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A Method to Determine the Kinetics of Multiple Proteins in Human Infants with Respiratory Distress Syndrome

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Abstract

We report a method to measure in vivo turnover of four proteins from sequential tracheal aspirates obtained from human newborn infants with respiratory distress syndrome using targeted proteomics. We detected enrichment for all targeted proteins approximately 3 hours from the start of infusion of [5,5,5-²H₃] leucine, secretion times that varied from 1.2 to 2.5 hours, and half lives that ranged between 10 and 21 hours. Complement factor B, a component of the alternative pathway of complement activation, had an ~2-fold longer half life than the other three proteins. In addition, the kinetics of mature and carboxy-terminal tryptic peptides from the same protein (surfactant protein B) were not statistically different (p=0.49).

Keywords

Protein Turnover; Respiratory Distress Syndrome; Selected Reaction Monitoring; SRM; Protein Kinetics; Protein Metabolism

Introduction

Mass spectrometry has become an indispensable tool for addressing complex biological problems in the multifaceted rapidly expanding field of proteomics. Common problems addressed by mass spectrometry include determination of primary sequences of proteins, identification of sites of post translational modifications, quantification of proteins across different physiological states, and evaluation of protein-protein interactions. Comparative studies of specific disease states have focused on differences in the presence, modification, and/or abundance of proteins at a single time point. Although these studies can be informative, a single time point provides only a “static snapshot” of protein abundance in a disease state. Multiple factors that alter protein synthesis or degradation including developmental, genetic, and environmental factors (e.g., diet, exercise, smoking) can change

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protein abundance. While longitudinal sampling and controlled systems^{1–2} can mitigate protein variability in comparative studies, these strategies do not address measurement of the “missing dimension in proteomics,”³ the rate of protein synthesis and degradation (i.e., protein turnover). Protein turnover can be used to characterize both normal physiology and disease states.^{4–6} In addition, the determination of differential kinetics between diseased and control would be helpful in the discovery of therapeutic and diagnostic targets and monitoring treatment efficacy. Furthermore, protein turnover data might offer new insights into the discrepancies observed between proteome and transcriptome data.^{7–8}

Traditionally, protein turnover is measured by enrichment of a labeled tracer with gas chromatography/mass spectrometry after infusion of a stable isotopically labeled amino acid, isolation of the proteins of interest from a complex biological fluid, hydrolysis and derivatization.^{9–11} However, this method is limited by low throughput¹² and variable efficacy of protein isolation which may reduce accuracy and permit analysis of only a single, abundant protein. Recently, studies have used SIL tracers and proteomics to determine comprehensive protein turnover. These experiments have focused on *in vitro* studies^{3, 13} or animal models for *in vivo* studies (e.g., mice,^{14–15} chicken¹⁶). In all studies the relative isotope abundance (RIA), a measure of enrichment of the tracer, was relatively high (>30%). To permit comprehensive concurrent assessment of turnover of multiple proteins for both systems biology studies and statistical evaluation, new methods to measure proteins with low levels of enrichment must be developed.

In this report, we extend a previous study¹⁷ to demonstrate the ability to measure low levels of enrichments (RIA = 1–4%) and calculate kinetic parameters for multiple proteins in tracheal aspirates from human neonates using selected reaction monitoring-mass spectrometry preceded by solid phase extraction to remove phospholipids. We chose four proteins, surfactant protein B, prosaposin, complement factor B, and lactotransferrin. We could reproducibly measure enrichments as low as 1% from proteotypic peptides from each protein. Half lives were similar between prosaposin, surfactant protein B, and lactotransferrin (10–12 hours); however, complement factor B had a half life of 21 hours. This longer half life suggests an alternate degradation pathway. In addition, we demonstrate that the kinetics of two different peptides from mature and carboxy-terminal surfactant protein B are indistinguishable.

Methods

Materials

Formic acid, ammonium bicarbonate, DTT, and iodoacetamide, were obtained from Sigma Aldrich (St. Louis, MO). Proteomics grade trypsin was purchased from Promega (Madison, WI). HPLC grade acetonitrile was from Burdick & Jackson (Muskegon, MI).

Infusion Protocol

The protocol for intravenous infusion of deuterated leucine has been described in detail elsewhere.¹⁷ In brief, each infant received a 5 min priming dose of 18 $\mu\text{mol/kg}$ followed by a 6-hour continuous infusion of 18 $\mu\text{mol/kg/hour}$ of [5,5,5-²H₃] leucine. Samples were collected via routine airway suctioning of the endotracheal tube after instillation of 0.5 mL of normal saline and aspiration into a sterile trap. Time points were collected every 3–6 hours over a 5-day period and stored at –80°C until analysis. All protocols for the sample collection, measurements and analysis were approved by Institutional Review Boards at both Washington University and the University of Washington, and parents of all infants provided informed consent for their infants to participate.

Sample Preparation

Using a Bradford assay, we measured total protein concentrations in tracheal aspirate samples and discarded samples with protein concentrations <150 µg/mL due to difficulty in obtaining adequate signal (6 of 77 samples). For sample preparation, 100 µL of aspirate was added to 100 µL of 50 mM ammonium bicarbonate (pH=7.8) with 0.2% (w/w) PPS silent surfactant (Protein Discovery Inc, Knoxville, TN). The mixture was incubated at 100°C for 5 minutes. DTT was added to a final concentration of 5 mM and the mixture was incubated at 60°C for 30 minutes to reduce disulfide bonds. Alkylation of free cysteines was performed by the addition of iodoacetamide (15 mM), and the mixture was then incubated at RT in the dark for 30 mins. Finally, 10 µg of trypsin was added to the mixture and the sample was digested for 2 hours at 37°C. After digestion, 10 µL of 5M HCl was added to each sample.

Prior to LC-MS analysis, aspirate samples were subjected to solid phase extractions (SPE) using a mixed-mode Oasis MCX cartridge (Product # 186000256 Waters Corporation, Boston, MA). Cartridges were conditioned with the buffers in the following order: 1) 1 mL of 100% methanol; 2) 1 mL of 3/97 ammonium hydroxide/water; 3) 2 mL of 100% methanol; 4) 3 mL of 0.1% formic acid in water. Samples were loaded on column and washed with 1 mL of 0.1% formic acid in water. Following removal of highly hydrophobic species (e.g., negatively charged lipids) with 0.1% formic acid in methanol, peptides were eluted with 1 mL of 3/97 ammonium hydroxide/methanol. The eluent was then lyophilized *in-vacuo* at 37°C and reconstituted in 50 µL water/acetonitrile (98/2) with 0.1% formic acid. We found this method critical for removing the large amounts of phospholipids that were present in the aspirate samples which are detrimental to the nano-flow LC-SRM-MS method.

Nano-flow LC-SRM-MS

A 2 µL aliquot of the reconstituted sample (~1–3 µg of protein) was flushed at a flow rate of 2 µL/min onto a 3 cm trap packed in house with C12 4 µm (Phenomenex, Torrance, CA, USA) stationary phase. After ~10 trap-volume washes, the 10-port valve switched and the gradient and data collection commenced. Buffers A and B were 99.9/0.1 water/formic acid and 99.9/0.1 acetonitrile/formic acid, respectively. The gradient ramped from 3% B at 3 mins to 32% B at 50 mins, and then increased to 80% B and held constant for 5 minutes. Initial conditions 97% A were restored for the final 14 minutes of the run. Total analytical run time was 70 minutes. For selected reaction monitoring mass spectrometry (SRM-MS), a ThermoFisher Vantage (San Jose, CA) was used with Q1 set to a resolution of 0.2 *m/z* and Q3 set to a resolution of 0.7 *m/z*. Three transitions were monitored for each peptide and each sample was measured in duplicate.

Method Development

Protein sequences in fasta format were imported into an in-house developed software program called Skyline.¹⁸ Skyline performed an in-silico tryptic digestion of all proteins, and peptides were filtered for uniqueness in Skyline by searching the NCBI Human Proteome Database. Unique peptides between 8–25 amino acids in length were retained. All proteotypic peptides that contained only a single leucine were monitored and the data was then exported back into Skyline¹⁸ where a single peptide was chosen as a proxy for each protein. For SP-B, the kinetics of two peptides from either the C-terminus or mature forms were evaluated. This final method was then exported directly from Skyline and samples were analyzed with a dwell time of 100 ms. These experiments enabled the determination of the best ionizing leucine containing peptide for maximum sensitivity for each protein.

Kinetic calculations

Raw files were exported back into Skyline for data analysis. The isotope ratio of each peptide was calculated by integrating the area of the M+3 isotope peak and dividing that by the area of the M+0 peak. This integration and ratio calculation were performed automatically in Skyline. For each infant, a time point prior to infusion of deuterated leucine (i.e., T=0) was analyzed. The isotope ratio was then converted to a measure of the enrichment by subtracting the isotope ratio at T=0 (R_0) from the ratio at T=t (R_t). This calculation of the tracer-to-tracee ratio ($R_t - R_0$) corrected for endogenous signal in the M+3 channel.

The delay between the start of the infusion and the appearance of the label in the peptide, known as the secretion time (T_s), was calculated by fitting a regression line to the linear part of the synthesis curve and extrapolating it to baseline enrichment.¹⁹ Secretion time represents the time required for d_3 -leucine transport from the blood stream into the lung cells, incorporation of label into proteins, and secretion of labeled peptides into the alveolar lumen of the lung. Fractional catabolic rates (FCR) were calculated for each peptide by taking the slope from the linear regression after natural logarithmic transformation of the clearance trend in each enrichment curve.²⁰ Fractional synthetic rates were not reported due to the need to accurately determine the amount of [5,5,5- 2H_3] leucine in the lung cells. However, if one assumes steady state (i.e., protein abundance does not change) then the FSR is equivalent to the FCR. Half lives were calculated by taking the natural log of 2 divided by FCR.^{17, 20}

Results and Discussion

Measuring Kinetics of Multiple Proteins

Table 1 displays the peptides monitored from each of the 4 proteins. We chose these proteins based on the detectability of a tryptic peptide containing a single leucine and the biological significance in human infants with respiratory distress syndrome. The ~10 kb surfactant protein B gene (*SFTPB*) encodes a 381 amino acid pre-proprotein that is sequentially proteolytically cleaved at amino and carboxy termini and glycosylated in pulmonary alveolar type 2 cells to yield the highly hydrophobic, 8.6 kDa mature protein. Surfactant protein B is structurally homologous to the saposin family of proteins²¹ and is critical for surfactant function and homeostasis.^{22–26} Infants with rare, recessive, loss of function *SFTPB* mutations develop lethal respiratory distress syndrome due to surfactant deficiency and survive only with lung transplantation.²⁷ Complement factor B is also expressed by alveolar type 2 cells²⁸, is an essential component in the alternative pathway of complement activation, and contributes to regulation of airway inflammation.²⁹ Its abundance has been reported to be lower in infants with respiratory distress syndrome compared to controls.³⁰ Prosaposin is a multifunctional protein and precursor to saposins A, B, C, and D which are involved in the degradation of sphingolipids.^{31,32–33} Lactotransferrin is an 80kDa glycoprotein³⁴ commonly found in various fluids including milk, saliva, and nasal secretions.³⁵ It is part of the innate human defense system where its capacity to sequester iron provides antibacterial activity – particularly in the mucus of human infants.³⁴

Figure 1A-D displays the enrichment curves from a representative infant for surfactant protein B, prosaposin, complement factor B, and lactotransferrin, respectively. The 2H_3 -leucine tracer enrichment could readily be detected in all 4 proteins three hours from the start of the infusion. Maximum enrichment levels for surfactant protein B and lactotransferrin were between 5–8% but significantly lower (1–3%) for prosaposin and complement factor B. For the most part, similar enrichment curves were seen for the four proteins with the time from baseline to peak enrichment and back all occurring in less than

48 hours. It is important to note that in some cases, we observed a secondary uptake after initial degradation (Figure 1B and 1C). This observation could be attributed to recycling of the labeled amino acid³⁶ as degradation of labeled proteins provides a minimal increase in the labeled amino acid precursor pool. This increase can then be detected in the enrichment curve as the label is reincorporated into newly synthesized proteins.

Table 2 summarizes the secretion time, rate constant, and calculated half live for each protein. Secretion times (T_s) varied between 1.2 and 2.5 hours consistent with a similar secretion pathway for the four proteins. Comparable half lives for surfactant protein B, prosaposin, and complement factor B suggest a similar degradation mechanism. However, we found that complement factor B had ~2-fold longer half life than the other proteins. For some infants, data quality did not permit confident calculation of kinetic parameters. Specifically, low overall signal intensity and low levels of enrichment compromised prosaposin kinetic parameters for 4 out of the 6 infants in the study.

Comparison of different peptides from surfactant protein B

When proteins exist in multiple isoforms or the peptide measured does not reside in the functional domain of the protein, using a single peptide as a proxy for the metabolism of the functional protein may be misleading. In this study and in a previous study¹⁷, the most abundant and reproducible peptide for surfactant protein B resides on the carboxy-terminus of the pro-protein³⁷ (Figure 2A). As a result, these studies measure the turnover of a surfactant protein B peptide that may not accurately reflect kinetics of mature surfactant protein B. In addition, via a combination of western blot analysis and protein-cleavage isotope dilution mass spectrometry, we have confirmed multiple isoforms of SP-B in tracheal aspirates from infants.^{17, 38}

To compare kinetics of different surfactant protein B peptides, we measured the enrichment of both the carboxy-terminus peptide and a peptide from the mature form (Figure 2A) of the protein in a subset of infants (n=4). Figure 2B displays a scatter plot of the enrichment values from these two surfactant protein B peptides. Overall, the enrichment curves look similar as the two lines represent a locally weighted scatter-plot smoothing (LOWESS) regression plotted through each respective data set. Using a paired t-test for differences in means of the half lives calculated from each individual curve for the two different peptides, we found no statistical difference (p=0.49). The indistinguishable kinetics of these two peptides suggest that either the main isoform present in these infants is a combination of the mature and carboxy-terminal peptides or that various isoforms have similar turnover rates.

Conclusion

We have detailed a method to measure the kinetics of multiple proteins in human infants with low levels of enrichment using targeted proteomics. While similar secretion times were observed in all 4 proteins, one (complement factor B) had twice the half life of the other 3 proteins. The similarity in kinetics of a carboxy-terminus peptide and a mature peptide from surfactant protein B suggests tight kinetic linkage of these isoforms or a combination of both peptides in measurement of enrichment of the major surfactant protein B isoform. Future studies will look at correlations between clinical and kinetic turnover data to identify peptide kinetic characteristics associated with disease acuity, progression, and response to therapeutic interventions.

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Figure 1.

Enrichment curves (Tracer-to-tracee ratio vs. time) corresponding to **A)** SP-B (C-terminus peptide); **B)** prosaposin; **C)** complement factor B; and **D)** lactotransferrin. Enrichment was readily observed 3 hours from the start of infusion. Significantly higher levels of enrichment were observed for SP-B and lactotransferrin. Infusion of [5,5,5- $^2\text{H}_3$] leucine ended after 6 hours.

A 1 MAESHLLQWL LLLLPTLCGP GTAAWTTSSL ACAQGPEFWC QSLEQALQCR ALGHCLQEVW
 61 GHVGADDLCQ ECEDIVHILN KMAKEAIFQD TMRKFLEQEC NVLPLKLLMP QCNQVLDYF
 121 PLVIDYFQNN TDSNGICMHL GLCKSRQPEP EQEPGMSDPL PKPLRDPLPD PLLDKLVLPV
 181 LPGALQARPG PHTQDLSEQQ FPIPLPYCWL CRALIKRIQA MIPKGALAVA VAQVCRVVPL
 241 VAGGICQCLA ERYSVILLDT LLGRMLPQLV CRLVLRCSMD DSAGPRSPTG EWLPRDSECH
 301 LCMSVTTQAG NSSEQAIPQA MLQACVGSWL DREKCKQFVE QHTPQLTLV PRGWDHHTC
 361 QALGVC GTMS SPLQCIHSPDL

Figure 2.

A) The protein sequence of Pro-SP-B displaying the N-terminus (blue), mature (black) and C-terminus (red) forms of the protein. The underlined peptides were targeted for analysis. B) A comparison between the enrichment curves of peptides from the C-terminus (i.e., nonfunctional form) and from the mature (i.e., functional form) form of SP-B. Both peptides demonstrate similar kinetics. The difference in the average calculated half life between the two peptides was insignificant ($p=0.49$).

Table 1

Peptides monitored as a proxy for each protein and their respective biological significance. The 2⁺ charge state was monitored for each precursor.

Proteins of Interest	Peptide Monitored	Transitions monitored	Biological Significance
surfactant protein B	R.SPTGEWLPR.D K.GALAVAVAQVCR.V	y ₆ ,y ₅ ,y ₄ y ₇ ,y ₆ ,y ₅	Surfactant Homeostasis
prosaposin	K.EILDAFDK.M	y ₆ ,y ₅ ,y ₄	Catabolism of sphingolipids
complement factor B	R.STGSWSTLK.T	y ₇ ,y ₆ ,y ₄	Inflammation
lactotransferrin	K.FQLFGSPSGQK.D	y ₉ ,y ₈ ,y ₇	Immune response

Table 2

A summary of the kinetics for the four targeted proteins.

	Secretion Time (hrs) Avg $\pm$ SE	FCR (%/hour) Avg $\pm$ SE	Half Live (hrs) Avg $\pm$ SE
surfactant protein B n=6	2.54 $\pm$ 0.15	0.058 $\pm$ 0.0010	12.1 $\pm$ 0.2
Prosaposin n=2	2.06 $\pm$ 0.70	0.062 $\pm$ 0.008	11.7 $\pm$ 1.7
compliment factor B n=5	1.46 $\pm$ 0.15	0.033 $\pm$ 0.0010	21.2 $\pm$ 0.6
Lactotransferrin n=5	1.23 $\pm$ 0.20	0.072 $\pm$.0037	10.1 $\pm$ 0.5