Proteomic Analysis of Diurnal Variation in Human Skeletal Muscle Performance

By

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Abstract

Phenotyping of human muscle based on its profile of myosin heavy chain isoforms is commonly used to help understand changes in muscle function. However, in many instances, measureable changes in force output or contractility occur in the absence of any change in myosin heavy chain profile. Therefore, more sophisticated analysis is required. Proteomic techniques including 2-dimensional gel electrophoresis, high-performance liquid chromatography and peptide mass spectrometry can be used to investigate changes in the abundance of hundreds of proteins simultaneously. To date, such techniques have not been used to specifically characterise the human myofibrillar proteome, or study how the myofibrillar proteome relates to muscle outputs such as peak isometric force or the velocity of contraction. This thesis presents a series of studies that develop proteomic techniques for the analysis of myofibrillar proteins as well as optimisation of techniques for measuring the range of muscle output from isometric through to velocity maximum of the human knee extensor muscles *in vivo*. After optimisation, the proteomic and muscle function measurement were employed to study diurnal variation. Time-of-day differences in sports performance and muscle function are widely reported, and typically, performance is ~10 % greater in the evening compared to the morning. This is consistent with our result in Chapter 3; we investigated this chapter by conducting a battery of muscle performance tests in a population of well-familiarised participants. Our data show that RFD exhibits the greatest diurnal variation (18 %) followed by isometric force (10.2 %). The diurnal variation in IKD data was less robust (range 8.1 - 9.8 %), which may have been due to the lesser precision of this technique compared to MVC and RFD. Therefore MVC and RFD were used in the final study. In final study, this thesis reports significantly ($P<0.05$) greater peak isometric force (11 %) and rate of force development (16 %) of knee extensor muscles of young strength-trained males in the evening compared to morning. Proteomic analysis of biopsy samples of the vastus lateralis profiled more than 100 myofibrillar protein species and detected 8 significant differences in protein abundance between morning and evening samples. The greatest difference was in the abundance of the slow isoform of myosin binding protein C (MyBPC1), which is known to modulate the activity of actin-bound myosin ATPases. MyBPC1 was resolved to 6 species; therefore the difference in abundance of one species reported here likely represents a change in post-translational modification. Therefore, this thesis provides associational evidence that post-translational modification of MyBPC1 contributes to the diurnal variation in muscle function.

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List of Abbreviations, Acronyms and Symbols

°.S-1	Degree per second
2DGE	Two dimensional gel electrophoresis
ACN	Acetonitrile
ADP	Adenosine diphosphate
ANOVA	Analysis of variance
APS	Ammonium persulfate
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
AV	Average power
BMAL1	Brain and muscle ARNT-like 1
BSA	Bovine serum albumin
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
CK	Creatine kinase
CLOCK	Circadian locomotor output cycles kaput
CO^2	Carbon dioxide
CRH	Corticotrophn releasing hormone
CSA	Cross sectional area
CV	Coefficient variation
Da	Dalton
ddH^2O	Distilled-deionised water
dH^2O	Distilled water
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EDL	Extensor digitorium longus
EDTA	Ethylenediaminetetraacetic acid
EM	Electron multiplier
EMG	Electromyography
ESI	Electrospray ionization
FA	Formic acid
FDR	False discovery rate
FG	Fast glycolytic
FOG	Fast oxidative glycolytic
FTICR	Fourier transform ion cyclotron resonance
f-v	Force-velocity
HCR	High capacity runner
HFABP	Heart-type fatty acid binding protein
HPLC	High-performance liquid chromatography
IAA	Iodoacetamide
IEF	Isoelectric focusing
IKD	Isokinetic dynamometry

IPG	Immobilized pH gradient
kDa	Kilo Dalton
LC	Liquid chromatography
LCR	Low capacity runner
LIT	Linear ion trap
LMW	Low molecular weight
m/z	mass-over-charge
MALDI	Matrix-assisted laser desorption/ionization
MOWSE	Molecular weight search
MRM	Multiple reaction monitoring
mRNA	Messenger RNA
MS	Mass spectrometry
MVC	Maximal voluntary contractions
MW	Molecular weight
MyHC	Myosin heavy chain
MyLC	Myosin light chain
N:NIH	Normalized/National Institutes of Health
PAGE	Polyacrylamide gel electrophoresis
PAR-Q	Physical readiness questionnaire
PDF	Portable Document Format (Adobe Acrobat)
pI	Isoelectric point
Pi	Inorganic phosphate
POMS	Profile of mood states
PT	Peak torque
PTM	Post translation modifications
QTM	Qualisys track manager
Rad	Radius
RFD	Rate of force development
RPE	Rate of perceived exertion
RT	Room temperature
RTU	Ready to use
SCN	Suprachiasmatic nuclei
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SO	Slow oxidative
SPSS	Statistical package for social sciences
SRM	Selective reaction monitoring
TC	Thermal comfort
TFA	Trifluoroaceic
T_m	Muscle temperature
TOF	Time of flight
T_{rec}	Rectal temperature
TRIS	Tris(Hydroxymethyl)aminomethane
TSH	Thyroid stimulating hormone

T_{sk}	Skin temperature
v/v	volume/volume
w/v	weight/volume
xg	*gravity*
YOTU	Young old trained untrained
ω	Angular speed

INTRODUCTION TO THESIS

This thesis aims to combine new techniques in muscle proteomics with optimised measures of human muscle force production *in vivo* and use diurnal variation as a model. In doing so, we hope to bring new understanding regarding the role of muscle proteins in underpinning muscle force production.

Proteomics is the study of proteins using high-throughput techniques and relies on a combination of genomics, mass spectrometry and protein biochemistry. The human genome contains of approximately 20,000 genes that are transcribed into mRNA and then can be translated in to proteins. Researchers can test hypotheses regarding individual mRNA or proteins using techniques such as Northern blots (for mRNA expression) or Western blots (for protein abundance) (Wackerhage, 2014). This

'reductionist' approach, where biology is reduced to individual questions, has been the mainstay of biological research. However, data arising from hypothesis-led studies clearly indicates that biology is not organised or controlled by isolated events. Rather, biological systems are organised as complex networks and multiple interactions occur to bring about physiological changes. Therefore, more comprehensive (e.g. '-omic') analysis techniques are required in order to advance our understanding of biological systems.

The proteome is cell-specific and dynamic, responding on a minute-by-minute basis to changes in cell environment. Consequently, the proteome reflects the particular stage of development and current environmental condition the cell finds itself

experiencing. With regard exercise proteomics, Burniston and Hoffman, (2011) report proteomic studies have mostly focused on striated muscle responses to endurance training, which are associated with health benefits underpinned by improvements in aerobic capacity. However, few studies have specifically used proteomic techniques to investigate the human myofibrillar sub-proteome, which will be the main focus of the work conducted in this thesis. Although limited in number of proteins, the myofibrillar sub-proteome is complex because each myofibrillar protein can be expressed as different isoforms and splice-variants, and many also undergo post-translational modifications, such as phosphorylation. Therefore, this thesis investigates different proteomic approaches for capturing this complex information so that it can be correlated with changes in muscle function.

Traditionally, the functional characteristics of a muscle have been related to the size and composition of its myofibres. Human muscle consists of 3 principal fibre types, type I, type IIa and type IIx and studies on the contractile properties of single human muscle fibres in vitro demonstrate each fibre type exhibits a different force-velocity profile (Larsson et al., 1997). While the peak isometric force produced by each fibre type is relatively similar, the maximal velocity of shortening differs markedly; the maximum velocity of shortening of IIa fibres is ~4-times greater than slow type I fibres, and the maximal velocity of shortening of IIx fibres is approximately double that of fibres that express myosin heavy chain IIa. Therefore, differences in the relative proportions of each fibre type can alter the gross force-velocity profile of the muscle. This has direct consequences on power output and athletic performance, but many fibres are hybrids of two fibre types and so attempts to explain changes in

muscle function based on changes to the proportion of type I, type IIa and type IIx fibres becomes difficult.

Muscle fibres are designated as begin type I, IIa or IIx based on their whether the express myosin heavy chain (MyHC) isoform I (protein code MYH7), MyHC IIa (protein code MYH2) or MyHC IIx (protein code MYH1). Each myofibre nucleus (i.e. myonucleus) contains the entire genetic information for Type I, IIa and IIx as well as all other related proteins, but the myofibrillar genes are selectively expressed based on the activity pattern of muscle use. Typically muscle of untrained individuals exhibits relative proportions of 40 % type I, 45 % type IIa and 15 % type IIx (e.g. Holloway et al., 2009). Resistance exercise training is associated with hypertrophy of each myofibre type and, in addition, results in a shift toward greater number of IIa fibres such that the contribution of type IIa fibres to gross muscle performance becomes greater (Widrick et al., 2002). In contrast, endurance training is associated with selective hypertrophy of type I fibres (Gollnick et al., 1973).

Phenotyping of muscle based on its relative expression of MyHC isoforms has provided important insights regarding muscle function and plasticity, but such characterisation is relatively simplistic. Skeletal muscle peak force is proportional to physiological cross sectional area (CSA) and myosin heavy chain isoform (Pearson et al., 2006), but muscle power output is a complex variable that may be influenced by numerous qualitative changes in other myofibrillar proteins. It is important to appreciate the diverse heterogeneity of human muscle fibres, which comprise different myosin heavy chains, as well as many other ancillary proteins (Schiaffino

& Reggiani, 1996). Indeed, studies (Cristea et al., 2008; McGuigan et al., 2003) report significant changes in muscle function induced by squat jumping (Malisoux et al., 2006) and sprint training (Holloway et al., 2009) in the absence of changes in MyHC content. Changes in isoform expression, splice variation or post-translational modification (PTM) of MyHC and other myofibrillar proteins, such as troponins and myosin light chains (MyLC), can alter contractile characteristics in the absence of changes in MyHC. Therefore, it is important to investigate the entire myofibrillar sub-proteome in order to gain further understanding of the proteins that dictate muscle function.

Recent technological developments including robust two-dimensional gel electrophoresis (2DGE), high-performance liquid chromatography (HPLC) and mass spectrometry (MS) enable comprehensive investigations and inductive 'discovery science' approaches at the protein level and have recently been applied to questions of muscle adaptation. This thesis begins by investigating the type of proteomic technique that is best suited to analysis of myofibrillar proteins. Specifically our aim was to advance the study of changes in myofibrillar proteins beyond MyHC in order to more accurately understand the mechanisms that underpin the force-velocity (f-v) characteristics of human muscle. After investigating different proteomic techniques our next aim was to establish techniques for robust measurement of human muscle performance *in vivo*. Finally, we attempted to bring these two areas of research (i.e. muscle proteomics and muscle function) together to investigate proteins that correlate with diurnal changes in muscle force production.

The power output of human muscle and human sports performance exhibit diurnal variation (Drust et al., 2005; Reilly and Waterhouse, 2009). Diurnal variation in muscle force and sports performance in young healthy individuals is widely reported but the mechanism underpinning this phenomenon are not yet understood. Muscle force production is least between the hours of 06.00 – 08.00 in the morning and greatest from 16.00 – 18.00 in the afternoon. Table 1 summaries research studies that have investigated the effects of diurnal variation on muscle performance (see Table 1). Regardless of the dependant variable measured (e.g. maximum isometric force, peak torque etc.) muscle performance is consistently better during the evening as opposed to the morning. The magnitude of the change in muscle output from morning to evening is reported to range between 5-20%. The underlying cause of this variation has been attributed to variations in metabolism, circulating levels of hormones, and differences in core or local muscle temperature, all of which have shown patterns of circadian rhythms and may therefore affect performance (Araujo et al., 2011; Racinais & Oksa, 2010).

Table 1. Studies reporting diurnal variation in muscle output

Test	Author	Difference (%)
MVC		
	Martin et.al (1999)	8.9%
	Racinais et al. (2005)	9.2%
	Reilly (2007).	9.0%
	Edwards et al. (2013)	12.6%
IKD		
60	Reilly (2007).	6.2%
90		4.6%
180		8.2%
60	Edwards et al. (2013)	9.6%
240		10.6%
Anaerobic		
Broad jump	Reilly (2007).	3.4%
Stair run		2.1%
Flight time		2.4%

Fluctuations in force production co-occur alongside biological rhythms in core temperature but we have found diurnal variation in muscle performance is not entirely explained by differences in muscle temperature. For example, passive heating in the morning to stimulate the warmer afternoon muscle temperature did not bring about similar elevation in muscle performance. Similarly, passive cooling in the afternoon to replicate the cooler muscle temperature of the morning was not associated with the decrease in performance. Thus intrinsic differences occur in the ability of skeletal muscle to produce force during the course of the day. The parameters of muscle performance (i.e. maximum force and maximum speed) are determined by collections of contractile proteins. Differences in genetic background, habitual activity or training status affect the relative amounts of each muscle protein and are one of the main reasons for the broadly different physique and physical performance of sprinters compared to marathon runners. The same contractile proteins can also be rapidly modified to cope with short-term changes in demand. This latter mechanism contributes to the positive effect of warming up prior to strenuous exercise, and similar mechanisms could able responsible for the greater output of skeletal muscle in the afternoon compared to the morning. Therefore further investigation involving proteomic analysis of muscle biopsies needs to be done.

Aim and objectives of the thesis

The overall aim of this thesis was to combine proteomic analysis of muscle biopsy samples with measurements of muscle performance in humans. Because the proteome is what defines a cell (or tissue) and dictates its functional properties we reasoned that changes in muscle function must be underpinned by changes in the muscle proteome. More specifically, changes in muscle force production will be underpinned by changes to the myofibrillar proteins and we used diurnal variation as a model. To achieve our overall aim we conducted a series of experiments to establish and optimise techniques for proteomic analysis of muscle biopsy samples and techniques for the analysis of human muscle function in vivo. The purpose of this chapter is to summarise the findings from each of the foregoing experimental chapters and detail how the main aims of this thesis were met. A secondary purpose of this chapter is to provide recommendations for further research based upon this body of work.

The specific aims of this thesis were:

1. To establish new proteomic techniques for the analysis of the myofibrillar sub-proteome of human muscle

2. i) To investigate whether measurements of isolated knee extension can be used to predict performance during more complex sport-related movements (i.e. vertical jump performance)

ii) To investigate the reliability of isolated knee extension measurements encompassing the entire range of the human force-velocity relationship in vivo

4. To determine which method of measurement or which feature of knee extensor performance exhibits the greatest diurnal variation

5. To discover diurnal differences in the myofibrillar sub-proteome of human vastus lateralis and investigate whether these correlate with differences in muscle function.

Chapter 1

Analysis of Animal and Human Skeletal Muscle Using Traditional and Proteomic Analysis

1.1 INTRODUCTION

Chapter background

Skeletal muscle

Skeletal muscle represents ~40 % of body weight in healthy adults and is important in metabolism, thermogenesis and locomotion. The ability of muscle to produce force underpins all sporting performance. Individual skeletal muscle fibres have two main protein structures that make up the myofilament and incorporate actin (thin filament) and myosin (thick filament). These proteins are assembled in a specific manner to form the basic repeating functional unit of a myofibril – the sarcomere. The sarcomere consists of thick myosin filaments that are anchored to a protein sheet (the M-band) and overlap with the thin actin filaments. This arrangement gives the skeletal muscle its characteristic striated appearance. The sarcomeres (Figure 1) are arranged in sequence along the myofibrils, and it is the interaction between actin and myosin within each sarcomere that enables a muscle to contract.

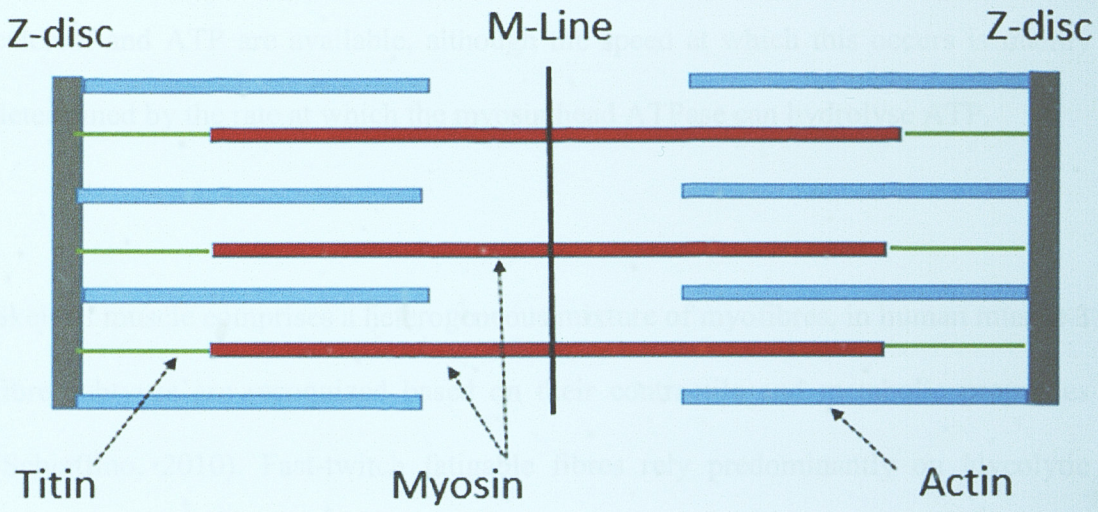

Figure 1. Figure of sarcomere component.

The myosin molecule is made up of 6 polypeptides – 2 heavy chains and 4 light chains. The myosin heavy chain contains a myosin head that binds to the actin molecule and forms the basis for muscle contraction. The myosin head region serves as the binding site for adenosine triphospate (ATP) and contains the enzyme adenosine triphosphatase (ATPase), which enables the hydrolysis of ATP into adenosine diphosphate (ADP) and inorganic phosphate (Pi), thus providing the energy for muscle contraction (Scott et al. 2001). The thin actin filament consists of 2 regulatory proteins – troponin and tropomyosin. Under resting conditions, tropomyosin binds to the active sites on actin, and prevents actin and myosin from binding together.

The stimulus for muscle contraction is an action potential, which is conducted along the sarcolemma. Depolarisation of the fibre leads to the release of calcium from the sarcoplasmic reticulum and the activation of the cross bridge cycle that results is muscle contraction and force generation. Cross-bridge cycling continues as long as calcium and ATP are available, although the speed at which this occurs is mainly determined by the rate at which the myosin head ATPase can hydrolyse ATP.

Skeletal muscle comprises a heterogeneous mixture of myofibres, in human muscle 3 fibre subtypes are recognized based on their contractile and metabolic properties (Schiaffino, 2010). Fast-twitch fatigable fibres rely predominantly on glycolytic metabolism and are designated FG (fast glycolytic), whereas, fast-twitch fatigue-resistant and slow-twitch fibres have relatively greater mitochondrial content and are designated FOG (fast oxidative glycolytic) and SO (slow oxidative), respectively.