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Fatigue biomarker index: An objective salivary measure of fatigue level

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ABSTRACT

Fatigue changed the composition of the small-molecular weight (sMW) proteome of saliva during a 10 h session of moderate (70% of maximum ventilatory threshold) physical exertion. Saliva samples were collected from nine recreationally trained cyclists participating in a cross-over study designed to simulate prolonged manual labor, a military operation or wildfire-suppression work. During each hour of the study, participants performed an exercise program that included upper and lower body exercises separated by short periods of recovery. Over the course of the study, fatigue level increased as suggested by a significant increase in the participants' relative perceived exertion. The composition of the sMW proteome was investigated using reversed-phase liquid chromatography with mass-spectrometric detection. Isotopes of acetic anhydride were used for mass-specific labeling of samples and subsequent identification of ions with significant changes in intensity. Cluster analysis was used to identify a pair of peptides with concentrations that changed in opposite directions with fatigue level, i.e. concentration of one peptide increased while concentration of the other decreased. The sequences of the two peptides were determined by high-resolution mass spectrometry. The ratio of the ion intensities of these two peptides, referred to as the fatigue biomarker index, was calculated for subjects throughout the study. The FBI values from the start of the study likely arose from a different distribution than the FBI values measured at the end of the study (Mann–Whitney test, $P < .05$). While this study is restricted to a small population of recreationally trained cyclists performing exercise under controlled conditions, it holds promise for the development of an objective salivary measurement of fatigue that is applicable to a much broader population performing in uncontrolled environments.

1. Introduction

Fatigue degrades both physical and cognitive performance. The impact of fatigue is best exemplified by its position as the leading identifiable and preventable cause of accidents in transportation operations, surpassing even alcohol and drugs (Akerstedt, 2000). While fatigue levels are frequently assessed by self-reporting, an individual's perception of fatigue is not a robust predictor of the ability to perform (Van Dongen, 2004). Moreover, compensation practices within some industries motivate individuals to distort their self-reported fatigue levels, even though the consequences of an error in those industries impact the lives of many others besides that of the impaired operator (Akerstedt, 2000). Clearly, there is a need for objective measures of fatigue, especially in situations when individuals are motivated to distort their self-reported level of fatigue (Caldwell et al., 2009).

Fatigue is caused by many different factors, including sleep deprivation, persistent mental activity and prolonged physical exertion. In the case of a sustained physical effort, fatigue is associated with a loss of power in peripheral muscles, as well as a perception of fatigue mediated by signaling pathways in the central nervous system (CNS) (Ament and Verkerke, 2009). The former is associated with the depletion of glycogen stores, the accumulation of metabolites and a perturbation in ionic balances within myocytes. The mechanism for the latter remains a topic of research, although the CNS-mediated perception of fatigue is associated with changes in levels of cytokines and/or neurotransmitters, such as interleukin (IL) 1, IL-6, tumor necrosis factor (TNF), serotonin, dopamine and tyrosine (Cannon and Kluger, 1983; Cannon et al., 1989; National Research Council, 2009). Prolonged physical exertion also triggers changes in the autonomic nervous system (ANS) marked by a simultaneous withdrawal of the parasympathetic nervous system (PSNS) and activation of the sympathetic nervous system (SNS) (Klein and Corwin, 2002). All of these physiological changes offer potential targets for monitoring fatigue level objectively, thereby removing the reliance on self-reporting of fatigue.

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There is no universally accepted objective measure of fatigue. Current measures are based either on mood state, e.g. Profile of Mood States (Mcnair et al., 1971), neurobehavioral assay, e.g. psychomotor vigilance test (Dorrian et al., 2005) or a physiological marker, e.g. IL-6 levels in blood (Purvis et al., 2010). An ideal measure of fatigue would provide real-time, non-invasive assessment with high sensitivity and selectivity. While many studies have examined changes in blood chemistry during prolonged exercise, few have examined changes in saliva composition (Khaustova et al., 2010). The latter is an attractive biological fluid for physiological monitoring, because saliva can be sampled easily and repeatedly. Moreover, human salivary glands are innervated by both the PSNS and SNS (Garrett, 1987; Proctor and Carpenter, 2007), suggesting that changes occurring within the ANS will lead directly to changes in the production and/or composition of saliva.

Here, we report a study of changes in human saliva during prolonged physical exertion. Using a combination of liquid chromatography and mass spectrometry (LC–MS), we examined changes in the small-molecular-weight components of saliva. Our results suggest that the ratio of concentrations for two peptide fragments derived from proline-rich proteins can be used to monitor changes in fatigue level.

2. Materials and methods

2.1. Study participants

The nine participants were recreationally trained male cyclists ranging in age from 19 to 46 years (28.1 ± 11.0 years, mean ± SD). Values for height, weight, percent body fat and fat-free mass (FFM) were as follows: 178.1 ± 5.0 cm, 75.1 ± 11.3 kg, 8.9 ± 5.4% and 68.0 ± 8.0 kg. Prior to all testing, subjects signed an informed consent form approved by the University of Montana Internal Review Board.

2.2. Extended exercise trials

The exercise program has been described in detail elsewhere (Harger-Domitrovich et al., 2007). Briefly, subjects completed a 10 h session of continual exercise twice. Each 10 h session consisted of three different modes of exercise repeated hourly. Subjects reported to the laboratory at 05:00 h and exercise sessions occurred roughly between 07:00 h and 17:45 h. Subjects began with 9 min of upper-body ergometry, followed by 19 min of cycling and then 20 min of walking. Subjects were allowed 1 min to transition between exercises and a 10 min break between each set of three exercises and 40 min for lunch between the fifth and sixth sets of exercises. Subjects were asked to maintain an effort equivalent to 70% of maximum ventilatory threshold.

Subjects completed the two exercise sessions in a double-blind, random crossover design with the two arms of the study on different days separated by no more than seven days. During each arm of the study, subjects received hourly fixed volumes of either carbohydrate (20% malodextrin at 0.6 g × kg[FFM]$^{-1}$) [CHO] drink or an artificially sweetened, non-caloric placebo drink [PLA] of the same flavor and temperature.

2.3. Relative perceived exertion

At time points 0, 4, 5 and 8 h, participants were asked to evaluate their relative perceived exertion (RPE) after each exercise type (Borg, 1982). Total RPE was calculated by summing all of the separate RPE values recorded during a single set of exercises.

2.4. Saliva samples

Processing and LC–MS analysis of saliva samples required the following LC–MS grade reagents: methanol, water and acetonitrile (Sigma, St. Louis, MO). Other reagents included the following: bicinchonic acid (BCA) assay kit (Pierce, Rockford, IL), triethylammonium bicarbonate buffer (pH 8.5 ± 0.1, Sigma), heptafluorobutyric acid (≥99.5%, Sigma), ethyl alcohol (≥99.5%, A.C.S. grade, Sigma), acetic anhydride-D_6 (99 atom% D, Sigma), acetic anhydride-H_6 (99 atom% H, Sigma).

Raw saliva samples (~4 ml) were collected by passive drooling after 0, 4, 5, 6, 7 and 8 h of exercise in cryovials containing a cocktail of enzyme inhibitors (final concentrations: AEBSF 2 mM, EDTA 1 mM, bestatin 130 μM, E-64 14 μM, leupeptin 1 μM, aprotinin 0.3 μM, Sigma). Samples were frozen, shipped on dry ice and remained in storage at −80 °C until analyzed. To remove insoluble debris, raw saliva was spun at 4000 *g* for 20 min at 4 °C. The resulting supernatant was spun through a 50 kDa molecular-weight cutoff (MWCO) filter (regenerated cellulose, Millipore, Bellerica, MA) at 3000 *g* for approximately one h at 22 °C. The resulting filtrate was loaded into a 10 kDa MWCO filter (regenerated cellulose, Millipore) and spun for 1 h at 3000 *g* at 22 °C. The concentration of peptide and protein in the resulting filtrate was then determined using a commercially available kit for the BCA assay calibrated using bovine serum albumin standards. To remove salts and increase the concentration of peptides, ~100 μg of protein was passed through a commercially available peptide trap (Michrom, Auburn, CA). The peptide concentration was again quantified using the BCA assay. The sample volume was then reduced using a heated centrifugal concentrator (Centrivap, Labconco, Kansas City, MO). Concentrated samples were labeled with a mass-specific variant of acetic anhydride, i.e. acetic anhydride with either methyl protons or methyl deuterons (Yu et al., 2004). The labeling mixture consisted of a 1:250 dilution of mass-specific acetic anhydride prepared in ethanol with 50 mM triethylammonium bicarbonate. Samples were incubated for one h at 37 °C and then concentrated as described above. Finally, two aliquots (~2.5 μg each) from the same sample, labeled separately with light and heavy forms of acetic anhydride, were combined and injected onto the LC–MS system.

2.5. Low-resolution LC–MS analysis of saliva

The various components of the processed saliva were separated using an ultra-high-pressure liquid chromatography (UPLC) system (Acquity, Waters, Milford, MA) with the outlet flowing directly into an ion-trap mass spectrometer (Bruker, Esquire 3000+, Bellerica, MA). The UPLC was configured with a reversed-phase column (BEH300 C18, 1.7 μm particle, 2.1 mm × 100 mm, Waters) and the components were eluted from the column by varying the concentration of methanol in the running buffer linearly over a range from 10% to 35% at a flow rate of 0.3 ml/min. MS scans were collected at 2–5 Hz.

2.6. Analysis of LC–MS data

A custom analysis program was written in LabVIEW (National Instruments, Austin, TX) to allow for the objective and automated identification of peak pairs within the data set separated by the expected mass-to-charge (m/z) differences appropriate for labeling with acetic anhydride, e.g. delta m/z of 3 for a singly charged ion that has been labeled with one acetate group, delta m/z of 6 for a singly charged ion that has been labeled with two acetate groups, etc. Cluster analysis was used to identify peak pairs common to the group at the beginning and end of the study.

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2.7. High-resolution MS sequencing of salivary peptides

To determine the sequences of ions of interest, fractions (~1 min wide) of eluent near the elution time of the target ion were collected from LC injections of labeled saliva. Five fractions were pooled and sent to the University of Illinois (Neil Kelleher and Paul Thomas, Center for Top-down Proteomics) for high-resolution mass spectrometric analysis (12 T LTQ-FT Ultra, ProSight PC).

2.8. Statistical analysis

Statistical analysis was conducted using R (version 2.11.1) (Team, 2010). Linear mixed-effects models were constructed using the *nlme* package (Pinheiro and Bates, 2000). For box plots, the horizontal line represents the median value while the boundaries of the rectangle indicate the range of the middle two quartiles. The whiskers indicate a distance 1.5× greater than the interquartile range from the nearest edge of the box. Open circles indicate points beyond the whiskers.

2.9. Linear mixed-effects models

$$\text{Model 1.}\quad \text{RPE}_{ijk} = (\beta_0 + \gamma_{02}\text{Trial}_{2j} + b_{0i}) + (\beta_1 + \gamma_{12}\text{Trial}_{2j} + b_{1i}) * \text{Time}_{ik} + \varepsilon_{ijk}$$

In Model 1, the index i refers to the Subject, the index j refers to the arm of the study and the index k refers to a particular time point. The coefficients β_0,β_1 represent the fixed-effects terms while the coefficients b_{0i},b_{1i} represent the random-effects terms. The coefficient γ_{02} refers to the offset to the intercept for subjects in Trial CHO, the coefficient γ_{12} refers to the offset to the slope for subjects in Trial CHO and Trial_{2j} is a binary variable with value of 1 when data are from subject i in Trial CHO and value 0 when data are from subject i in Trial PLA.

$$\text{Model 2.}\quad \text{log\_FBI}_{ijk} = (\beta_0 + \gamma_{02}\text{Trial}_{2j} + b_{0i}) + (\beta_1 + \gamma_{12}\text{Trial}_{2j}) * \text{Time}_{ik} + (\beta_2 + \gamma_{22}\text{Trial}_{2j}) * \text{Time}_{ik}^2 + \varepsilon_{ijk}$$

In Model 2, the index i refers to the Subject, the index j refers to the arm of the study and the index k refers to a particular time point. The coefficients β_0,β_1,β_2 represent the fixed-effects terms for the population while the coefficient b_{0i} represents the random-effects term associated with a particular subject. The coefficients γ_{02},γ_{12},γ_{22} refer, respectively, to the offsets to the intercept, first- and second-order Time terms for subjects in Trial CHO. Trial_{2j} is a binary variable with value of 1 when data are from subject i in Trial CHO and value 0 when data are from subject i in Trial PLA.

3. Results

3.1. Increases in relative perceived exertion suggest increased levels of fatigue

During both arms of the crossover trial, participants experienced significant increases in RPE as the trial progressed, suggesting that the continuous exercise led to increased levels of fatigue (Fig. 1). The significance level of the apparent increase in RPE levels with time was assessed using linear mixed-effects (LME) modeling (see Model 1 in Section 2.9) (Pinheiro and Bates, 2000).

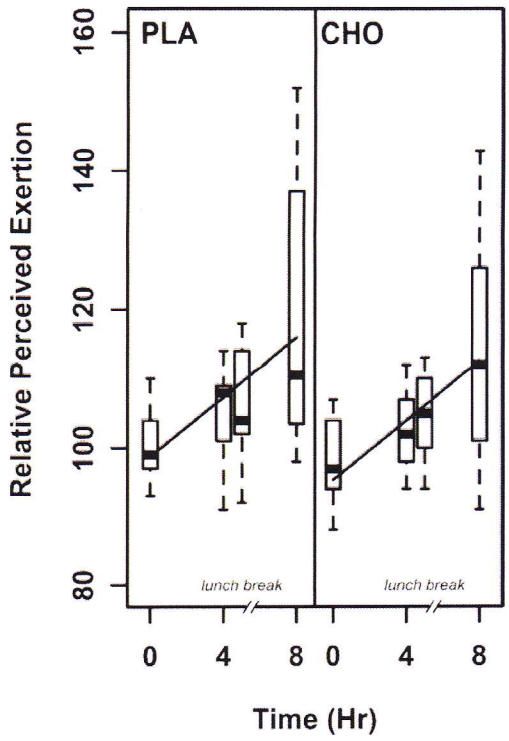

Fig. 1. An increase in relative perceived exertion (RPE) suggests an increase in fatigue as both arms of the study progress. Total RPE (see Section 2.3) is plotted as a function of time. Data from the different arms of the study are shown in the adjacent panels. The solid line in each panel shows the best-fit line of the linear mixed-effects model shown in Model 1. The best-fit LME model for the data included a single slope term, but trial-specific intercepts (Trial PLA: 2.1 ± 0.6, 98.8 ± 1.6; Trial CHO: 2.1 ± 0.6, 95.4 ± 1.6; slope ± SE, intercept ± SE, resp.; $P < .05$ for all parameters). (See Section 2.8 for a description of box plots.)

3.2. LC–MS analysis of saliva samples

In general, each of the LC–MS data sets from a single saliva sample contained thousands of different ion peaks, as shown for a representative data set in Fig. 2A. To compare the ion composition of saliva samples collected near the beginning and end of each arm of the study, we employed a mass-specific labeling approach using acetic anhydride with protonated or deuterated methyl groups to label free amine groups in solution. When aliquots from a single saliva sample were labeled separately with the different isotopic forms of acetic anhydride and then recombined, ions labeled with acetic anhydride appeared within the LC–MS data set as ion pairs with predictable differences in mass-to-charge ratio and similar retention times.

Software was used to scan the LC–MS datasets for the presence of ion pairs, denoting the locations of acetylated ions. The lists of acetylated ions for all samples collected at the same time point were then compared to determine which ions were common to the subjects at a given time. The length of the list of ions in common to the group was controlled by adjusting the fraction of subjects needing to have an ion present before it was considered common to the group. In Fig. 2B, the positions of the ion pairs are shown for the lists generated using a criterion of five-out-of-nine subjects.

Overall, more ion pairs were identified in the samples from $t = 8$ h than in the samples from $t = 0$ h. This difference could be explained by a larger average amount of material in the samples from $t = 8$ h, even though our methods were designed to yield samples with identical amounts of protein. Given this difference, it might not be surprising to find ion pairs present in the samples from $t = 8$ h which were not present in the samples from $t = 0$ h. Therefore, we

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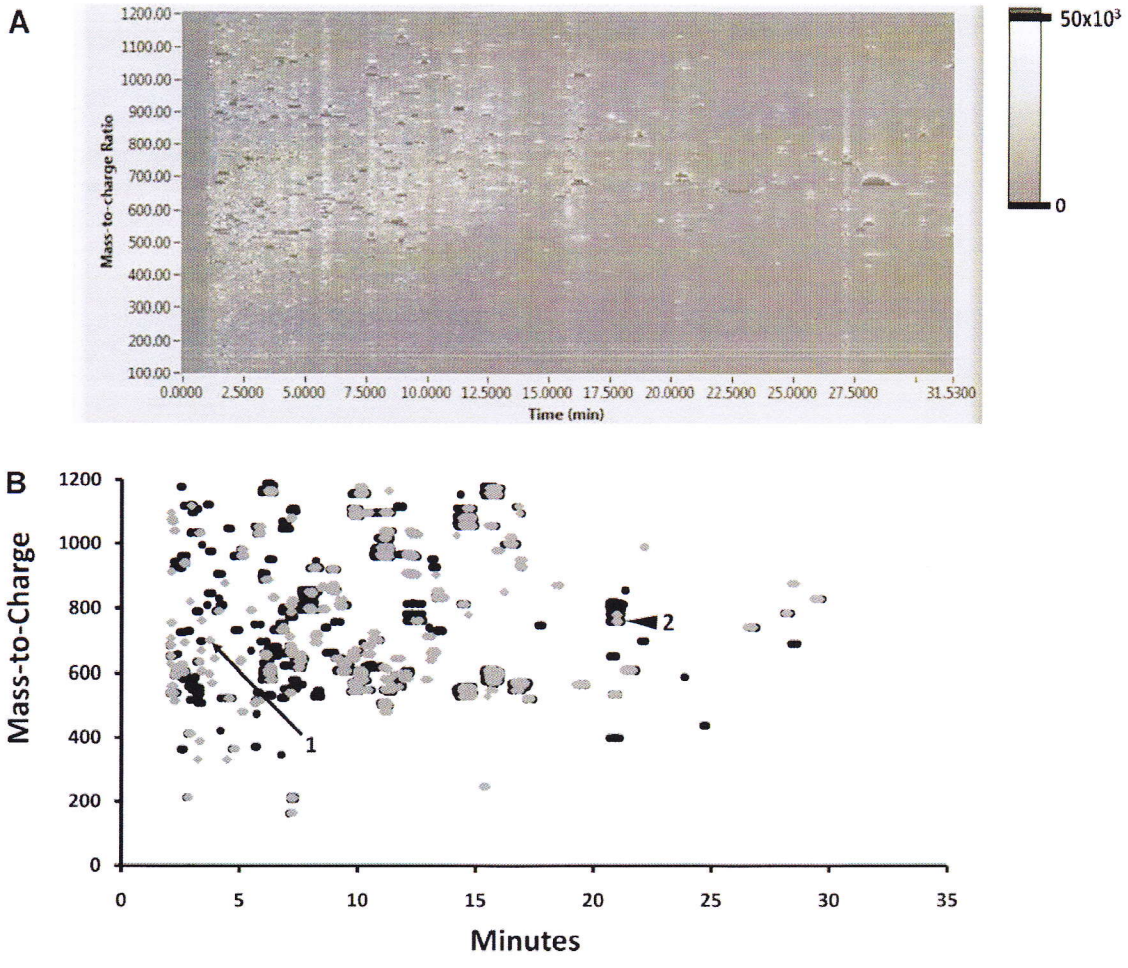

Fig. 2. (A) Three-dimensional display of LC–MS data from a representative saliva sample. The ion current intensity is encoded according to the scale shown to the right of the image. The data shown have been filtered with respect to time and frequency using low-pass filters. (B) Clustering of acetylated ions in saliva samples collected at 0 and 8 h. Data from time = 0 h (grey) and 8 h (black) are overlaid. The arrows labeled "1" and "2" indicate the locations of the ion used for the FBI numerator and FBI denominator, respectively.

focused first on the ion pairs that were present in the samples from $t = 0$ h but not in the samples from $t = 8$ h. An example of such an ion has been highlighted in Fig. 2B (Arrow #1). To reduce the likelihood of identifying a marker associated with a trivial source of change such as the amount of material in the sample, we sought to identify a second ion pair with levels that either remained relatively constant between time points or appeared more abundant in the samples collected at $t = 8$ h compared to samples collected at $t = 0$ h. The second ion we selected is indicated in Fig. 2B (Arrow #2). We then used the ratio of intensities for the first and second ions as our biomarker; we refer to the ratio as the fatigue biomarker index (FBI) in the remainder of this paper.

3.3. Fatigue biomarker index

Levels of the two peptide components of the FBI were measured for all samples and the FBI was calculated as described above. Because absolute values of FBI spanned a range of more than five orders of magnitude, we report FBI values on a logarithmic scale. The FBI values from the start of the study likely arose from a different distribution than the FBI values measured near the end of the study (Fig. 3, Mann–Whitney test, $P < .05$, study arms tested independently; $t = 0$ h versus $t = 8$ h).

3.4. Sequencing of FBI peptides

The amino acid sequences of the two component peptides for the FBI were determined by high-resolution mass spectrometry. The following sequences were determined for the peptide components of the FBI: GGHPPPP (numerator) and ESPSLIA (denominator). LC–MS analysis of synthesized peptides was used to confirm these sequences.

4. Discussion

4.1. Summary

We used low- and high-resolution LC–MS analysis, as well as custom software, to evaluate changes in the small-molecular-weight components of saliva during prolonged physical exertion. A ratio of ion intensities for two novel peptides, derived from acidic and basic proline-rich proteins (see Section 4.2), was identified as an objective marker for the evaluation of fatigue. While LC–MS methods were used to identify these peptides, efforts are underway to raise antibodies against them, allowing for simpler and less costly methods of detection.

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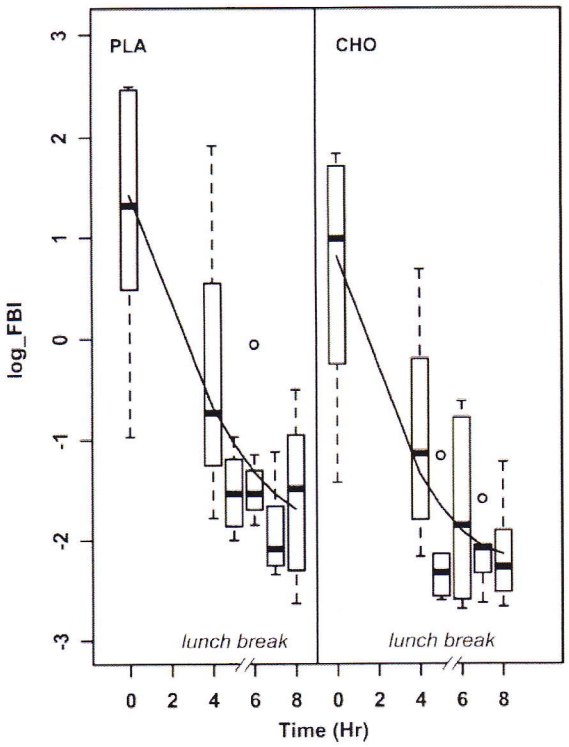

Fig. 3. Kinetics of FBI. Panels show data from the two trials. The solid black line shows the best-fit line of the linear-mixed effects model shown in Model 2. While there was a significant offset to the intercept term across the two trials, there was no difference in the curvature of the fits, suggesting that the kinetics of change were similar. The best-fit parameters for Model 2 were as follows: $\beta_0 = 1.42 \pm 0.32^*$, $\beta_1 = -0.66 \pm 0.15^*$, $\beta_2 = 0.034 \pm 0.019^*$, $\gamma_{02} = -0.61 \pm 0.40^*$, $\gamma_{12} = -0.047 \pm 0.21$, $\gamma_{22} = 0.008 \pm 0.03$ ($\pm$ SE, $^*P < .05$).

4.2. Peptide sequences and potential parent proteins

Each of the amino acid sequences of the two heptapeptides used in the FBI ratio is found in only one class of human proteins. The heptapeptide, GGHPPPP, is found in the acidic proline-rich proteins (salivary acidic proline-rich phosphoprotein 1/2, SwissProt P02810.2) while the heptapeptide, ESPSLIA, is found in the basic proline-rich proteins (basic salivary proline-rich protein 1, SwissProt P04280.2; basic salivary proline-rich protein 2, SwissProt P02812.3). We are unaware of any proteases known to recognize the sequence surrounding either of these heptaptides. However, several recent reports have identified similar peptide fragments from several different salivary proteins in both whole and parotid saliva from humans (Hardt et al., 2005; Helmerhorst and Oppenheim, 2007; Campese et al., 2009).

4.3. Changes within the autonomic nervous system (ANS) accompanying fatigue

Prolonged physical exercise is a form of stress known to induce changes in the ANS. Classically, the stressor leads to activation of the sympathetic nervous system (SNS) and withdrawal of the parasympathetic nervous system (PSNS) (Klein and Corwin, 2002). Salivary glands are innervated by neurons from both the SNS and PSNS. The roles of these nerves remain an active area of research, although there is general agreement on some of their functions (Proctor and Carpenter, 2007). In general, PSNS inputs are believed to drive constitutive secretion of a fluid-rich saliva containing only moderate amounts of protein, while SNS inputs influence the amount and distribution of protein secreted (Garrett, 1987). Morphological evidence also supports the different roles of sympathetic and parasympathetic neurons, as demonstrated by significant differences in acinar secretory granule appearance following secretion driven by sympathetic or parasympathetic neurons (Johnson et al., 1987; Proctor and Carpenter, 2007). Moreover, pharmacological studies have demonstrated that the catecholamines secreted by SNS neurons influence the synthesis of salivary peptides (Abe and Dawes, 1978; Abe et al., 1980; Garrett and Thulin, 1975; Johnson, 1984; Johnson et al., 1987; Johnson and Cortez, 1988; Nederfors et al., 1994, 1995). Considering the roles of the ANS in responding to prolonged physical exertion and in regulating salivary gland physiology, it should not be surprising that changes in fatigue level gave rise to measurable changes in the composition of saliva.

Indeed, salivary alpha-amylase (sAA) levels have previously been discussed as an indicator of ANS activity (Granger et al., 2007). However, it is important to note that pharmacological studies have demonstrated that chronic exposure to sympathomimetic agonists leads to decreases in sAA (Johnson, 1984) while acute exposure to the same agents leads to increases in sAA (Amsterdam et al., 1969). This bi-directional response in sAA levels associated with increases in catecholamines makes it unclear whether sAA would still be useful for measuring changes in ANS activity.

4.4. Previous MS investigations of small-molecular-weight peptides in saliva

Early investigations of salivary proteins demonstrated the importance of proteolytic processing in determining the final composition of saliva. Because whole saliva is exposed to the environment, both endogenous and exogenous proteases were recognized as the enzymes which could be responsible for processing of salivary proteins. The role of the peptide fragments generated remains unclear, although subsequent studies have demonstrated that many of the peptides have anti-microbial activity (Hardt et al., 2005). More recent studies of parotid saliva have revealed that post-translational proteolytic processing occurs within secretory vesicles and/or within the salivary ducts during transport to the oral cavity. Studies using a similar LC–MS approach have described numerous peptide fragments arising from salivary proteins, including histatin and proline-rich proteins (Hardt et al., 2005; Helmerhorst and Oppenheim, 2007). More traditional approaches have also demonstrated the instability of salivary proteins in the oral environment (Campese et al., 2009).

5. Conclusion and limitations

We have described changes in the small-molecular-weight proteome of saliva associated with prolonged physical exercise, leading us to propose an objective biomarker index for the analysis of fatigue level. While our study included only a small cohort of male recreationally trained cyclists, future studies will include much larger populations with a mix of genders, a wider range of basal fitness levels and a greater range of ages. Future studies also will employ more frequent sampling regimes and examine whether the changes observed occur when fatigue arises from other stressors such as dehydration or sleep deprivation. Moreover, it is important to determine whether the circadian cycle influences the levels of these particular peptides. Finally, the development of antibodies for detection of the individual peptide components of the FBI should allow for faster and lower cost measurements.

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