Biomarkers, 2010; 15(3): 249–258

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ORIGINAL ARTICLE

Potential biomarkers of muscle injury after eccentric exercise

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Abstract

Proteomics was utilized to identify novel potential plasma biomarkers of exercise-induced muscle injury. Muscle injury was induced in nine human volunteers by eccentric upper extremity exercise. Liquid chromatography–mass spectrometry identified 30 peptides derived from nine proteins which showed significant change in abundance post-exercise. Four of these proteins, haemoglobin α chain, haemoglobin β chain, α1-antichymotrypsin (ACT) and plasma C-1 protease inhibitor (C1 Inh), met the criterion for inclusion based on changes in at least two distinct peptides. ACT and C1 Inh peptides peaked earlier post-exercise than creatine kinase, and thus appear to provide new information on muscle response to injury.

Keywords: *Exercise; α1-antichymotrypsin; C-1 protease inhibitor; proteomics*

Introduction

Elevated blood concentrations of normal intracellular myocyte constituents, such as creatine kinase (CK) and myoglobin, have served as biomarkers of muscle injury (Sorichter et al. 1999). The linkage of these markers or their pattern of appearance to specific injury aetiologies is incompletely defined. As a result, the interpretation of an elevated serum CK concentration is challenging in a patient in whom the aetiology of muscle injury is unclear. The CK elevation may have resulted from a self-limited process like the transient injury of recent exercise (Nicholson et al. 1985), underlying muscle disease (Jackson 2008) or drug-induced muscle injury (Bannwarth 2002, Guis et al. 2003). Additionally, an elevated CK does not indicate the age of the injury, or if the process is ongoing and thus exposing the patient to a risk of subsequent rhabdomyolysis (Veenstra et al. 1994). Thus, new biomarkers are needed to differentiate these important clinical scenarios.

Unaccustomed eccentric exercise of the upper extremities is a model of muscle injury that has been used widely and allows the onset of injury to be identified at a discrete point in time. Injuries related to eccentric exercise include mechanical disruption of muscle identifiable acutely on histopathology (Friden & Lieber 2001, Lieber et al. 2002), evidence of inflammation and oxidative stress within 24–48 h (Hirose et al. 2004) and characteristic changes in gene expression (Chen et al. 2003).

As a first step in a programme designed to discover biomarkers that can identify and differentiate the mechanism of muscle injury, the current study was designed to test the hypothesis that plasma proteomics could be used to identify potential biomarkers of muscle injury and adaptive response to eccentric exercise.

Methods

Subjects

Subjects were recruited for study by local advertising. To increase the likelihood that any biomarkers identified would be broadly applicable to eccentric exercise injury and responses, a diverse study cohort was recruited,

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(Received 08 October 2009; revised 19 November 2009; accepted 21 November 2009)

ISSN 1354-750X print/ISSN 1366-5804 online © 2010 Informa UK Ltd
DOI: 10.3109/13547500903502802

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including both healthy men and women and subjects covering a wide age range. In this way it was hoped that any potential biomarkers identified would have applicability in diverse patient populations. To include a representation of chronic disease, individuals with diabetes were also recruited. Diabetic subjects were required to be on stable medical regimens for at least a month prior to the study, but no medications were exclusionary. Healthy subjects were taking no prescription medications. Subjects were excluded if they had any history of muscle disease, or had significant abnormalities on screening blood chemistries, which included serum electrolytes, CK and thyroid-stimulating hormone concentrations. Subjects were also excluded if they regularly performed upper extremity resistance exercise, and participants were asked to refrain from any vigorous exercise during study participation. Subjects abstained from taking nutritional supplements, non-steroidal anti-inflammatory agents or over-the-counter medications during study participation. The protocol was reviewed and approved by the Institutional Review Board of The Los Angeles Biomedical Research Institute at Harbor-UCLA Medical Center, and all subjects gave informed consent prior to participation.

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Exercise stimulus

Subjects were instructed by an exercise trainer on performance of seated biceps curls (elbow flexion and extension) using a bench with a padded support positioning the upper arm at a 45° angle, and independent left and right lever bars of adjustable weight. The goal of the exercise session was to induce sufficient muscle injury to cause mild to moderate degrees of soreness and elevations in muscle markers in most or all subjects. Each participant's individual 8–10-repetition maximum (maximal weight that could be lifted and lowered with maintenance of good form only 8–10 times) was first estimated by minimal trial-and-error lifting. Following this, three sets of 10 curls at the determined weight were then performed using each arm individually, alternating sets between arms. Because capacity for the lowering (eccentric) phase of a task is higher than for the raising (concentric) phase it was expected that subjects would be able to complete the 30 lowering movements even though they would likely be unable to complete the corresponding number of lifting phases. Thus, to ensure the complete eccentric protocol could be completed, assistance was given with the lifting phase and/or the weight was adjusted downward during the exercise session as needed to allow the subject to complete the prescribed three-exercise sets. Subjects were coached to maintain correct form and to perform each lowering movement in a controlled fashion over a period of 3–4 s. Time between sets was limited to the time needed to reposition the subject for exercise of the other arm so that sessions were completed within a total time of 12–18 min. Blood was sampled at 1 h after completion of the exercise. Subjects then returned to the General Clinical Research Center (GCRC) on each of three successive days after the exercise session for additional blood sampling.

Muscle soreness

The occurrence of subjective muscle soreness was assessed at all study visits by use of a visual analogue scale (VAS). Subjects marked a 10 cm line whose left end was labelled 'no soreness' and right end 'maximum soreness' to indicate the soreness of their non-dominant arm. Numeric scores were assigned corresponding to the distance in centimetres, to the nearest centimetre, from the left end of the line.

Blood sampling

Venous blood was sampled from an antecubital vein by experienced phlebotomists in the GCRC at each of five time points: at the time of screening (2–7 days prior to exercise), and 1, 24, 48 and 72 h post-exercise. For the last three time points, sampling was accomplished within a window of ± 2 h of the indicated times. A total of 45 ml of blood was collected at the baseline, and 35 ml of blood at each of the subsequent time points.

For the proteomic analysis, blood samples were collected in EDTA tubes (Vacutainer; Becton Dickinson, Franklin Lakes, NJ, USA) and processed by immediate centrifugation with separation of plasma and plasma stored at -80°C for later analysis. Technical limitations precluded sample availability from all subjects at all time points. Subjects with adequate samples from baseline and at least three of the four subsequent time points were included in the proteomics cohort, comprising a total of 41 samples from nine subjects (9, 8, 8, 7 and 9 samples at baseline, 1 h, 24 h, 48 h and 72 h, respectively).

Clinical laboratory assays

Serum specimens from each sampling time were processed by a clinical laboratory using standard procedures for quantitation of electrolytes, creatinine and glucose, as well as several conventional markers of muscle injury. The latter included CK, myoglobin, aldolase, lactate dehydrogenase (LDH), alanine aminotransferase (ALT) and aspartate aminotransferase (AST).

Proteomic analysis

Proteomic analyses were performed in duplicate on all 41 available samples as previously described (Meng

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et al. 2007, Zhang et al. 2003, 2005). On a TECAN robot (Tecan, Männedorf, Switzerland), 25 µl plasma aliquots were processed in a 96-well plate format using an N-linked glycopeptide enrichment procedure (Zhang et al. 2003, 2005). N-linked glycoproteins were covalently coupled to hydrazide beads, washed, reduced by 8 mM TCEP (Pierce, Rockford, IL, USA) and then alkylated by 10 mM iodoacetamide (Sigma, St Louis, MO, USA). The proteins were digested using trypsin (Promega, Madison, WI, USA), and the resulting glycopeptides (while still covalently linked to the beads) were washed again to remove the non-glycopeptides. Finally, the glycopeptides were released from the beads using PNGase F (New England Biolabs, Ipswich, MA, USA). All 82 processed samples (41 in duplicate) were dried and then resuspended in 250 µl of 0.1 M acetic acid.

Processed samples (1 µl) were analysed by liquid chromatography–mass spectrometry (LC-MS) as previously described (Meng et al. 2007). Samples were loaded onto the reverse-phase capillary columns and then separated by a linear solvent gradient (solvent A: 0.1 M acetic acid; solvent B: 0.1 M acetic acid in 90% acetonitrile). As the peptides eluted from the capillary spraying column, their mass to charge (*m/z*) values and signal intensities were recorded on a LTQ-Fourier-transformed mass spectrometer (LTQ-FTMS; Thermo Electron, San Jose, CA, USA) in FT full-scan mode, which were later used for peptide quantitation. In parallel, three data-dependent ion trap MS/MS datasets were recorded, which were used to deduce the amino acid sequences of the studied peptides. Samples were processed and analysed in a randomized order to minimize systematic effects of chromatographic and mass spectrometric performance changes over time.

Data analysis and statistical model

Analysis of the LC-MS data was performed using a commercially available software suite, Elucidator™ (Rosetta Biosoftware, Seattle, WA, USA). The PeakTeller algorithm was used to analyse the complex LC-MS dataset and identify MS features which were defined by an *m/z* and a retention time value (i.e. an observed isotope peak). Key PeakTeller parameters used for this analysis were: peak time score, 0.7; peak *m/z* score, 0.8; alignment, adaptive alignment; and max. charge, 7. Chromatographic peak heights were designated as the intensity (or abundance) of the MS feature. A linear mixed-effect model (see below for details) was used to find MS features that showed statistically significant changes in abundance over time. For each MS feature, we computed its abundance at each time point by taking the average of the two technical replicates for the sample. The resulting dataset comprised 41 abundance values for each MS feature, and the data were fitted to the mixed-effect model. All the statistically significant MS features (using $p < 0.10$) were then manually reviewed. The peptides that showed consistent abundance profiles for multiple MS features and which displayed good chromatographic and mass spectrometric properties were then downselected. The amino acid sequences of the manually selected peptides were determined by searching their MS/MS against a human protein database using SEQUEST (TurboSEQUEST v.27 revision 12) (Eng et al. 1994). All the peptide sequence results were manually verified.

The linear mixed-effect statistical model used to analyse proteomics data (Cnaan et al. 1997) treated time as the fixed effect and the subjects as random effects. The null hypothesis was that there was no difference between the baseline and subsequent sampling times. This model accounts for the serial sampling experimental design (i.e. samples taken from the same subjects at different time points), and for 'missing' samples at certain time points. To ensure the robustness of putative biomarkers, identification of a plasma protein concentration as having increased following exercise required at least two distinct peptide components of that protein to be independently identified as having time-dependent changes by the statistical model. Concordance of time-dependent changes for distinct peptides from the same protein was assessed using pair-wise correlation of peptide abundance for all subjects at each time point. Further, an additional mixed-effect model was developed with time as the fixed effect and incorporating all peptide data for a given protein. This model allowed assessment of potential peptide–time interactions as evidence of discordance between peptides.

Conventional markers of muscle injury measured for purposes of comparison with the proteomic values were summarized using median and ranges of values at each point in time. Significant change of these markers in response to the exercise was identified using Wilcoxon matched-pairs signed ranks test comparing baseline to 72 h measures, based on prior reports of the time course of increases in these measures following eccentric injury. Stability of the measures made for safety monitoring (electrolytes, glucose and creatinine) was tested by repeated measures analysis of variance over the sample times. Correlations between the magnitudes of change for selected conventional and proteomic markers were analysed by Pearson product moment correlation coefficients; *p*-values $\leq$0.05 were considered significant with details provided with the reporting of specific analyses.

Results

Subject characteristics

Sixteen subjects volunteered to participate in the study. Three were excluded at screening (one each due to

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elevated CK on the baseline laboratory sample, history of prior drug-induced myalgias and inability to perform the exercise adequately secondary to obese body habitus). The remaining 13 subjects completed the study procedures and of these, nine had sufficient plasma samples for inclusion in the proteomics analyses. The age of the nine subjects ranged from 19 to 65 (mean 44) years; five were female, and five were diabetic (Table 1). The diabetic subjects were each regularly taking an average of seven prescription medications, including some with potential for effects on muscle, such as HMG-CoA reductase inhibitors (statins) ($n = 3$) and thyroid replacement ($n = 1$).

Muscle soreness

All subjects reported a baseline VAS score for soreness of 0, and all reported some degree of delayed-onset muscle soreness. Their ratings of soreness were variable, with peak VAS scores ranging from 1 to 10 out of 10, with a median score of 7. As expected, soreness scores peaked most frequently at 24 or 48 h post-exercise (data not shown).

Clinical chemistry

There were no significant changes in serum concentrations of electrolytes, creatinine or glucose in the post-exercise measures compared with the baseline values (data not shown). Serum concentrations of conventional markers of muscle injury are illustrated by data from a subject showing a robust biomarker response in Figure 1, and changes for the group as a whole at each sampling time point are shown in Table 2. Consistent with previous reports (Sorichter et al. 1999), the most marked changes were in the concentrations of CK and myoglobin. Relatively smaller changes were observed for aldolase, LDH and AST. No significant change in ALT was observed. The highest values for each of the conventional markers measured were observed at the 72 h time point. Also consistent with prior reports (Nosaka & Clarkson 1996), there was wide variability in the magnitude of increase in concentration of injury markers among the subjects, with peak CK values ranging from 137 to over 22 000 mU l^{-1}. There was strong concordance among the conventional serum markers (e.g. for CK and myoglobin concentrations across all time points, $r = 0.87$). The correlation between serum markers and subjective reports of muscle soreness was weaker (e.g. for CK and VAS scores across all time points, $r = 0.31$).

LC-MS based proteomics analysis

The statistical analyses and manual data review identified 95 observed ions that showed time-dependent abundance profiles in the serial samples. Among these, 30 peptides were identified that correspond to nine human proteins (Table 3). Four of these proteins met the criterion of being identified by at least two distinct peptides. These were α1-antichymotrypsin precursor (ACT), plasma protease C-1 inhibitor (C1 Inh), haemoglobin α chain (Hbα) and haemoglobin β chain (Hbβ). Time-dependent abundance profiles for a representative peptide for each of the four proteins are displayed in Figure 2. ACT and C1 Inh both peaked at 48 h, whereas the two haemoglobin proteins showed highest abundance at the 72 h time point. The concordance of the time-dependent changes for the distinct peptides identified for each protein was supported by two additional analyses. First, the correlation between each pair of distinct peptides for each protein was assessed across the subjects at each time point. This analysis confirmed concordance based on correlation coefficients (for example median pair-wise correlation coefficients of 0.967, 0.944, 0.964,

Table 1. Demographic and clinical characteristics of subjects. Note that the subject numbers listed here are used consistently in the article for reference purposes.

Subject	Age (years)	Sex	Height (cm)	Weight (kg)	Highest VAS score	Diabetes mellitus	Medications
1	24	Female	156	76	7	No	None
2	59	Female	165	104	5	Yes	Aspirin, simvastatin, insulin, metformin, glipizide, lisinopril, metoprolol
3	65	Female	170	68	5	Yes	Aspirin, lovastatin, niacin, gemfibrozil, metformin, glyburide, lisinopril, levothyroxin
4	65	Female	159	67	8	Yes	Aspirin, simvastatin, pioglitazone, glipizide, benazepril, lansoprazole
5	45	Male	174	123	9	Yes	Insulin, lisinopril, omeprazole, docusate
6	19	Male	186	72	5	No	None
7	32	Female	160	63	1	No	None
8	47	Male	176	85	10	Yes	Aspirin, gemfibrozil, metformin, insulin, lisinopril, clonazepam, amitriptyline, risperidone, ranitidine
9	39	Male	180	96	9	No	None

VAS, visual analogue scale.

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Table 2. Plasma concentrations for conventional markers of muscle injury at baseline and each post-exercise sampling time.

	Baseline	1 h	24 h	48 h	72 h
CK (mU ml^{-1})*	99 (44–208)	102 (43–364)	215 (70–608)	520 (74–4993)	1583 (137–22 290)
Myoglobin (μg l^{-1})**	28 (26–30)	28 (28–152)	43 (28–152)	102 (28–1540)	258 (28–2100)
Aldolase (mU ml^{-1})#	4 (2–6.5)	4 (2.1–8.6)	4 (2.5–9.2)	7 (3.7–23.4)	14 (2.8–93.8)
LDH (mU ml^{-1})#	124 (99–180)	129 (96–188)	136 (115–171)	142 (119–254)	196 (121–563)
AST (mU ml^{-1})#	19 (15–40)	23 (14–44)	23 (18–36)	29 (20–82)	39 (18–247)
ALT (mU ml^{-1})	23 (15–59)	25 (11–54)	25 (15–48)	27 (20–55)	32 (17–49)

Values are median (range); $n = 9$; *$p < 0.004$; **$p < 0.008$; #$p = 0.05$ for the change from baseline at 72 h. CK, creatine kinase; LDH, lactate dehydrogenase; AST, aspartate aminotransferase; ALT, alanine aminotransferase.

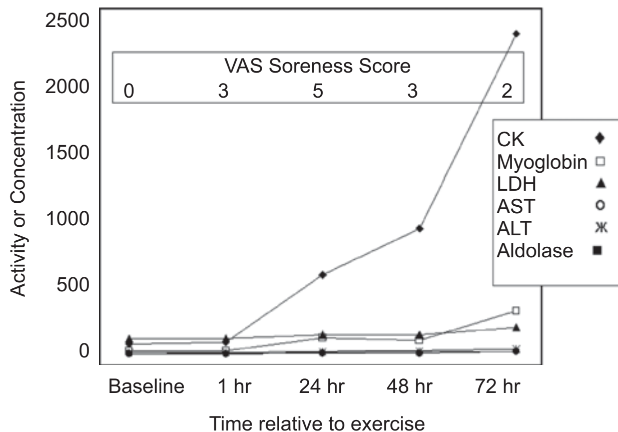

Figure 1. Conventional markers of muscle injury and visual analogue scale (VAS) soreness scores for one subject before (baseline), and at 1, 24, 48 and 72 h following an eccentric exercise stimulus. Values of serum markers are expressed as μg l^{-1} for myoglobin, and U l^{-1} for all others. Soreness was scored on a unitless VAS of 0–10 with higher numbers indicating greater soreness. Data are from subject 3 in Table 1. Note the non-linear x-axis. CK, creatine kinase; LDH, lactate dehydrogenase; AST, aspartate aminotransferase; ALT, alanine aminotransferase.

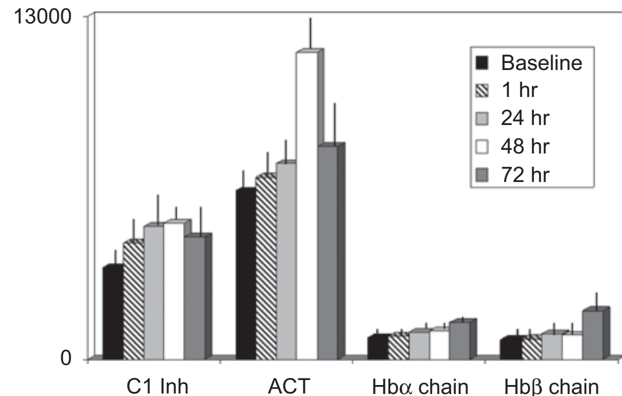

Figure 2. Relative abundance of a representative peptide for each of four proteins identified to increase in plasma following eccentric exercise. Peptides and the proteins they represent are: α1-antichymotrypsin (ACT): NLAVSQVVHK; plasma C-1 protease inhibitor (C1 Inh): NSVIKVPMMNSK; haemoglobin α chain (Hbα): VGAHAGEYGAEALER; and haemoglobin β chain (Hbβ): FFESFGDLSTPDAVM*GNPK. Group mean intensities, in arbitrary units (+ SEM), for the most abundant isotope of each peptide are shown at pre-exercise baseline ($n = 9$) and at 1 h ($n = 8$), 24 h ($n = 8$), 48 h ($n = 7$) and 72 h ($n = 9$) after the exercise session. Peak abundance was highest at the 72 h time point for the haemoglobin proteins, but earlier for the two proteases.

0.882 and 0.959 at baseline, 1, 24, 48 and 72 h, respectively, for the 14 peptides derived from ACT). Second, a mixed-effect model was used incorporating all peptide data for a specific protein to assess any interaction between peptide and time. This analysis demonstrated no interaction between peptide and time (all $p > 0.40$), thus confirming no discordance between peptides from the same protein over time.

As was the case for the traditional biomarkers, there was substantial between-subject heterogeneity in the magnitude of responses of peptides from the four proteins identified using proteomics (Table 4). On average, the fold-increase relative to baseline for these peptides was less than that for CK, but was in the range of some of the other traditional markers (Table 2). Across subjects, the fold changes in abundance for the four proteins (calculated by averaging fold changes for their peptides) did not correlate with the peak CK response (Table 5). In contrast, there were strong correlations between changes measured for the peptides from the two haemoglobin chains, and between the two protease inhibitors ($r = 0.99$ and 0.93, respectively). This is further illustrated in Figure 3 which shows the relationship between ACT and C1 Inh abundances for the nine subjects at all post-exercise sampling points.

Although the number of subjects was small, no obvious patterns of difference were observed in conventional markers or proteomics findings when comparing the healthy subjects with those with diabetes, or as related to gender or age.

Discussion

In this study a proteomics approach was used to seek novel markers of muscle injury which might be helpful in detecting or characterizing such injury in clinical and research applications. In a diverse cohort of subjects four circulating proteins, ACT, C1 Inh, Hbα chain and Hbβ chain, previously unsuspected of being markers of muscle injury or adaptation were identified. The results

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Table 3. Plasma proteins identified by component peptides which demonstrated time-dependent profiles following eccentric exercise injury. Under each protein name in bold are the peptides from which they were identified, the first four proteins listed being identified by more than one peptide.

Protein name (SwissProt accession number) and component peptide sequences	*m/z*	Charge state[m/z]	Elution time (min)
Plasma protease C1 inhibitor (SW:P05155)[b]			
GVTSVSQIFHSPDLAIRDTFVN#ASR[c]	680.61	4+	38.2
HRLEDMEQALSPSVFK	629.66	3+	29.8
	472.49	4+	29.8
KYPVAHFIDQTLK	520.63	3+	24.2
LEDMEQALSPSVFK	531.93	3+	32.2
NSVIKVPMMNSK	449.91	3+	21.1
TNLESILSYPKDFTCVHQALK	617.07	4+	36.1
Haemoglobin β chain (SW:P68871)[b]			
FFESFGDLSTPDAVM*GNPK[d]	692.66	3+	33.5
VNVDEVGGEALGR	657.84	2+	20.9
Haemoglobin α chain (SW:P69905)[b]			
MFLSFPTTK	536.28	2+	29.1
TYFPHFDLSHGSAQVK	459.48	4+	28.1
VGAHAGEYGAEALER	510.59	3+	17.3
α1-Antichymotrypsin precursor (SW:P01011)[b]			
ADLSGITGAR	480.76	2+	16.3
AKWEM*PFDPQDTHQSR[d]	497.98	4+	22.2
	663.64	3+	22.2
	995.46	2+	22.2
AVLDVFEEGTEASAATAVK	954.49	2+	32.7
EIGELYLPK	531.30	2+	24.7
EQLSLLDRFTEDAK	555.62	3+	30.7
EQLSLLDRFTEDAKR	455.99	4+	29.2
	607.66	3+	29.1
KLINDYVK	331.53	3+	15.2
	496.79	2+	15.2
LINDYVK	432.75	2+	15.8
LYGSEAFATDFQDSAAAK	946.44	2+	30.4
LYGSEAFATDFQDSAAAKK	674.00	3+	28.5
NLAVSQVVHK	365.55	3+	15.1
RLYGSEAFATDFQDSAAAKK	544.77	4+	26.2
WRDSLEFR	370.19	3+	21.5
	554.78	2+	21.5
LINDYVKN#GTR[c]	647.35	2+	16.3
Proteins identified by a single peptide			
Complement C2 precursor (SW:P06681)[b]			
LTDTICGVGN#M*SAN#ASDQER[c,d]	719.64	3+	21.1
Haptoglobin-related protein precursor (SW:P00739)[b]			
QLVEIEK	429.75	2+	15.0
Antithrombin-III precursor (SW:P01008)[b]			
RVWELSK	459.27	2+	15.4
α1-Antitrypsin precursor (SW:P01009)[b]			
RLGM*FNIQHCK[d]	355.68	4+	15.9
Fibrinopeptide (SW:P02675)[b]			
QDGSVDFGR	490.73	2+	14.5

[a]*m/z*, mass to charge ratio. [b]Peptides from plasma C-1 protease inhibitor are all statistically significant ($p < 0.10$) for 48 h vs pre-exercise; peptides from haemoglobin β chain are all statistically significant ($p < 0.10$) for 72 h vs pre-exercise; peptides from haemoglobin α chain are all statistically significant ($p < 0.10$) for 72 h vs pre-exercise; and the peptides from α1-antichymotrypsin are all statistically significant ($p < 0.10$) for 48 h vs pre-exercise. Only one peptide was found for complement C2, haptoglobin, antithrombin-III, α1-antitrypsin and fibrinopeptide, and they are all statistically significant ($p < 0.10$) for 48 h vs pre-exercise. [c]N#X(S/T) represents the motif for N-link glycopeptides. After the enrichment procedure, amino acid residue N at that site will be converted to amino acid residue D. [d]M* represents oxidized M.

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Table 4. Relative change from baseline to peak values for creatine kinase (CK) and four proteins identified from the proteomics analysis. For each protein, peak changes (independent of time point observed) are expressed as the fold change relative to baseline values; for the proteomics-identified proteins, fold changes are expressed as the average for its component peptides. Subject numbers correspond to those in Table 1.

Subject	CK	ACT (average of 14 peptides)	C1 Inh (average of 6 peptides)	Hbβ (average of 2 peptides)	Hbα (average of 3 peptides)
1	34	1.2	1.3	1.1	1.1
2	1.8	1.2	1.3	1.3	1.1
3	21	4.5	6.2	> 10[a]	> 10[a]
4	21	1.2	1.1	1.1	1.2
5	142	1.0	2.3	1.5	1.3
6	30	6.2[b]	5.5	3.8	3.7
7	1.1	2.4	2.3	4.5	3.9
8	32	1.1	1.5	4.0	2.6
9	7.6	1.3	1.6	4.4	3.8
Geometric mean	14.6	1.8	2.1	2.7	2.4

[a]Quantitative fold changes calculated for these samples may be relatively unreliable due to low values for baseline abundance.
[b]Fold change for this sample based on abundance of 13 of the 14 identified peptides, because the baseline abundance for peptide LINDYVKN#GTR was too low for accurate assessment.
CK, creatine kinase; ACT, α1-antichymotrypsin; C1 Inh, plasma C-1 protease inhibitor; Hb, haemoglobin.

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Table 5. Correlation coefficients between peak changes of five proteins. For each protein, the peak change was expressed as fold increase relative to baseline values (see Table 4) and linear correlation coefficients (r) calculated between pairs of proteins for the nine subjects. For the four proteins identified by proteomics analysis, peaks were characterized as the mean of the peak values for its discrete peptides as in Table 4.

	CK	Hbα	Hbβ	C1 Inh
Hbα	-0.24			
Hbβ	-0.26	0.99		
C1 Inh	0.01	0.81	0.75	
ACT	-0.17	0.63	0.58	0.93

CK, creatine kinase; Hb, haemoglobin; C1 Inh, plasma C-1 protease inhibitor; ACT, α1-antichymotrypsin.

indicate that plasma proteomics has potential not only for identifying markers of injury, but also for providing a basis for new insights into the injury and adaptive processes associated with eccentric exercise.

In the samples from the nine subjects studied multiple peptides were identified by proteomic analysis that changed significantly within the 72 h following the exercise stimulus. Among these, two or more distinct peptide fragments were mapped to the four proteins identified. Importantly, the peptides from the same protein showed consistent time-dependent abundance profiles. These observations provide strong evidence that there were true changes in the concentrations of these proteins in blood, and that the changes were consequent to the exercise stress, and not spurious variations. In contrast to conventional markers of muscle injury which are intracellular constituents which leak into the blood, the four proteins identified by proteomics are recognized plasma constituents. The peptides from the two protease enzymes identified, ACT and C1 Inh, demonstrated a time profile different from conventional markers as they peaked earlier, and the magnitude of changes for the identified peptides were independent of the magnitude of change in conventional markers, suggesting that they reflected different processes and may provide differential information on the response to eccentric exercise.

The value of the new potential biomarkers identified in differentiating exercise-induced injury from other causes of muscle injury is currently unknown. However, the traditional biomarker data from the current study illustrate how a panel of biomarkers might be used to differentiate causes of CK elevations, for example. The minimal change in ALT despite large changes in CK observed (Table 2) contrasts with reports of muscle injury due to other causes (Nathwani et al. 2005), including statin-associated injury in animals (Seachrist et al. 2005) and man (Alexandridis et al. 2000, Ricaurte et al. 2006). In these reports, the magnitude of the elevation in ALT concentrations relative to the increase in CK was greater than in the present study. Clarkson and colleagues also reported relatively small changes in ALT relative despite large increases in CK after eccentric exercise (Clarkson et al. 2006). Thus, it can be hypothesized that a lack of an increase in ALT in the setting of a large increase in CK might be helpful for identifying eccentric exercise as the probable aetiology of a muscle injury. Similarly consistent with stimulus differentiation, despite the clear increase in the eccentric exercise model, C1 Inh has been reported not to increase after aerobic exercise (Semple et al. 2006). Observations that statins modify the response to eccentric exercise in humans (Urso et al. 2005) provide further relevance for the data from this model in the current report. Further research including ACT, C1 Inh and the haemoglobin chains in assessing muscle responses to stimuli have the potential to provide further discrimination.

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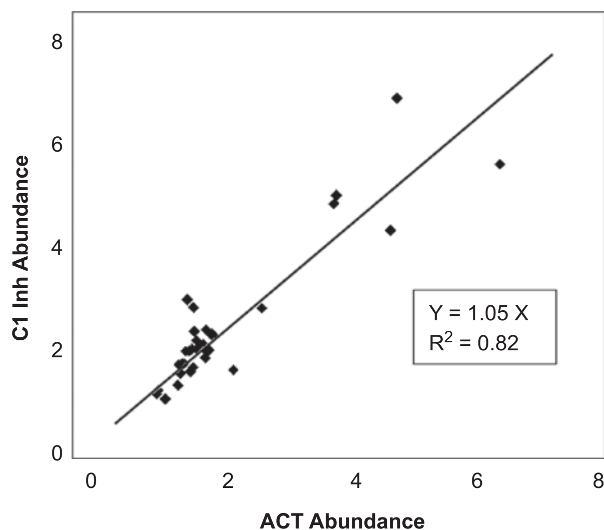

Figure 3. Concordance of post-exercise changes in abundance of proteins α1-antichymotrypsin (ACT) and plasma C-1 protease inhibitor (C1 Inh). Data points represents the abundance of ACT (14 peptides) and C1 Inh (six peptides), expressed relative to their abundance in the baseline sample, at each of the available four post-exercise time points for each of nine subjects.

The biological basis for the changes in abundance of the identified peptides cannot be concluded from these data. ACT and C1 Inh are both inflammatory markers but may have other functions. Eccentric exercise and other noxious stimuli are well recognized as evoking an inflammatory response in muscle (Peake et al. 2005). In this context it is interesting to note that other proteins preliminarily identified on the basis of single peptide changes post-exercise (Table 3) are also related to inflammatory responses. However, many aspects of this inflammatory response may also be components of the muscles' adaptation to the stimulus, including remodelling and hypertrophy (Akaaboune et al. 1995, Arnold et al. 2007). The observed changes in plasma ACT and C1 Inh may be of relevance to these processes. Increases in circulating ACT concentrations have been associated with preservation of muscle mass in elderly subjects (Schaap et al. 2006). ACT and other protease inhibitors have also been suggested to protect muscle under conditions such as denervation or ischaemia-reperfusion (Businaro et al. 2001, Murohara et al. 1995). This contrasts with another finding, that in the setting of chronic disease, ACT may be associated with decreases in muscle mass (Friis et al. 2002), which may reflect its identity as an acute-phase reactant, and therefore a simple marker of inflammation. In this study, the increases in ACT and C1 Inh preceded those of the traditional markers of muscle injury, which may be consistent with the early inflammatory processes associated with eccentric injury.

Plasma concentrations of haemoglobin peptides presumably reflect haemolysis, and were found to increase in a similar time course as the conventional markers of muscle injury. This finding is unlikely to represent an artefact of phlebotomy or sample preparation as the sampling and processing of samples were carried out in an identical manner across the study. The time course of the haemoglobin increases differed from that of acute intravascular haemolysis which is sometimes associated with strenuous exercise and attributed to either mechanical trauma or altered membrane fragility of red cells (Smith 1995). The increase in circulating haemoglobin in this study probably reflects erythrolysis occurring concomitant with late effects of muscle injury and remodelling, possibly due to local vascular effects in the injured muscle.

In general the responses of traditional biomarkers in this study were similar to those reported from previous studies using comparable kinds of exercise stimuli (Clarkson et al. 2006). Variability in the increase of these injury markers among subjects performing the same exercise protocol is well recognized (Nosaka & Clarkson 1996) and not an unexpected finding. Unexplained variability was also observed in the responses to ACT and C1 Inh (Table 4). Some degree of muscle soreness was reported by all subjects in this study, although the relationship between subjective VAS scores and the biochemical markers was weak. This is also consistent with prior observations from a larger cohort of subjects showing poor correlation between delayed soreness and other indices of muscle injury due to eccentric exercise (Nosaka et al. 2002). Although no differences were evident in the findings for diabetic compared with healthy volunteers in this study, this could also reflect the small sample size and inherent variability in responses within each cohort, rather than a true lack of differences in susceptibility to, or patterns of, muscle injury. Similarly, age may be a factor in determining responses to eccentric exercise (Lavender & Nosaka 2006), but the number of subjects studied here precludes assessing the affect of age on the responses observed.

It is important to note that there are inherent limitations of LC-MS-based proteomics (Mann et al. 2001). Although modern mass spectrometers have absolute limit of detection at the attomole levels, the relative abundance of the analyte of interest is of importance in its detection (Mann et al. 2001). As reported previously, detection of high-abundance proteins is favoured when analysing complex protein mixtures such as plasma (Anderson & Anderson 2002, Hack 2004). To address this, a number of biochemical fractionation techniques can be applied to samples prior to LC-MS profiling. The glycopeptide enrichment method was used to simplify the complexity of the plasma samples in the present study. Alternative approaches may also be used, depending on the focus of the investigation, such as ultrafiltration to facilitate analysis of low molecular

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weight proteins, or immunodepletion to reduce selected abundant proteins from samples (Pieper et al. 2003). Such alternative approaches might change the signal-to-noise for certain peptides and thus allow the identification of other proteins affected by eccentric exercise. Another limitation of these data is that not all features profiled in the LC-MS were identified by the MS/MS technique (collisional activated dissociation) used in this study. Newer MS/MS techniques (such as electron-transfer dissociation (Coon et al. 2005)) are evolving that may further increase the capability to identify peptides of interest. Finally, the relatively small number of subjects may have precluded identification of other proteins that changed post-exercise, but with greater variability or a smaller magnitude of change. None of these limitations, however, detract from the conclusion that the plasma concentrations of the four proteins identified were increased following eccentric exercise.

In summary, proteomics provides a tool for identifying changes in the plasma compartment with relevance to processes occurring at the level of the muscle and systemically. Using an eccentric exercise model of muscle injury proteomics identified unanticipated increases in four plasma proteins that reflected responses other than leakage of myocyte constituents. Despite the small number of subjects, the requirement that each protein be identified based on at least two independent peptides and the observed correlation between the functionally related proteins suggest that the identified proteins are associated with the response to eccentric exercise. These findings suggest that application of proteomics to other models of muscle injury and further study of the four proteins identified may lead to insights into muscle responses and response profiles that differentiate the cause of injury. Thus, increases in plasma ACT and C1 Inh concentrations occur after eccentric exercise in man. These proteins appear to reflect different processes other than myocyte leakage and thus may represent useful potential biomarkers of muscle injury in future research.

Acknowledgements

Declaration of interest

This study was supported by the General Clinical Research Center of Los Angeles Biomedical Research Institute at Harbor UCLA Medical Center, with funds provided by the National Center for Research Resources, grant no. 5 M01 RR00425, US Public Health Service, and by Rosetta Inpharmatics, LLC, a wholly owned subsidiary of Merck & Co, Inc. K.E.S. and E.P.B. were consultants to Rosetta InPharmatics/Merck Research Laboratories at the time the work was completed. F.M., N.A.Y., R.C.H., A.L., Q.S. and R.G.U. were employees of Rosetta InPharmatics/Merck Research Laboratories at the time the work was completed.

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