POSTER SESSION

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(1) A New Sample Preparation Method for Tissue Proteomics

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Introduction: Tissue proteomics is increasingly recognized for its role in biomarker discovery and disease mechanism investigation. However, the high dynamic range in protein abundances and solubility still present tremendous challenges. Herein, we have developed a new sample preparation method featuring a sequential extraction and an MS-compatible surfactant which has significantly improved the detection of low abundance proteins and membrane proteins. **Methods:** Swine heart tissue samples were excised from healthy Yorkshire domestic pigs, snap frozen in liquid N2, and stored under -80°C before use. The first extraction (E1) was obtained by homogenizing the tissues at 4°C in HEPES buffer. The remaining pellet was extracted with HEPES buffer again to obtain the secondary extraction (E2). Meanwhile, an equal amount of pellet sample was homogenized in HEPES buffer, MS-compatible surfactant (E2S) as well as SDS. The protein samples were further trypsinized via in-solution digestion follow by LC/MS/MS analyses for protein identification. **Results:** The protein assay data showed 8 and 4 times the protein amounts in E1 and E2S, respectively, as compared to E2. There were no differences between E2S and E2 with SDS. The SDS-PAGE results also revealed that protein amounts were significantly higher in E1 and E2S than E2. A total of 200, 223, and 254 proteins were identified in E1, E2, and E2S, respectively, in triplicate analyses. The reproducibility of proteins identification in separate runs was 75%, 84%, and 82% in E1, E2, and E2S, respectively. The E1 was dominated by high abundance blood proteins such as albumin and hemoglobin which presumably came from the blood remaining in tissue samples. Notably, the highly abundant blood proteins were significantly reduced in E2, and E2S based on spectra count quantification. In contrast to E1, unique proteins could be identified in secondary extraction without (E2) or with surfactant (E2S). Overall, there were a total of 102 unique proteins identified in E2S, in comparison to the 53 in E1 and 64 in E2. According to the Gene Ontology Database, 35.8%, 40.6%, and 31.4% of the proteins belong to cytosol (soluble proteins) in E1, E2, and E2S, respectively. Additionally, 20.8%, 20.3%, and 24.5% of the proteins were located on membrane in E1, E2, and E2S, respectively. The results confirm that our sequential extraction method reveals an abundance of low abundant proteins.

(2) Mapping the Distribution of Intranasally Administered Arginine Vasopressin (AVP) in Rat Brain Using MALDI Imaging Mass Spectrometry

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Mapping of distribution of intranasally administered arginine-vasopressin (AVP) in rat brain by MALDI imaging mass spectrometry (MS) provides a novel means to evaluate the efficacy of drug delivery. AVP is a nonapeptidic hormone that is secreted from hypothalamic nuclei. AVP plays a role in modulating social behaviors, including memory formation and pair bonding [1], so it may have potential as a therapeutic[1]. Peripheral AVP crosses the blood-brain barrier poorly, so intranasal administration has been used to target it to the brain[2]. By applying MALDI imaging MS on drug treated tissue sections, the distribution of unlabeled drug and associated metabolites can be well characterized[3], which is critical for interpreting AVP's pharmacodynamics and pharmacokinetic profiles.

In this study, AVP was intranasally administered to adult, female rats, followed by upper body saline perfusion and brain dissection. For a brain, one hemisphere was separated into 10 sections (cerebellum, spinal cord, brain stem, mid brain, frontal pole, occipital pole, amygdala, hypothalamus, pituitary gland and olfactory bulb) for liquid-phase protein extraction; the other hemisphere was embedded in gelatin solution and frozen in -80°C. Tissue sections were extracted in acidified methanol for MS analysis. Frozen hemisphere was cut into 12m sagittal sections for profiling and imaging analysis on Ultraflex MALDI-TOF/TOF mass spectrometer (Bruker Daltonics, Billerica, MA) and MALDI-LTQ-Orbitrap mass spectrometer (Thermo Scientific, San Jose, CA) with spatial resolution of 50-100m. Rats without AVP treatment were used as controls.

Preliminary results on tissue extractions revealed that intranasally administered AVP appears to reach several areas of the rat brain including amygdala, spinal cord/brain stem and hypothalamus; while in control rat, endogenous AVP signal was only detected in pituitary gland. Tissue washing and matrix application methods have been optimized for tissue profiling and imaging. Preliminary profiling and imaging experiments have detected AVP signals in the hypothalamus region. More imaging experiments are planned with the optimized tissue preparation procedures. These preliminary results suggest that intranasally administered AVP is rapidly delivered to several brain regions. Along with imaging results of the distribution of AVP signal in rat brain, these results may also contribute to better understanding of the pathways for intranasal drug targeting to the brain as well as AVP's pharmacokinetics.

(3) Dissecting Human Skeletal Muscle Troponin Proteoforms by Top-down Mass Spectrometry

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Skeletal muscle, one of the most abundant tissues in the human body, is composed of a heterogeneous collection of muscle fibers, which perform various functions. Skeletal muscle troponin (sTn) regulates skeletal muscle contraction. It consists of 3 subunits, troponin I (TnI), troponin C (TnC), and troponin T (TnT). TnI inhibits the actomyosin Mg^{2+}-ATPase and TnT is the tropomyosin (Tm)-binding subunit. Cardiac muscle and skeletal muscle share many similarities and modifications of cTn are known to affect the muscle contractility mechanisms. We hypothesize that modifications of sTn might be related to neuromuscular diseases. However, the modification status of sTn remains unknown. Herein, we have employed top-down mass spectrometry (MS) to decipher the modifications of human sTnT and sTnI. In this study, we determined the masses of sTnT and sTnI with high mass accuracy, and characterized the sequences of the observed isoforms of sTnT and sTnI. In this study, the mono-phosphorylated form of slow sTnT(II) (ssTnT(II)), ssTnT(III), mono-phosphrylated form of ssTnT(III), fsTnI, and ssTnI were identified. Furthermore, the phosphorylation site of sTnT(III) was mapped to Ser1 (with removal of the N-terminal methionine) via MS/MS. This is the first study to comprehensively characterize human sTn, and also the first to identify phosphorylation site for the human sTnT.

(4) DiLeu Isobaric Tagging Reagents for Complex Quantitative Proteomics

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Multiplexed isobaric labeling has become a routine method for mass spectrometry-based relative quantitation of proteins and endogenous peptides, and several commercially available products are available for such experiments. However, the high cost of these reagents is often prohibitive. We have developed our own set of multiplexed isobaric labeling reagents, DiLeu, featuring N, N-dimethylated leucines as reporters that can be readily synthesized using commercially available chemicals at greatly reduced cost. The DiLeu reagent resembles the general structure of other isobaric labeling reagents in that it contains a reporter group, a balance group, and an amine-reactive group for targeting the N-terminus and Lysine side chain of a peptide. Each incorporated label results in a mass shift of 145.1 Da, and intense reporter ions at m/z 115.1, 116.1, 117.1, and 118.1 are observed for the pooled samples upon tandem-mass fragmentation. To demonstrate the reagents' utility in complex proteomic studies, four aliquots of a *Saccharomyces cerevisiae* yeast lysate tryptic digest reference sample from Promega (Madison, WI) were labeled in triplicate with 4-plex DiLeu reagents and combined in 1:1:1:1 and 1:5:2:10 ratios. Samples were cleaned up via strong cation exchange (SCX), and LC-MS/MS analysis was performed

on a Thermo Scientific Orbitrap Elite system using HCD fragmentation to identify proteins and demonstrate quantitative performance of 4-plex DiLeu reagents with complex proteomics samples. Using ProteomeDiscoverer proteomics software suite for data processing, quantitation, and Mascot database searching, DiLeu labeled yeast peptide quantitative accuracy was shown to be accurate to within 11% for 351 proteins identified with 1% FDR and a Mascot score >50 across triplicate runs.

(5) Development of novel 8-plex N,N-dimethylated Leucine (DiLeu) Isobaric Labels for Quantitative Proteomics and Peptidomics

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Mass spectrometry-based analyses typically do not produce reliable quantitative data. Numerous label-based quantitation approaches for mass spectrometry exist, but isobaric labeling methods provide both identification and relative quantitation during a single MS/MS experiment. One popular but costly type, iTRAQ, utilizes up to eight isobaric labels with reporter ions spaced one Da apart upon MS/MS fragmentation. Our lab previously developed a cost-effective set of 4-plex isobaric reagents, dimethyl leucine (DiLeu), with comparable performance to 4-plex iTRAQ. The development of 8-plex DiLeu labels could increase the number of biological replicates or states compared in one experiment while retaining the low cost of the 4-plex labels. Mass spectra revealed that the activated 8-plex DiLeu products were correctly synthesized (m/z 336.3). Bradykinin standards were completely labeled in EtOH by a 100x molar excess of each DiLeu reagent for two hours. Reverse phase chromatography retention times of labeled bradykinin standards were all ~25 min and appeared to be unaffected by the incorporation of deuterium isotopes into the labels due to their placement around polar functional groups. MS/MS spectra of labeled bradykinin showed intense reporter ions at m/z 114.1-119.1, and 121.1-122.1 while still allowing for peptide identification from b and y product ion fragments. The dynamic range of bradykinin quantitation increased when the 8-plex reagents were used as a 4-plex set spaced 2 Da apart. A BSA tryptic digest (20 µg) was dissolved in water and labeled with a 25x weight excess of 8-plex DiLeu reagent for two hours. Analysis of the MALDI mass spectra revealed that only two of the identified peptides were unlabeled. Of the 25 labeled, identified peptides, 18 were completely labeled. Synthesis conditions are currently being optimized to provide higher purity DiLeu reagents and increase labeling efficiency for more complex peptidomic and proteomic samples.

(6) Probing the Molecular Heterogeneity of the Heart Using Top-down Mass Spectrometry

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Although the heart must function as a single contractile unit, there is increasing evidence that significant molecular heterogeneity exists. This heterogeneity is not only responsible for optimal cardiac function but also influences myocardial remodeling following cardiac injury or in heart failure. However, to date, there has not been an in-depth study of the molecular heterogeneity in healthy and diseased hearts. In this study, we utilized a top-down targeted proteomics approach, combining high-resolution mass spectrometry (MS) with affinity purification, to study the molecular heterogeneity of the swine heart using cardiac troponin I (cTnI) and troponin T (cTnT) as probes. We found that cTnI phosphorylation exhibited significant regional heterogeneity with higher levels of cTnI phosphorylation observed in the ventricles, in comparison to the atria, in healthy swine hearts. In addition, cTnI phosphorylation was significantly lower in the epicardium, in comparison to the myocardium, of the left ventricle of healthy swine; however, while cTnI phosphorylation varied both regionally and transmurally, the extent of cTnT phosphorylation was constant both across the heart and transmurally. Interestingly, in ischemic swine hearts there was also a proximity-associated decline in cTnI phosphorylation. Our data supports the existence of both molecular heterogeneity in healthy hearts and the altered post-translational modification of key myofilament proteins in diseased hearts.

(7) A Quantitative Map of the Liver Mitochondrial Phosphoproteome Reveals Post-translational Control of Ketogenesis

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Mitochondria are dynamic organelles that play central roles in a diverse array of metabolic processes. Elucidating mitochondrial adaptations to changing metabolic demands and the pathogenic alterations that underlie metabolic disorders represent principal challenges in cell biology. Here, we performed multiplexed quantitative mass spectrometry-based proteomics with isobaric tags to chart the remodeling of the liver mitochondrial proteome and phosphoproteome in more than 50 mice during both acute and chronic physiological transformations. In particular, we analyzed mitochondria from both lean (wild type) and obese (leptin deficient) mice at two different ages (4 weeks or 10 weeks), each from two common strains (C57BL/6J (B6) and BTBR). Using this same approach, we assessed the reversibility of these modifications in wild type B6 mice during acute fasting and refeeding. Collectively, we identified 811 phosphosites — including more than 100 not previously reported — on 295 mitochondrial proteins, and revealed that a large proportion of these modifications are dynamic and reversible during these acute and chronic transformations. Our analyses further reveal that mitochondrial protein phosphorylation is a key mechanism for regulating ketogenesis in obesity and type 2 diabetes. Specifically, we demonstrated that phosphorylation of a conserved serine on Hmgcs2 significantly enhances its catalytic activity in response to increased ketogenic demand. Collectively, our work describes the plasticity of the mitochondrion at high resolution and provides a framework for investigating the roles of proteome restructuring and reversible phosphorylation in mitochondrial adaptation. This data is freely available through our online resource, MitoMod (http://mitomod.biochem.wisc.edu).

(8) A Novel Strategy for Quantification of Primary Amine-containing Metabolites Using N,N-dimethyl Leucine Reagents via Capillary Electrophoresis-electrospray Ionization-mass Spectrometry

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Metabolites can directly indicate the states of cells in biological systems. The changes of their expression level are associated with various processes such as aging, disease and drug administration. Therefore, the quantitative analysis of metabolites is of great significance to understand those complex processes. Previously, our lab-developed DiLeu isobaric labels have been mainly employed to quantify peptides by efficiently reacting with the N-terminus of peptides. Here, we investigate the applicability of 4-plex DiLeu tags to label amine-containing metabolites. To accommodate the small sample amount in some biological applications, CE-ESI-MS is utilized to analyze DiLeu-labeled metabolite standards spiked in the mouse urine sample as a proof-of-principle study. Mouse urine sample went through molecular weight cut-off filters and Strong Cation Exchange (SCX) ziptip before the labeling reaction to remove urinary protein, salts and other impurities. Detection sensitivity of the labeled metabolites in CE-ESI-MS was more than 50 fold greater compared to the unlabeled counterparts because of the increased ionization efficiency of analytes by the demethylated leucine. By using DiLeu isobaric tags, up to four samples can be analyzed simultaneously. Relative quantification of metabolites was achieved by calculating the intensity ratios of DiLeu reporter ions *m/z* 115, 116, 117, 118 in MS2 fragmentation. When labeled sample were combined at equal ratio, the ratio between reporter ions was within 15% accuracy, demonstrating the applicability and accuracy of DiLeu labeling technique in small molecule quantitation. Currently, 4-plex DiLeu reagents are employed for labeling mouse urine samples without metabolites standard spiking after sample pretreatment procedures. On-going studies aim to develop this technique for accurate and high-throughput quantification of metabolites for different kinds of complex biological samples.

(9) Development of Monolithic Columns with Immobilized Enzymes for Enhanced Proteomic and Peptidomic Analysis

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Mass spectrometry coupled to micro-separation techniques has become a primary tool for peptidomics and proteomics studies. In recent years, monolithic materials have been developed as not only chromatographic media but also biologically related support for immobilizing multiples types of reagents. Monoliths possess several advantages over particulate chromatography system, including high porosity, low back pressure at high flow rate, a high loading capacity and straightforward preparation in microfluidic devices. Herein we fabricated the N-acryloxysuccinimide (NAS)-based monolithic column with immobilized enzyme (trypsin) onto the column within silanized 200 µmi.d. fused-silica capillaries. Performance of immobilized enzyme reactor (IMER) was tested by directly passing 10µM intact bovine serum albumin (BSA) dissolved in ammonium bicarbonate buffer through the column, followed by fraction collectionprior to MALDI-TOF/TOF analysis. Comparison was made between the IMER digestion (seconds to minutes) with conventional in solution digestion (overnight); similar MS signals and protein sequence coverage were observed. Protein digestions from this enzyme reactor exhibited comparable peak intensities and a very similar enzyme activity was observed after two weeks storage. With mouse serum we observed a total of 160 proteins using IMER digestion coupled to LC-ESI-Orbitrap and 141 proteins from in solution digestion. Collectively, we demonstrate improved protein identification results with monolithic enzyme reactor followed by MS analysis. These results highlighted the potential of employing NAS-based monolithic column for fast and highly efficient sample preparation prior to MS-based analysis in proteomics studies.

(10) Mass Spectral Characterization of the Neuropeptidome of the Crayfish *Orconectes rusticus*

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The rusty crayfish, *Orconectes rusticus (O.rusticus)*, is a widely used model organism in basic behavioral neuroscience study. In addition, *O. rusticus* has become a formidable invasive species in the Midwest United States and caused various negative environmental and economic impacts. Neuropeptide (NP) has been indicated playing important roles in aggression, adaptation to environmental changes in other decapod crustacean species. Crayfish *O. rusticus* obtained from Trout Lake, Wisconsin was used. Here, we employed a multi-faceted mass spectral (MS)-based methodology and two complementary data processing strategies to determine the NP content in the sinus gland (SG) and brain in *O.rusticus*.

Database construction was accomplished by using both in-house compiled information and publicly available data, which contains known crustacean neuropeptides from published papers of Li lab and others.

The National Center for Biotechnology Information (NCBI) protein database was also used to obtain relevant known protein sequences. Brain and eyestalk samples were analyzed in parallel. In total, 145 high quality PSMs were made from MS/MS spectra. 98 peptides from 8 different neuropeptide families observed in previous mass spectral study of decapod crustaceans were successfully matched. Five isoforms of crustacean hyperglycemic hormone (CHH) precursor-related peptide (CPRP) and three of pigment dispersing hormone (PDH) have been reported in another crayfish *Orconectes limosus*. Extensive coverage of CPRPs from eyestalk extract was achieved, indicating that the CPRPs were highly conserved between these two *Orconectes* species. Several orcokinins were also observed in both species. NPs from other families, such as B-type allatostatins (AST-Bs), FMRFamide-like peptides (FLPs), SIFamides, and myosuppressin have previously reported in other crustacean species.

Moreover, 47 putative NPs from 12 families were also mapped, which were either from non-decapod crustaceans or from genetic studies. Matches were found in the linker-peptide regions of the crustacean cardioactive peptide (CCAP), FMRFamide-like peptides, red pigment concentrating hormone (RPCH),

orcokinin, SIFamide, and tachyknin. These results greatly increase the number of known signaling peptides in crustacean species and highlight the advantage of MS-based approaches toward neuropeptide discovery.

(11) Global Peptide Collision Cross-section Profiling on a Travelling Wave Ion Mobility Mass Spectrometer

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Since the introduction of commercial instrumentation, travelling wave ion mobility-mass spectrometry (TWIM-MS) has been applied to a variety of scientific inquiries. However, an unfortunate aspect of TWIM-MS is the need to use calibration standards to measure an ion's collision cross-section (CCS). Although great progress has been made in the development of accurate and straightforward calibration strategies, very few have attempted to couple TWIM CCS measurements with liquid chromatography proteomic/peptidomic experiments. A primary concern is that the instrument settings that favor ion transmission, such as higher bias and injection voltages in the ion optics, may be detrimental to CCS measurements. Herein, our aim is to develop and validate a large-scale TWIM-MS CCS calibration/measurement strategy compatible with global proteomic and peptidomic investigations.

Global CCS profiling has been available on home-built IM-MS instrumentation for over a decade. Our primary goal is to demonstrate this on commercial TWIM instrumentation by expanding recently published calibration strategies. While such capabilities may not be of much use for structural analysis of ions sprayed from native-like solvents, it could be utilized to improve proteomic/peptidomic identifications or study gas-phase structure of large peptide populations. The preliminary results give a positive indication for the accuracy of profiling complex mixtures. Further development will include CCS measurement in parallel with HDMSE and the use of lockspray for CCS calibration.

(12) The Investigation of Neuropeptides in Crustacean to Reveal the Neurotoxicity of Nanoparticles by MALDI-MS

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With the wide-spread use of nano-products in our daily life, many health concerns have been raised due to exposure to various nanoparticles. However, detailed molecular mechanisms are not well understood. Given the extensive regulatory role of neuropeptides in various physiological processes, we seek to measure the expression level changes of neuropeptides upon exposure to Ag nanoparticles in crustacean model organisms. Herein we will implement a multi-faceted strategy based on mass spectrometry to qualitatively and quantitatively analyze the chemical messengers involved under nanotoxicity stress in the nervous system. By mapping the spatial distribution of various neuropeptide families and assessing their relative abundance levels in several major neuroendocrine organs, peptidergic regulation of nanotoxicity can be probed with unprecedented detail. In this study, blue crab *Callinectes sapidus* is adopted as the animal model system. Different amounts (20�L, 50�L, 100�L, 200�L) of in-house synthesized Ag nanoparticles (50ppm) were injected into blue crab *Callinectes sapidus* at their leg joint while the control animal was injected with the same volume crab saline. Neuropeptides were extracted from dissected sinus gland (SG) and pericardial organ (PO). After isotopic dimethyl labeling using formaldehyde, MALDI-TOF/TOF (Bruker UltrafleXtreme) was utilized to measure the relative expression level changes of neuropeptides caused by the exposure to Ag nanoparticles. The relative abundance changes of different neuropeptide families were detected with peptide identification by searching against our in-house neuropeptide database. The ratios of peak intensity from heavily and lightly labeled neuropeptide pairs were calculated. Results were averaged over 3 biological replicates. Neuropeptides from the same family exhibit similar trend under each exposure level while different families display distinct patterns of changes at different exposure conditions. On-going research aims to map the change of abundance and localization of neuropeptides after exposure to nanoparticles in the entire central nervous system by imaging mass spectrometry.

(13) In-depth Characterization of Intact Myofilament Proteoforms by Top-down LC/MS-based Proteomics

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Introduction

Myofilaments are the filaments of myofibrils, which play essential roles in cardiac contractility and signal reception/transduction during the onset and progression of heart failure (HF). Multiple myofilament post-translational modifications (PTMs) are believed to act synergistically in regulating cardiac function, whereas mutations of specific myofilaments are reported to be associated with left ventricle dysfunction in HF. However, a comprehensive analysis of myofilament proteoforms (all modified forms of intact proteins) is still lacking. Herein, we develop a novel top-down LC/MS method to rapidly separate and simultaneously characterize intact myofilament proteins extracted from myocardial tissues, allowing the identification of disease-related changes in myofilament proteoforms for better understanding and diagnosis of HF.

Methods

Swine heart tissue samples were excised from juvenile Yorkshire domestic pigs, snap frozen in liquid N_2, and stored under -80°C before tissue homogenization and myofilament protein extraction. All procedures were approved by UW-Madison Animal Care and Use Committee. Approximately 1-4 mg of myocardial tissue was homogenized in HEPES buffer with protease and phosphotase inhibitors. The protein mixture was then centrifuged and myofilament proteins were extracted with TFA buffer. The supernatant after centrifugation was injected into LC/MS with 2D-nanoLC system (Eksigent®) coupled on-line to LTQ/MS (Thermo) and targeted proteins were fraction collected and comprehensively characterized by LTQ/FT MS (LTQ/FT Ultra™, Thermo Scientific). Protein traps/columns were either packed in-house or from New Objective®.

Preliminary Results

We have successfully established a novel top-down LC/MS method to separate and identify intact myofilament proteins extracted from myocardial tissue and comprehensively characterized these intact proteins by fraction-collection method. Full on-line separation of myofilament proteins has been achieved including cardiac tropoin T (cTnT, RT~25min), cardiac troponin I (cTnI, RT~28min), tropomyosin(Tm, RT~32min), myosin light chain1 (LC1, RT~43min), myosin light chain 2 (LC2, RT~45min), and actin (RT~47min). The off-line top-down identification of these myofilament proteins by fraction-collection method simultaneously with on-line separation provides highly accurate molecular weight measurement, a global view of all detected proteoforms of myofilament proteins and amino acid sequence confirmation with full sequence coverage.

Recently, we have established a fraction-collection method combined with high-resolution MS for comprehensive characterization of proteoforms of myofilament proteins. Notably, MS/MS data revealed that deep-sequencing of myofilament proteins has been achieved. Swine cTnI (MW: 23951.89 Da) was N-terminally acetylated and the phosphorylation sites were unambiguously localized at Ser22/Ser23. Meanwhile, swine α-Tm (MW: 32735.92 Da) was identified to be the predominant isoform in swine cardiac muscle and an isoform of swine Tm (β-Tm, MW: 32815.90 Da) was discovered and characterized with high-resolution MS. MS/MS data of α-Tm also revealed two amino acid polymorphisms at R38Q and P64L in the sequence. Moreover, high-resolution MS demonstrated neither α-actin (MW: 41840.03 Da) nor LC1 (MW: 21766.04 Da) is phosphorylated, while LC2 (MW: 18801.54 Da) is mono-phosphorylated. The top-down LC/MS provides precise quantification (LC/MS) and characterization of myofilament proteoforms (high-resolution MS) for diagnosis of heart failure. We are now in the progress of screening a large cohort of human samples as well as animal models to establish a causal relationship between altered myofilament proteoforms and HF.

Novelty

First comprehensive analysis of intact myofilament proteoforms extracted from myocardial tissues.

(14) Increased SILAC Multiplexing via Neutron Encoding (NeuCode) Tracks Global Proteome Remodeling during Murine Myogenic Differentiation

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Cellular differentiation induces large proteome changes, often controlled by alterations to post-translational modification (PTM) patterns. Yet achieving deep quantification of these changes is challenging. Stable isotope labeling in cell culture (SILAC) is a powerful tool for protein quantification, yet has limited multiplexing ability. This prevents the inclusion of the biological replicates necessary for confident, wide-spread assessment of PTMs in particular. We developed a strategy to greatly extend the multiplexing of SILAC, up to 18-plex, by harnessing mass defects caused by differences in neutron binding energies of carbon, nitrogen, and hydrogen isotopes (NeuCode SILAC). This method allowed us to quantify protein and phosphorylation changes occurring due to differentiation in C2C12 myoblasts in biological triplicate in one NeuCode SILAC experiment. We identified 6,261 proteins, 4,869 of which contained a lysine and were quantifiable. Of those proteins, 2,393 did not contain an arginine and were quantified with only one replicate, while 2,475 contained arginine and could be quantified across three replicates. In total, 691 proteins showed a change in abundance of >2-fold, although several proteins, 40S ribosomal protein S25 for example, showed a large change with only 1 replicate (normalized log2 fold change of -4) but a much smaller change when quantified with 3 replicates (fold change of 0.7), highlighting the importance of replicates for accurate analysis. We conclude that NeuCode SILAC offers the high plexing capacity of isobaric tagging while eliminating both the need for an MS/MS scan for quantification and the pervasive problem of precursor interference that cripples quantitative accuracy in isobaric tagging. And, as mass resolution continues to improve, so will the multi-plexing capacity of NeuCode.

(15) Using Parallel Reaction Monitoring to Target Neuropeptide Y from Murine Islets: Investigating the Onset of Type II Diabetes

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Type II diabetes results when a dampened response to insulin causes an increased requirement for the hormone, coupled to a concurrent failure to produce of enough of it to meet this demand; consequently, understanding the regulation of insulin secretion is important to understanding the underlying mechanism of the disease. In mice, the common inbred strain B6 exhibits diet-induced insulin resistance, while CAST, a wild-derived strain, remains insulin sensitive. Differences between the two strains at the genetic and proteomic levels in islets, the insulin-producing cells of the pancreas, may yield clues to how these two mouse strains respond to the dietary challenge. Islets from several mice from each strain were pooled and compared using quantitative shotgun proteomics via isobaric tags. The relative abundance of Neuropeptide Y (NPY), a known suppressor of insulin secretion, was over 10-fold higher in the islets from CAST mice compared to B6 mice. Pooling provides average relative abundance levels between the two strains, but the statistical power of comparing multiple sets of individual animals is necessary to further characterize the role of NPY in type II diabetes; thus, the new challenge lies in reproducibly analyzing and absolutely quantifying islet NPY levels from individual mice. Here we present our work using the sensitivity and reproducibility of targeted proteomics and parallel reaction monitoring (PRM) to further investigate and quantify NPY changes in the islets from B6 and CAST mice. PRM is a robust platform to simultaneously co-detect and distinguish all transitions in targeted proteomics, utilizing the high resolution and accurate mass capabilities of an orbitrap. PRM couples the strengths of traditional SRM methods performed on triple quadrupole instruments to higher selectivity and increased dynamic range provided by the orbitrap, enabling the reproducible quantification of NPY in islets of individual mice.

(16) A Versatile Automated Sample Preparation Platform for Reproducible, High-throughput Protein Digestion, Peptide Fractionation, and Peptide Cleanup for LC/MS

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Hundreds of putative protein biomarkers for disease have been revealed by LC/MS assays and documented in the literature. Yet, the vast majority of these biomarker studies have not progressed beyond the initial investigation involving a small set of patient samples. These sample sets are often orders-of-magnitude shy of the number of samples required for validating an assay for routine clinical use. While significant technological advances have been made recently in LC/MS instrumentation and informatics, far slower progress has been made in the area of protein sample preparation which continues to be highly manual and difficult to multiplex. Manual sample preparation can lead to variable, user-dependent results problematic for protocol transfers, cross-laboratory comparisons, and throughput scaling. The success of protein analysis by LC/MS is critically dependent on reproducible sample preparation, but the lack of reliable, sample-scalable automation has limited the adoption of LC/MS assays requiring large sample campaigns or high throughput. Sample preparation methods must be developed that can scale from hypothesis-driven surveys to full clinical validations and large-scale testing. Such scaling can be achieved if built upon a foundation of automation engineered specifically for protein sample preparation and the needs of proteomicists. We present an automated sample preparation platform for protein analysis by LC/MS facilitating in-solution protein digestion, peptide cleanup, and fractionation of peptide mixtures. This platform permits parallel processing of up to 96 samples in a microtiter plate formate enabled by a precision liquid handler adapted to use disposable microchromatography cartridges operated through a simple user interface. We report key analytical figures-of-merit for this platform using model systems for protein and peptide quantification and characterization by LC/MS. Signals from target peptides typically yield % CVs < 5% for a digestion/cleanup workflow and < 10% for a digestion/cleanup/fractionation/cleanup workflow without the use of internal standards.

(17) Comparative Analysis of the Proteomic Changes of Amniotic Fluid in Different Gestational Age for Lung Development

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The chemical composition of amniotic fluid (AF) varies with different gestational age. It has been shown that the number and amount of differentiation markers and transcription factors in gene expression of lung epithelium increase significantly in pre-term than those in near-term. Furthermore, the in vitro lung explants exhibit more maturity in pre-term AF, where more peripheral airways have been established and the sizes of the peripheral airways are usually smaller. It is highly possible that there are important molecular factors in the pre-term AF that promotes the organ development, especially for lungs. We therefore characterized the proteome of AF in gestational stage 15.5 and 17.5 respectively by mass spectrometry-based quantitative proteomics. We employed immunodepletion to remove the seven most abundant proteins in AF. After immunodepletion, the number of protein identification improved significantly. A total of 163 and 201 proteins were identified from AF 15.5 and AF 17.5 respectively and quantified based on spectral counting, and 154 proteins were identified from both conditions. Furthermore, several proteins, such as fibronectin, plasminogen, and collagen alpha-2, exhibiting significant fold changes or only existing in pre-term, such as ankyrin-2, annexin A1 and protein KIAA0664 by spectral counting technique are considered as important factors that improve epithelial cell differentiation and mesenchyme thinning in lungs. For example, we observed more than twice elevated levels of fibronectin in AF 17.5 by spectral counting. This protein is known to play an important role in improving cell adhesion, migration and proliferation. Additionally, the MS-based quantitative proteomics result has been validated by Western Blotting.

(18) Characterization of Human Tropomyosin Molecular Heterogeneities Using Top-Down Mass Spectrometry

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Introduction:

Tropomyosin (Tm) is an important myofilament regulatory protein. It binds to troponin complex to inhibit myosin from binding to actin at low level of intracellular Ca^{2+} whereas it exposes the binding site to allow the binding of myosin to actin at higher intracellular Ca^{2+} concentration, playing a crucial role in regulating muscle contraction. To comprehensively understand its role in heart failure, the structure and molecular heterogeneities of Tm in human heart must be first characterized. Herein, we have developed a method to rapidly purify Tm from 5 mg human heart muscle tissue and employed top-down mass spectrometry (MS) to characterize the isoforms, posttranslational modifications (PTMs) and sequence of Tm in human heart.

Methods

Postmortem and transplant human heart tissue samples were from UW hospital and stored under -80°C. Around 5 mg heart tissue was homogenized manually using a Teflon homogenizer and then underwent extraction using 50 µl of HEPES and TFA buffer consecutively. The sample was further purified using 10% ammonium hydroxide to precipitate small proteins and desalted with a MWCO filter. A linear ion trap quadrupole /Fourier Transform ion cyclotron resonance mass spectrometer (LTQ/FT Ultra™, Thermo Scientific) was used to collect the MS spectra of purified Tm and then the precursor ions at individual charge states were isolated and fragmented by ECD and CAD respectively. MS and MS/MS spectra were processed using in-house developed software, MASH Suite, and manually validated.

Preliminary results

A simple and efficient method has been developed to extract and purify Tm from human heart tissue. The amount of tissue used in this method was dramatically reduced compared to previous methods. The high-resolution MS characterization of human Tm revealed interesting molecular heterogeneity, presumably as a result of alternative splicing and/or PTMs. According to MS data, the major Tm isoforms present in human heart were identified to be α-Tm (MW = 32749.94 Da) and β-Tm (MW= 32891.85 Da); monophosphorylated α-Tm ($_{p}$α-Tm with MW=32829.89 Da), the major Tm degradation product was α-Tm [1-282] with MW=32549. 82 Da. Comparing the spectra of Tm from failing human heart with those from healthy human heart, we found that the major forms of Tm were present in both healthy and failing heart whereas the relative abundance monophosphorylated α-Tm was significantly decreased in the failing heart.

For MS/MS data, α-Tm ions were isolated as precursor ions for comprehensive sequence characterization of Tm and for further localization of PTMs. Both ECD and CAD data of the major Tm isoforms demonstrated multiple mismatches between experimental human Tm fragments and the calculated values predicted from human α-Tm chain. Almost no ECD fragments and CAD fragments matched with the original α-Tm chain sequence at the N-terminal. Conversely, the high resolution MS/MS unambiguously revealed the acetylation of the first amino acid at the N-terminus (42.01) in the sequence of α-Tm. After incorporating these modifications, the experimental MW of Tm (32749.94) matched exactly with calculated value (32749.74). Moreover, the bond cleavages from ECD and CAD data matched well with those of modified sequence. Hence, we have explicitly characterized the amino acid sequence of the major Tm forms in human heart.

(19) Mass Spectrometric Characterization of the Neuropeptidome in the Dungeness Crab (*Cancer magister*)

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Introduction:

The Dungeness crab *Cancer magister* has been widely used in the study of regulation of cardiac activity and other physiological processes. However, the characterization of its neuropeptidome has not been reported. In this study, we employed three LC MS/MS instrument platforms, Waters Synapt G2 HDMS quadrupole time-of-flight instrument and Thermo Scientific Q Exactive hybrid quadrupole-orbitrap and Orbitrap Elite mass spectrometers, to obtain a complete description of the neuropeptidome of *C. magister*, including both small neuropeptides (e.g. RFamide, Orcokinin) and large neuropeptides (e.g. Crustacean Hyperglycemic Hormone (CHH)). In addition, neuropeptidome changes during feeding behavior were evaluated.

Methods:

First, to achieve complete characterization of CHH peptides in crab nervous system, a multi-faceted MS-based methodology was employed, including offline-HPLC-MALDIMS, Orbitrap Elite, nanoLC-Q-Exactive MS/MS coupled with CID and HCD fragmentation strategies, combining top-down and bottom-up methods. The data was analyzed with Mascot database search and PEAKS *de novo* sequencing, which provided complementary information for characterization of these neuropeptides. Second, offline-HPLC was coupled with Synapt G2 LC-ESI-Q-TOF to reduce the sample complexity and assist *de novo* sequencing of small neuropeptides with low abundance. Third, to study the global neuropeptide release in the sinus glands after feeding behavior, a label-free quantitative MS method was employed. The peptides in crude tissue extracts were analyzed by LC-MS/MS, followed by data analysis using SIEVE software package.

Preliminary Results:

- One novel CHH peptide was identified and sequenced in *C. magister* by combining top-down and bottom-up mass spectrometric strategies.
- 31 novel neuropeptides were identified and sequenced in *C. magister* by analyzing fractionated crab organ extracts on LC-ESI-Q-TOF instrument.
- The preliminary results of quantifying neuropeptide changes during feeding behavior by using label-free method and SIEVE quantitation platform were collected for further study.

Novel Aspect:

Discovery of novel neuropeptides in *Cancer magister* and evaluation of a hybrid quadrupole-orbitrap instrument for *de novo* sequencing of neuropeptides.

(20)Carbonyl-Reactive Tandem Mass Tags Facilitating Separation and Characterization of Carbohydrates by CE-ESI-MS

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Glycan characterization and quantification play important roles in biological research, clinical analysis and biopharmaceutical production. Most of the derivatization strategies currently applied in glycan analysis are designed for optical detection, but few of them are compatible with electrospray ionization-mass spectrometry(ESI-MS) analysis.

Recently, two novel stable isotope labelled carbonyl-reactive tandem mass tags (Glyco-TMTs), namely hydrazide and aminooxy TMTs, were reported to be used for quantitative analysis of N-glycans by MALDI-TOF. We observed that the ESI signals of Glyo-TMT labelled carbohydrates are enhanced by about 50 fold compared to those of the native carbohydrates. Also, for Glyo-TMTs labelled carbohydrate, the reporter ions generated by ESI-CID-MS have higher signal-to-noise ratio than that from MALDI-Post-Source Decay-TOF (MALDI-PSD-TOF).

In this work, we applied capillary electrophoresis (CE)-electrospray ionization (ESI)-mass spectrometry (MS) in separation, characterization and relative quantification of the Glyco-TMTs labelled carbohydrates. As the tertiary amine groups in the Glyco-TMTs can be positively charged in acidic pH buffer, it is possible to separate the TMTs-labelled carbohydrates by electrophoresis, and meanwhile perform relative quantification by comparing the intensities of reporter ions generated from tandem MS. Neutral and acidic sugar standards, as well as N-glycans released from chicken egg ovalbumin were used demonstrate analysis of Glyco-TMTs labelled carbohydrate by CE-ESI-MS.

(21) Cloud-base Systems Accelerate Researches in Proteomics and Systems Biology

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In the last decade, data creation technologies in life sciences have outpaced traditional data management and processing capabilities. Cloud-based technologies have been widely used in managing big datasets, processing data online automatically, and accelerating research publications. In the last few years, we have developed unique web-based technologies specifically for life science big data processing and visualization. We have applied these technologies in proteomic research by collaborating with multiple research groups. For example, Coon group at UW-Madison identified thousands of phosphopeptides in Medicago truncatula (Plant Physiol. 2010, 152(1): 19-28). We used our cloud-based technologies to help build the Medicago PhosphoProtein Database (MPPD, http://phospho.medicago.wisc.edu). Through the web-based interface, users are allowed to browse identified proteins or search for proteins of interest. Furthermore, we allow users to conduct BLAST searches of the database using both peptide sequences and phosphorylation motifs as queries. In a separate study, the Attie and Pagliarini groups in UW-Madison performed multiplexed quantitative mass spectrometry-based proteomics to chart the remodeling of the mouse liver mitochondrial proteome and phosphoproteome during both acute and chronic physiological transformations in more than 50 mice (Cell Metab. 2012, 16(5): 672-83). To analyze the large sets of data generated from MS/MS, we helped develop the Mitomod system (http://mitomod.biochem.wisc.edu). The database in this system serves as a resource for investigating the levels of site-specific phosphorylation on mitochondrial proteins. The system contains integrated tools for searching, comparing, correlating, and graphically visualizing data from different experiments and conditions. The analysis results revealed that reversible phosphorylation is widespread in mitochondria, and is a key mechanism for regulating ketogenesis during the onset of obesity and type 2 diabetes. Taken together with our previous success in applying similar technologies in genomics (*e.g.,* Genome Res. 2008, 18(5): 706-16. http://diabetes.wisc.edu/), we have demonstrated that cloud-based technologies can apply to various scientific areas with big datasets, and can greatly accelerate researches in proteomics and system biology.