conditioning, and therefore may help in the improved design of stimulation protocols used in biomedical applications such as dynamic cardiomyoplasty [3,4]. In contrast to studying individual proteins by conventional biochemical techniques, the proteomics method allows a more comprehensive survey of a great variety of different muscle protein species using one experimental approach. In order to avoid the potential artifactual enrichment or loss of muscle proteins during extensive subcellular fractionation procedures, this comparative study employed total muscle extracts. In contrast to the traditional approach of focusing on individual proteins, related groups of proteins or subcellular fractions, we have applied mass spectrometry-based proteomics technology to investigate the entire muscle protein complement that is accessible by gel electrophoretic separation.

2. Materials and methods

2.1. Materials

Antibodies were purchased from Affinity Bioreagents, Golden, CO (mAb $VIIID1_2$ to the fast CSQ_f isoform of calsequestrin, mAb IIH11 to the fast SERCA1 isoform of the Ca^{2+}-ATPase, and mAb IID8 to the slow SERCA 2 isoform of the Ca^{2+}-ATPase), US Biological, Swampscott, MA (pA to the slow CSQ_s isoform of calsequestrin), Sigma, Dorset, UK (mAb MY-32 to the fast MHC_f isoforms IIa, IIb, IId and neonatal of the myosin heavy chain and mAb NOQ7.54D to the slow MHC_s isoform of the myosin heavy chain), Novacastra Laboratories Ltd., Newcastle upon Tyne, UK (mAb against αB-crystallin/HSP-B5), and Abcam Ltd., Cambridge, UK (mAb 8343 to myoglobin and mAb 14225 against albumin). Peroxidase-conjugated secondary antibodies were from Chemicon International, Temecula, CA. Protease inhibitors were purchased from Roche Diagnostics GmbH, Mannheim, Germany. Ultrapure Protogel acrylamide stock solutions were obtained from National Diagnostics (Atlanta, GA). Electrophoresis grade chemicals, protein dyes, immobilized pH gradient (IPG) strips of pH 3–10 (linear) and IPG buffer of pH 3–10 for isoelectric focusing and acetonitrile were obtained from Amersham Biosciences, Little Chalfont, Bucks., UK. A matrix kit containing α-cyano-4-hydroxycinnamic acid was from Laserbiolabs, Sophia-Antipolis, France. Immobilon NC nitrocellulose membranes and chemiluminescence substrates were obtained from Millipore, Bedford, MA and Pierce and Warriner, Chester, UK, respectively. All other chemicals used were of analytical grade and purchased from Sigma Chemical Company, Dorset, UK.

2.2. Animal model

Unstimulated control specimens and chronic–low-frequency stimulated muscle samples were obtained from adult male New Zealand white rabbits and made available to our laboratory by Dr. Dirk Pette (Department of Biology, University of Konstanz, Germany). Laboratory visits to Germany by the Maynooth team were generously supported by the Irish Higher Education Authority and the Deutscher Akademischer Austauschdienst. The chronic electro-stimulated rabbit tibialis anterior muscle represents the standard model for fast-to-slow fibre transformation [1,2,5,6]. This muscle can be conveniently stimulated through chronic low-frequency telestimulation of the peroneal nerve [7] and is a highly reproducible animal model for muscle conditioning. The normal unstimulated tibialis anterior is a predominantly

fast-twitching skeletal muscle. Histochemical and biochemical fibre analyses have shown that the rabbit tibialis anterior muscle is composed of four defined fibre types (I, IIC, IIA and IIB) [8]. For proteomics screening, the left hind limb was stimulated continuously at 10 Hz for 42 days. Three animals per time point of stimulation were used in this study and six control and six stimulated samples from one animal were used for the statistical analysis of the electrophoretically separated muscle proteome. For immunoblotting applications, muscles were stimulated for 5 to 78 days. Following chronic-low frequency stimulation, the conditioned left and the untreated right tibialis anterior muscles were dissected and quick frozen in liquid nitrogen. Muscle samples were then stored at −80 °C prior to proteomics analysis.

2.3. Preparation of total muscle extracts

In order to carry out a proteomics analysis of stimulation-induced fibre type transformation, total protein extracts from fresh muscle homogenates were prepared as recently described by our laboratory [30]. Most studies on the fate of low-abundance muscle proteins in conditioned fibres have been conducted with subcellular fractions. However, we could recently show that the differential centrifugation of skeletal muscle homogenates may introduce artifacts in comparative approaches. While the immunoblotting and mass spectrometric analysis of two-dimensionally separated crude extracts from dystrophic fibres revealed a drastic reduction in calsequestrin [31], the previous immunoblot survey of microsomal membranes did not reveal this difference of calsequestrin expression in muscular dystrophy [30]. We therefore used total muscle extracts to avoid potential problems with artifactual loss or enrichment of proteins during subcellular fractionation. All preparative steps were performed at 0–4 °C in the presence of a protease inhibitor to prevent proteolytic degradation. The protein concentration of the crude tissue samples was determined by the Bradford dye-binding assay system [32]. Protein samples were used immediately for isoelectric focusing or otherwise quick-frozen in liquid nitrogen and stored at −80 °C. For one-dimensional immunoblot analysis, microsomal muscle preparations were isolated by an established subcellular fractionation technique [33].

2.4. Comparative two-dimensional gel electrophoresis

Isoelectric focusing was carried out in the first dimension using an IPGphor focusing system (Amersham Biosciences, Little Chalfont, Bucks., UK). IPG strips of pH 3–10 (linear) were run at 50 μA per strip [34]. Initial screening was performed with first dimensional gels of 13 cm length, followed by a direct comparison of unstimulated and conditioned specimens with 7-cm-long strips. To facilitate the focusing effect, a lysis buffer containing 8 M urea, 2 M thiourea, 4% (w/v) Chaps, 1% (v/v) Triton X-100, 10 mM Tris-base, 65 mM DTT and 0.8% (w/v) carrier ampholytes was used. Total muscle extracts were focused at a final concentration of 100 μg of protein per strip [35], whereby protein concentration was determined by standard methodology [32]. Following rehydration for 9 h, the following running conditions were used: 120 min at 30V, 60 min at 100V, 60 min at 500V, 60 min at 1000V, 60 min at 2000V, 60 min at 4000V, 60 min at 6000V and finally 120 min at 8000V. Electrophoretic separation in the second dimension was performed on a 12% sodium dodecyl sulfate polyalcrylamide slab gel using the Protean Xi-II Cell from Bio-Rad Laboratories, Hemel Hempstead, UK [36]. Prior to the 2D separation step, the focused IPG strips were firstly equilibrated for 10 min in equilibration buffer (30% (w/v) glycerol, 20% (w/v) sucrose, 3% (w/v) SDS, 50 mM Tris–HCl, pH 8.8) supplemented with 100 mM DTT and then incubated for 10 min in equilibration buffer containing 0.25 M iodoacetamide. Equilibrated strips were briefly washed in SDS running buffer and carefully layered on top of the second-dimension gel. IPG strips were held in place using a 1% (w/v) agarose sealing gel poured on top of the strip and left to solidify. In order to avoid gel-to-gel variations in the comparative analysis of control and stimulated samples, 7-cm IPG strips were positioned next to each other on the same second dimensional gel system. Gels were run at a constant voltage of 100 V for 1 h, followed by 300 V for 3 h and a final step of 500 V for 2 h.

2.5. Immunoblot analysis

Two-dimensional immunoblotting with total muscle extracts [30] and one-dimensional immunoblotting with microsomal preparations [15] was carried out with protein gel replicas that had been electrophoretically transferred for 1h at 100V onto Immobilon-NC nitrocellulose membranes according to Towbin et al. [37] using a Protean blotting system from Bio-Rad Laboratories, Hemel Hempstead, Herts., UK. Blocking, washing and incubation with primary and peroxidase-conjugated secondary antibodies was performed as previously described [10]. Immuno-decorated protein spots or protein bands were visualised using the Super Signal enhanced chemiluminescence kit from Pierce and Warriner, Cheshire, UK [38]. Densitometric scanning of developed immunoblots was carried out with an Epson Perfection 1200S colour scanner from Seiko Epson Corporation, Japan.

2.6. Protein visualisation and image analysis

Protein spot patterns were visualised by hot Coomassie staining using PhastGel Coomassie Blue R-350 [30] or by silver staining [39]. Volume integration of 2D spots from Coomassie-stained gels and statistical analysis of expression levels in control versus stimulated samples were performed with the Image Master 2D Platinum software programme from Amersham Biosciences, Little Chalfont, Bucks., UK. The analysis software was used for the evaluation of

expression changes in candidate protein spots, whereby six normal and six stimulated protein complement preparations were used to determine a marked up- or down-regulation of specific protein species following chronic–low-frequency stimulation. Gels were stored in 10% (v/v) acetic acid for further analysis by mass spectrometry.

2.7. Protein identification by mass spectrometry.

Coomassie-stained protein spots, excised from the slab gel using 1 ml pipette tips, were transferred to sterile 1.5 ml Eppendorf tubes for destaining, chemical reduction and alkylation [30]. Individual gel plugs were shrunk using acetonitrile followed by rehydration in 50 mM ammonium bicarbonate. An equal volume of acetonitrile was then added. Following a 15-min incubation period, all solution was removed and the sample dried using a Heto type vacuum centrifuge (Jouan Nordic A/S, Allerod, Denmark). The sample was reduced and alkylated by incubation in 10 mM dithiotreitol and 50 mM ammonium bicarbonate for 45 min at 37 °C, followed by a 30-min incubation in 55 mM iodoacetamide and 50 mM ammonium bicarbonate in the dark. Gel plugs were then washed in 50 mM ammonium bicarbonate and shrunk by acetonitrile. The sample was dried as before in the vacuum centrifuge. Enzymatic digestion was achieved by the addition of 1 μl of trypsin and 20 μl of 50 mM ammonium bicarbonate to each gel plug. Exhaustive incubation with trypsin was carried out overnight at 37 °C. The tryptic digest was then centrifuged at 14,000×*g* in a bench top 5417R Eppendorf centrifuge for 20 min. The supernatant was carefully removed from each sample and was then immediately used for MALDI-ToF mass spectrometric analysis or otherwise stored at −20 °C. For spectrometric analysis, the digested sample was mixed with a preparation of α-cyano-4-hydroxycinnamic acid and spotted onto the sample plate. The matrix was prepared using a concentration of 50% acetonitrile:50% 0.1% trifluoroacetic acid and also spiked with internal standards of known peptide mass, Angiotensin III (897.5 *m*/*z*) and ACTH (2465.19 *m*/*z*). To aid protein identification, the first two target spots contained a mix of matrix and calibrants only, giving a known range of different peptide masses from 1046.54 *m*/*z* to 2465.19 *m*/*z*. Mass spectra were recorded by an Amersham MALDI-ToF Pro instrument in the positive reflector mode with an accelerating voltage of 20 kV and pulsed extraction. Mass spectra analysis was performed by MALDI evaluation software from Amersham Biosciences. Using the generated peptide mass fingerprint, protein identification was achieved with the PMF Pro-Found search engine and the NR database.

3. Results

The main objective of this study was to determine the differential expression profile of the fast-twitching skeletal muscle proteome during the fast-to-slow transition process. We have used two-dimensional gel electrophoresis, mass spectrometry and immunoblotting in our comparative proteomics analysis of unstimulated control muscle fibres versus chronic low-frequency stimulated fast muscle fibres.

3.1. Comparative two-dimensional immunoblotting of successful fast-to-slow fibre transformation

Prior to the proteomics screening, fibre transformation was confirmed by comparative two-dimensional immunoblotting of total muscle extracts. Fig. 1 illustrates the immuno-decoration of established protein markers that exist in distinct slow and fast isoforms, i.e., the myosin heavy

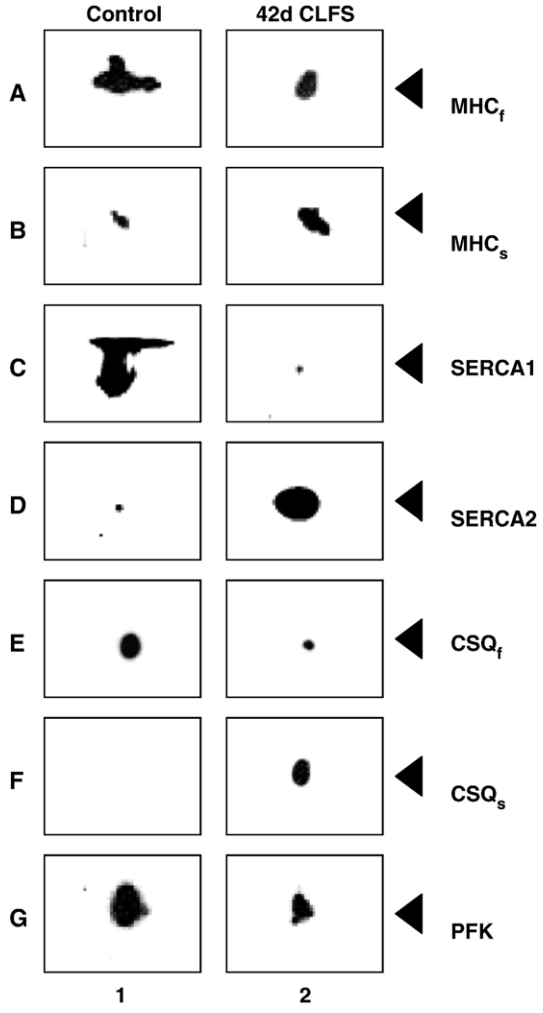

Fig. 1. Immunoblot analysis of muscle marker isoform shifting following chronic low-frequency stimulation. Shown are identical 2D immunoblots which were labeled with antibodies to the fast myosin heavy chain (MHC_f, A), the slow myosin heavy chain (MHC_s, B), the fast sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA1, C), the slow sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA2, D), fast calsequestrin (CSQ_f, E), slow calsequestrin (CSQ_s, F) and phosphofructokinase (PFK, G). Proteins were separated in the first dimension by isoelectric focusing and in the second dimension by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Immunoblotting panels correspond to the Coomassie-stained gels shown below in Fig. 3. Lanes 1 and 2 represent total protein extracts from unstimulated and 42 days chronic low-frequency (10 Hz) electro-stimulated skeletal muscle fibres, respectively.

chain [8], the sarcoplasmic reticulum Ca^{2+}-ATPase [40] and calsequestrin [41]. The representative blots show the concomitant decrease of the fast isoforms of the myosin heavy chain, the Ca^{2+}-ATPase and calsequestrin (Fig. 1A, C, E) and increase of their respective slow counterparts (Fig. 1B, D, F). Phosphofructokinase, a marker enzyme of glycolysis, was also shown to decrease in its abundance following chronic electro-stimulation (Fig. 1G). This clearly demonstrated a successful fast-to-slow transition process.

3.2. Two-dimensional gel electrophoretic mapping analysis of stimulated fast muscle

The characteristic protein spot pattern of 42 days electro-stimulated skeletal muscles following isoelectric focusing in the first dimension and sodium dodecyl sulfate polyacrylamide gel electrophoresis in the second dimension is shown in Fig. 2. This representative gel was run with 13-cm-long IPGphor isoelectric focusing gels for optimum resolution. The relative position of the main muscle protein spots represented in this expression profile agrees with published results and international databanks [20–22]. Major muscle protein spots corresponded to albumin (pI 5.8; 70 kDa), abundant glycolytic enzymes such as enolase (pI 7.8; 47 kDa), aldolase (pI 8.6; 40 kDa), phosphoglycerate mutase (pI 9.1; 29 kDa) and triosephosphate isomerase (pI 7.1; 27 kDa), contractile elements such as tropomyosin 2 (pI 4.7; 33 kDa), slow troponin T (pI 6.1; 30 kDa), fast troponin T (pI 6.7; 31 kDa), slow myosin light chain 2 (pI 4.8; 18.9 kDa), fast myosin light chain 2 (pI 4.8; 19 kDa), fast myosin light chain 1 (pI 5.0; 21 kDa) and actin (pI 5.2; 42 kDa), key enzymes such as mitochondrial creatine kinase (pI 6.6; 43 kDa), cytosolic creatine kinase (pI 6.6; 43 kDa) and adenylate kinase (pI 9.1; 22 kDa), as well as the small heat shock protein HSP-B5/crystallin (pI 6.8; 20 kDa) and the cytoplasmic hemoprotein myoglobin (pI 6.7; 17 kDa).

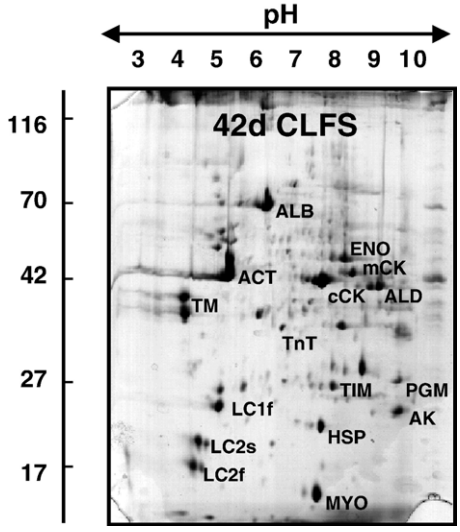

Fig. 2. Two-dimensional gel electrophoretic analysis of chronic low-frequency stimulated fast skeletal muscle. Shown is a representative silver-stained gel of 42 days chronic low-frequency (10 Hz) electro-stimulated fast muscle. Major spots are labeled with the name of their respective protein species: ACT, actin; AK, adenylate kinase; ALB, albumin; ALD, aldolase; cCK, cytosolic creatine kinase; ENO, enolase; HSP, heat shock protein (HSP-B5/αB-crystalline); LC, myosin light chain; mCK, mitochondrial creatine kinase; MYO, myoglobin; TIM, triosephosphate isomerase; TM, tropomyosin; TnT, troponin T; and PGM, phosphoglycerate mutase. The pH-values of the first dimension gel system and molecular mass standards (in kDa) of the second dimension are indicated on the top and on the left of the panels, respectively.

3.3. Comparative two-dimensional gel electrophoretic analysis of stimulated fast muscle

Although the combination of the isoelectric point and the molecular mass is relatively unique, in order to unequivocally identify the specific protein species present in a gel spot, in proteomics approaches the protein spots are usually directly sequenced, immuno-decorated or analysed by mass spectrometry. In order to reduce potential analytic errors due to gel-to-gel variations in this comparative study, we have placed the first-dimensional focused gel strips from both the unstimulated control sample and the conditioned specimen next to each other on the same second-dimension gel. To accommodate both isoelectric focusing strips on the same slab gel, a reduced length of 7 cm was used. A shorter first-dimension gel causes a reduced resolution and thereby potentially a loss in proper separation of minor muscle proteins. However, the overall spot pattern is relatively comparable between both types of first-dimensional gels (Fig. 2 versus Fig. 3), allowing a direct mass spectrometric analysis of protein spots from control and stimulated samples separated on the same polyacrylamide slab gel (Fig. 3A, B).

Using Image Master 2D Platinum analysis software, the two-dimensional spot pattern of Fig. 3A and B, representing an unstimulated control sample and a 42-day electro-stimulated specimen, respectively, revealed drastic changes in the expression levels of distinct protein spots. A total of 596 spots were detected and the densitometric analysis was performed with 6 separate gels from normal versus stimulated muscles. Interesting candidates were labeled as spots 1 to 21 and the excised spots were then analysed by mass spectrometry.

3.4. Mass spectrometry-based proteomics analysis of stimulated fast muscle

Protein spots with a marked reduction or increase in density were digested with trypsin and the generated peptide fragments analysed by mass spectrometry. Table 1 summarises the information gained from this proteomics approach. Sixteen protein species were found to exhibit changes in the 1- to 6-fold range, of which 15 could be clearly identified as distinct muscle proteins. Another five protein spots with unchanged abundance were identified for control purposes. Table 1 gives information on the spot number of proteins with a changed density, so that they can be correlated to the

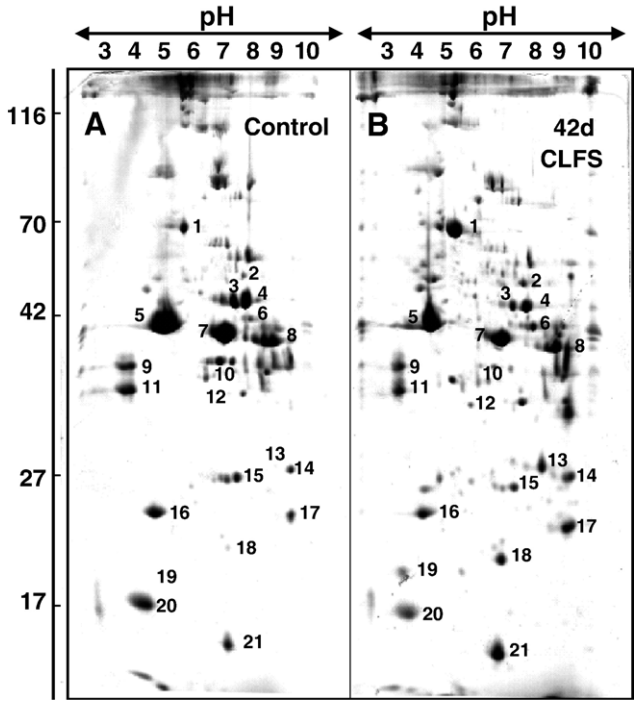

Fig. 3. Comparative two-dimensional gel electrophoretic analysis of total muscle extracts following chronic low-frequency stimulation. Shown are Coomassie-stained 2D gels of total protein extracts from unstimulated (A) and 42 days chronic low-frequency (10 Hz) electro-stimulated (B) skeletal muscle. The first-dimensional focused gel strips from both the unstimulated control sample and the conditioned specimen were placed next to each other on the same second-dimension gel in order to reduce potential analytic errors due to gel-to-gel variations in this comparative study. To accommodate both isoelectric focusing strips on the same slab gel, a reduced length of 7 cm was employed. Protein spots with a drastic change in expression level were identified by mass spectrometry. The numbering of spots correlates to the listing of identified protein species in Table 1. The pH-values of the first dimension gel system and molecular mass standards (in kDa) of the second dimension are indicated on the top and on the left of the panels, respectively.

numbering in Fig. 3. The table lists the percentage coverage of the identified peptide fragments with respect to the total primary protein sequence, the isoelectric point of the identified protein, its apparent molecular mass and the fold change in density.

As can be seen in Table 1, the extracellular fatty acid transport protein albumin (spot 1) and the mitochondrial ATP synthase (spot 2) exhibited an approximate 3.8-fold and 3.5-fold increase in abundance, respectively. In contrast, glycolytic enzymes such as the two enzymes forming the enolase complex (spots 3 and 4) and aldolase (spot 8) showed a decreased expression. The enzymes phosphoglycerate mutase (spot 14) and triosephosphate isomerase (spot 15) did not change their abundance following 42 days of electro-stimulation. The densitometric analysis of adenylate kinase (spot 17) revealed a small increase. Concomitant changes were observed with marker elements of the contractile apparatus. While the fast isoforms of troponin T (spot 10) and myosin light chain 2 (spot 20) were drastically down-regulated, their slow counterparts (spot 12 and 19) were up-regulated. Tropomyosin 2 (spot 9) was slightly reduced. The density of actin (spot 5), the fast myosin light chain 1 (spot 16) and alpha tropomyosin (spot 11) was not changed during the fast-to-slow transformation (Fig. 3). Interestingly, mitochondrial creatine kinase (spot 6) expression increased while the cytosolic isoform (spot 7) of this key muscle enzyme decreased. The expression of the small heat shock protein HSP-B5 (spot 18), often referred to as αB-crystallin, and the oxygen carrier protein myoglobin (spot 21) were both increased 2-fold following chronic low-frequency stimulation. An unidentified protein (spot 13) showed a dramatically increased density in conditioned fibres, but mass spectrometry of its peptide fragments failed to give reliable data for proper identification.

To document the reliability of the comparative densitometric analysis of two-dimensional spot patterns and to confirm the protein identification via mass spectrometry-based proteomics, we picked two representative proteins of the group of up-regulated muscle components. For internal standardisation of one-dimensional blots, Fig. 4A–D shows the immuno-decoration of established markers of fibre

Table 1
Muscle proteins with a changed expression level following chronic electro-stimulation as identified by mass spectrometry-based proteomic

Spot number of identified protein	Protein information	Coverage (%)	pI	Mass (kDa)	Up/Down Fold +/−
1. Albumin	gi29653363	21.7	5.8	70.89	+3.76
2. ATP synthase, mitochondrial F1 complex	gi6680748	21.3	9.3	59.85	+3.51
3. Beta Enolase	gi20141354 gi109215	36.4	7.8	47.39	−1.92
4. Phosphopyruvate Hydratase	gi109215	18	8.4	47.19	−1.46
6. Creatine Kinase, mitochondrial	gi3647324	27.4	8.9	47.90	+3.30
7. Creatine Kinase, cytosolic	gi66920	27.3	6.6	43.32	−1.35
8. Aldolase	gi13096354	52.1	8.6	39.65	−1.29
9. Tropomyosin 2	gi207497	29.9	4.7	32.93	−1.40
10. Troponin T Fast Isoform	gi2144841	21.4	6.7	31.41	−5.21
12. Troponin T Slow Isoform	gi339783	27.9	6.1	30.08	>+6.0
13. Unknown	Unidentified	–	–	–	+4.81
17. Adenylate Kinase	gi66933	14.9	9.1	21.73	+1.38
18. HSP-B5/Crystallin	gi1177579	28	6.8	20.09	+1.99
19. Myosin Light Chain 2, slow	gi47523262	51.8	4.8	18.9	+4.81
20. Myosin Light Chain 2, fast	gi127176	35.3	4.8	19.14	−3.09
21. Myoglobin	gi127691	56.2	6.7	17.07	+1.91

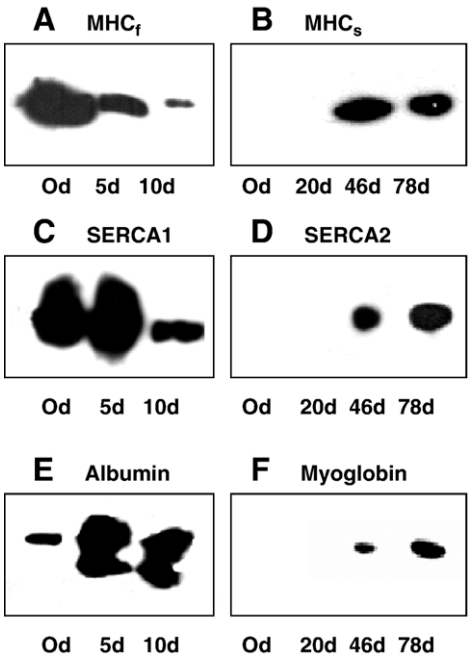

Fig. 4. Immunoblot analysis of albumin, myoglobin and muscle markers following chronic low-frequency stimulation. Shown are 1D immunoblots labeled with antibodies to the fast myosin heavy chain (MHC_f, A), the slow myosin heavy chain (MHC_s, B), the fast sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA1, C), the slow sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA2, D), albumin (E) and myoglobin (F). Lanes 1 to 7 in panels A and B, C and D, and E and F represent microsomal preparations from unstimulated and 5 days, 10 days, 20 days, 46 days and 78 days chronic low-frequency (10 Hz) electro-stimulated skeletal muscle fibres.

transformation. The fast and slow isoforms of the myosin heavy chain and the sarcoplasmic reticulum Ca^{2+}-pump were shown for both proteins to exhibit a switch in expression following chronic electro-stimulation. In analogy, the two representative proteins identified by proteomics showed a drastically changed expression pattern when analysed by immunoblotting. Albumin exhibited an increase in 5 days and 10 days chronic low-frequency stimulated muscle specimens (Fig. 4E), which agrees with the 4-fold increase determined by proteomics (Fig. 3, Table 1). In contrast, the immuno-decoration signal for myoglobin increased after 46 days and 78 days of muscle conditioning (Fig. 4F). This correlates well with the 2-fold increase of myoglobin expression as shown by proteomics analysis (Fig. 3; Table 1).

4. Discussion

Two-dimensional gel electrophoresis represents an extremely powerful tool for the separation of proteins based on the relatively unique combination of their molecular masses and isoelectric points. Here, we have employed mass spectrometry-based proteomics technology for the identification of two-dimensionally separated muscle protein spots. The analysis has identified several protein species that are differentially expressed following chronic low-frequency stimulation. During the optimization of the gel electrophoretic procedure for the analysis of skeletal muscle extracts, we could confirm that certain classes of proteins are more difficult to resolve on two-dimensional gels as compared to soluble proteins. This includes very acidic proteins, integral proteins and components of very high molecular mass, as well as low-abundance proteins. In addition, the presence of proteins with a high density can distort the two-dimensional separation pattern and potentially contaminate other protein spots. Proteolytic degradation products of high-molecular-mass proteins may also complicate the analysis of the gel image. However, despite these technical limitations, we were able to perform a successful proteome analysis of transformed fast muscle fibres.

Previous biochemical and cell biological investigations have established that chronic electro-stimulation protocols induce a variety of distinct cellular changes. This includes trans-differentiation resulting in a decreased caliber and appearance of transformed fibres [6], the early degeneration of fast glycolytic fibres [42], and the activation of the organotypic muscle stem cell pool, the satellite cells [2]. Metabolically, conditioned muscles exhibit an elevation of their aerobic-oxidative capacity [11,17]. Physiologically, an increase in the time to peak twitch tension and half-relaxation time documents the transition of stimulated fibres to a stage of improved resistant to fatigue [7]. Complementary to these well-established characteristics of conditioned skeletal muscles, our proteomics analysis of the fast-to-slow fibre transition process has identified changes in a variety of key muscle components. This included an up-regulation of the interstitial component albumin, the F1 complex of the mitochondrial ATP synthase, the heat shock protein αB-crystalline/HSP-B5, the oxygen carrier myoglobin, the mitochondrial creatine kinase isozyme and slow contractile elements such as the LC2s isoform of the myosin light chain.

The observed changes indicate that the conversion into fatigue-resistant red fibres depends on the optimum utilization of free fatty acids via albumin transportation, an increase in mitochondrial enzymes and a rearrangement of the creatine kinase isozyme pattern for enhanced mitochondrial activity, an increased availability of oxygen for aerobic metabolism via myoglobin transport, the conversion of the contractile apparatus to isoforms with slower twitch characteristics, and the up-regulation of chaperone-like proteins for stabilising myofibrillar proteins during the fast-to-slow transition process. While the down-regulation of certain glycolytic enzymes, such as enolase and aldolase, appears to play an integral part during muscle transformation following sustained contractile activity, the metabolic enzyme adenylate kinase and its function in ATP replenishment is enhanced.

Albumin represents the major plasma protein that exhibits a high degree of multi-functionality. In skeletal muscle, albumin serves as a temporary amino acid storage site,

maintains osmotic pressure and acts as a transporter for free fatty acids [43]. Since the majority of serum albumin exchanges with the extravascular compartment of adipose tissue and muscles [44], the identified albumin is presumed to be of interstitial localization. In agreement with an earlier study by Heilig and Pette [45], our proteomics approach showed that chronic electro-stimulation clearly up-regulates the concentration of extravascular albumin in transformed muscle fibres. It is not known whether this increase in albumin is due to increased expression or possibly because of a greater capacity of stimulated fibres in retaining albumin. This could mean that there is a potential increase of the extracellular space in conditioned muscles. With respect to fatty acid utilization, terminal substrate oxidation during sustained contractile activity may be limited by the availability and optimum transportation of free fatty acids. Both the extracellular fatty acid transportation via albumin and the intracellular fatty acid transportation due to the FABP protein [16] seem to be important factors to augment the aerobic-oxidative metabolic potential of slower contracting fibres.

Chronic electro-stimulation induces drastic increases in mitochondrial enzyme activities of terminal substrate oxidation [11,17]. The drastic increase in the F1 complex of the mitochondrial ATP synthase, as shown here by proteomics, agrees with this finding. The ATP synthase is the main enzyme responsible for ATP formation in aerobic cells [46]. Stimulation-induced changes also include an enhanced mitochondrial creatine kinase activity [47]. However, overall creatine kinase activity is lower in slow-twitching fibres as compared to fast muscles [48]. The densitometric analysis of unstimulated versus chronic electro-stimulated fibres agrees with these two findings and has demonstrated a concomitant increase in mitochondrial creatine kinase and a decrease in its extra-mitochondrial isozyme. The stimulation-induced rearrangement of the creatine kinase isozyme pattern reflects the central importance of the mitochondrial isoform of this enzyme in the creatine kinase shuttle system [49]. Phosphocreatine acts as a metabolic ATP-buffering system in skeletal muscles. Increased levels of mitochondrial creatine kinase correlate with the enhanced aerobic-oxidative potential of the transformed fibre population [50]. The proper transfer of high-energy phosphate between the extra-mitochondrial compartment and the intra-mitochondrial domains seems to be an integral part of slow fibres, since maximally exerting muscles have to efficiently regenerate ATP units. The increased levels of adenylate kinase stresses the importance of proper ATP replenishment for intermediary metabolic functions. Besides substrate-level phosphorylation and oxidative phosphorylation, the conversion of AMP to ADP by this enzyme, and subsequent conversion to ATP, appears to play a more pronounced role in slow as compared to fast muscles.

The fast-to-slow transition process induces a shift to aerobic–oxidative metabolism and thereby an increase in cellular oxygen consumption. In agreement with this is the drastic elevation of myoglobin concentration in transformed fibres. Myoglobin represents a cytoplasmic hemoprotein that reversibly binds oxygen via its porphyrin ring–iron ion complex and acts as an oxygen-storage protein in oxidative fibres [51]. During increased muscle activity, the buffering of the intracellular oxygen concentration and facilitation of oxygen diffusion by myoglobin concurs with our finding of higher myoglobin levels in chronic stimulated fibres. Indeed, myoglobin may be one of the limiting factors of aerobic-oxidative metabolism in slow-twitching muscles [16]. The alterations in energy metabolism are also clearly reflected by the down-regulation of key glycolytic enzymes such as enolase and aldolase. While aldolase catalyzes the cleavage of fructose-1,6-biphosphate to form two trios molecules, enolase is involved in the dehydration of 2-phosphoglycerate to phosphoenolpyruvate [52]. In addition, our immunoblotting survey showed a reduction in phosphofructokinase, the glycolytic enzyme that catalyses the conversion of fructose-6-phosphate to fructose-1,6-biphosphate. The findings from our proteomics screening of transformed muscle fibres agree with the extensive analysis of metabolic enzyme activity following chronic electro-stimulation [6,53]. The change in the glycolytic enzyme complement suggests a drastic metabolic change towards a reduced capacity of glycolytic substrate phosphorylation.

The mass spectrometry-based proteomics analysis of chronic low-frequency stimulated fast fibres, presented here, confirms the conversion of the contractile apparatus to isoforms with slower twitch characteristics [8,14,54]. Mammalian skeletal muscle represents an extremely heterogeneous type of tissue with a great variety of different fibre types that are capable of responding to altered functional demands. In analogy to the well-established switch between fast and slow myosin heavy chain isoforms following stimulation [14], as shown here by immunoblotting, the expression profile of myosin light chains is also affected during fibre transitions [54,55]. Our densitometric analysis of distinct two-dimensional protein spots, that represent the fast and slow isoform of myosin light chain 2 and troponin T, showed a dramatic switch to a slower molecular phenotype of the transformed contractile apparatus. In this context it is important to stress, that the re-modeling of the myosin assembly and its regulatory elements following extended periods of contractile activity is accompanied by the up-regulation of chaperone-like proteins [56]. Here, we could demonstrate, by proteomics, a drastic increase in the levels of the small heat shock protein αB-crystallin/HSP-B5, which is in agreement with a previous cell biological and immunoblotting study [57]. Oxidative stress caused by continuous contractile activity appears to trigger a clear response by heat shock proteins for stabilising myofibrillar proteins during the fast-to-slow transition process.

In conclusion, this report showed that mass spectrometry-based proteomics technology could be successfully applied to the biochemical analysis of major changes in the fast muscle protein complement following sustained contractile activity. Fig. 5 summarises the findings from the

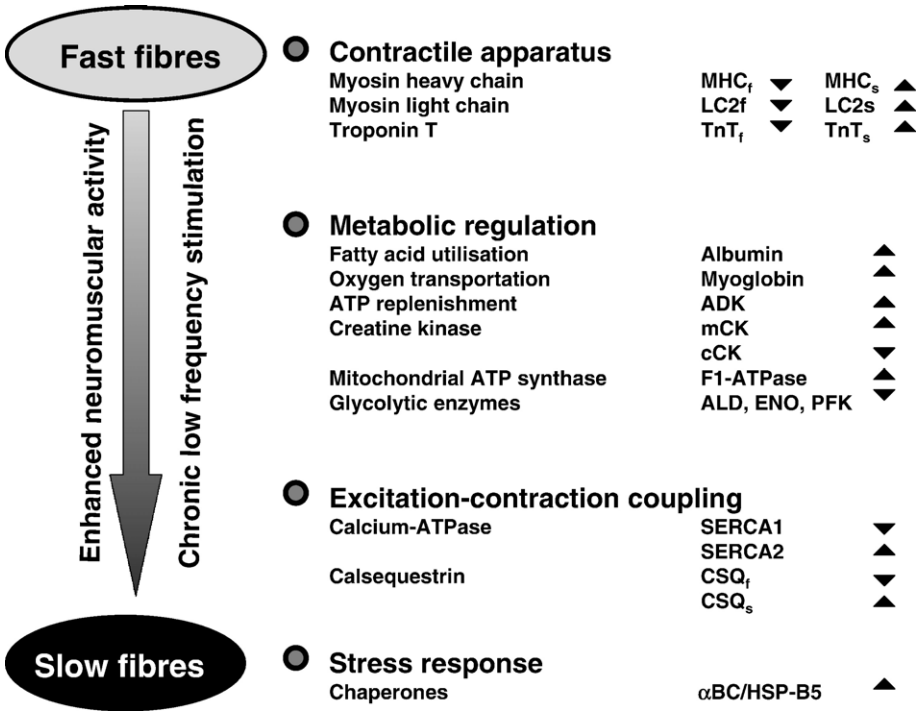

Fig. 5. Overview of proteomics profile of chronic low-frequency stimulated fast muscle. Shown are key muscle proteins involved in contraction, metabolism, excitation–contraction coupling and stress response that have been identified by mass spectrometry and immunoblotting. The drastic increase or decrease in these proteins reflects the molecular adaptation of transformed muscle fibres following chronic low-frequency electro-stimulation.

proteomics screening of chronic low-frequency stimulated fibres. It shows that the stimulation-induced transition to aerobic-oxidative metabolism is accompanied by major changes in the abundance and/or isoform expression pattern of various enzymes and carrier molecules of energy metabolism, the contractile machinery and its regulatory elements, the excitation–contraction coupling and muscle relaxation apparatus and chaperone-like heat shock proteins. Our proteomics findings of the fast-to-slow transition process agrees with previous studies which have established that electro-stimulation induces, in addition to the degeneration of fast glycolytic fibres, and the activation of muscle precursor cells, the trans-differentiation of the fast muscle phenotype into a slower-contracting cell type. Interestingly, the drastic up-regulation of metabolic factors such as the fatty acid-transporter albumin, the oxygen-carrier myoglobin and the mitochondrial creatine kinase suggest that the expression levels of these key muscle proteins might represent limiting factors in the shift to aerobic–oxidative metabolism. In this respect, the mass spectrometric analysis of two-dimensionally separated muscle proteins could be shown to be able to provide important biochemical data to better understand complex physiological processes.

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