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Quantitative proteomic profiling reveals hepatic lipogenesis and liver X receptor activation in the PANDER transgenic model

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ABSTRACT

PANcreatic-DERived factor (PANDER) is a member of a superfamily of FAM3 proteins modulating glycemic levels by metabolic regulation of the liver and pancreas. The precise PANDER-induced hepatic signaling mechanism is still being elucidated and has been very complex due to the pleiotropic nature of this novel hormone. Our PANDER transgenic (PANTG) mouse displays a selective hepatic insulin resistant (SHIR) phenotype whereby insulin signaling is blunted yet lipogenesis is increased, a phenomena observed in type 2 diabetes. To examine the complex PANDER-induced mechanism of SHIR, we utilized quantitative mass spectrometry-based proteomic analysis using Stable Isotope Labeling by Amino Acids in Cell Culture (SILAC) to reveal the global hepatic proteome differences within the PANTG under the metabolic states of fasting, fed and insulin-stimulated conditions. Proteomic analysis identified lipid metabolism as one of the top cellular functions differentially altered in all metabolic states. Differentially expressed proteins within the PANTG having a lipid metabolic role included ACC, ACLY, CD36, CYP7A1, FASN and SCD1. Central to the differentially expressed proteins involved in lipid metabolism was the predicted activation of the liver X receptor (LXR) pathway. Western analysis validated the increased hepatic expression of LXRα along with LXR-directed targets such as FASN and CYP7A1 within the PANTG liver. Furthermore, recombinant PANDER was capable of inducing LXR promoter activity *in-vitro* as determined by luciferase reporter assays. Taken together, PANDER strongly impacts hepatic lipid metabolism across metabolic states and may induce a SHIR phenotype via the LXR pathway.

1. Introduction

Over the past decade PANcreatic-DERived factor (PANDER or FAM3B) has been investigated with regard to secretion from the endocrine pancreas and biological impact on glycemic regulation both *in-vitro* and *in-vivo* (Zhu et al., 2002; Burkhardt et al., 2008; Burkhardt et al., 2006; Cao et al., 2005; Hou et al., 2011; Mou et al., 2013; Robert et al., 2005; Robert-Cooperman et al., 2010; Wang et al., 2008; Xiang et al., 2012; Xu et al., 2005; Yang et al., 2005a,b; Zhuang et al., 2011). Our recently generated pancreas-specific overexpressing transgenic mouse model (PANTG) exhibits both fasting and fed glucose intolerance primarily attributed to impaired hepatic insulin signaling concordantly coupled with both increased gluconeogenesis and lipogenesis (Robert-Cooperman et al., 2014). This result is consistent with other PANDER animal models that acutely express PANDER within the liver via adenoviral delivery (Li et al., 2011). The mechanism by which PANDER inhibits hepatic insulin signaling has been attributed to suppressed phosphorylation of Akt (Yang et al., 2009) and AMPK (Robert-Cooperman et al., 2014), both of which serve as major regulators of gluconeogenesis. However, a major paradox to PANDER signaling has been the documented increase in hepatic lipogenesis despite inhibited insulin signaling (Robert-Cooperman et al., 2014; Li et al., 2011). This bifurcation of signaling results in a selective insulin resistant state that mimics what is observed in T2D animal models and humans (Biddinger et al., 2008; Brown and Goldstein, 2008). Encompassing prior PANDER research, an emerging hypothesis suggests that the pathophysiological conditions of T2D could potentially induce increased circulating PANDER levels contributing to selective hepatic insulin resistance (SHIR) resulting in increased hepatic glucose output and lipogenesis (Wilson et al., 2011; Wang et al., 2012), as precisely observed in our PANTG model. Recent evidence has now indicated that circulating PANDER

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levels are elevated and associated with metabolic syndrome components in a Chinese population (Cao et al., 2016). Plasma PANDER levels significantly correlated with fasting plasma glucose, 2 h plasma glucose, and triglyceride levels. Between animal model results and recent clinical studies, an emerging theme with PANDER is the possible role of this novel hormone in the promotion of hepatic insulin resistance and lipogenesis. Despite this importance, the precise PANDER-induced signaling mechanism in the liver has yet to be determined.

To elucidate PANDER-induced hepatic molecular mechanisms, we utilized quantitative mass spectrometry-based proteomic analysis via a stable isotope labeling by amino acids in cell culture (SILAC) approach to characterize hepatic proteomic differences between the PANTG murine liver with that of wild-type mice under three metabolic states: fasting, fed, and insulin-stimulated. To achieve this, stable isotope-labeled liver protein lysate from mice that were metabolically labeled with $^{13}C_6$-Lys was utilized as an internal standard for relative quantification of global proteome differences in the liver, a technique rarely used to study metabolic disorders yet previously validated from examination of insulin signaling and liver proteomic characterization. Differentially expressed proteins using this approach can be analyzed with bioinformatics tools such as Ingenuity Pathway Analysis (IPA) in order to reveal altered molecular networks and their function as well as differences in canonical pathways that can be later validated via additional molecular approaches. This unbiased, global-scale approach has led to novel insight into PANDER-induced hepatic pathway alterations in our PANTG model, in particular, those related to increased lipogenesis.

2. Materials and methods

2.1. Transgenic mouse generation and genotyping

Generation and original phenotyping of the PANDER transgenic mouse was described previously (Robert-Cooperman et al., 2014). In brief, this murine model overexpresses PANDER from the endocrine pancreas resulting in increased levels of circulating PANDER with a phenotype of impaired glucose tolerance and hepatic insulin sensitivity.

2.2. Proteomic Experimental design

The purpose of this study was to examine the PANDER-induced hepatic proteome in various metabolic states. As outlined in Fig. 1, the PANTG mouse was exposed to fasted, fed, and insulin stimulatory conditions prior to liver extraction and subsequent proteomic examination. All treatments were performed on PANTG and wild-type mice at six weeks of age. Fed and fasted mice were withheld from food for approximately 4 and 16 h, respectively. Following the 4 h fast, fed mice were provided with chow *ad-libitum* for 2 h. Insulin-stimulated mice were fasted for 4 h prior to insulin injection (Humulin®, 1 unit/kg). Insulin was diluted to 20 units/ml and injected intraperitoneally with exposure for 15 min. Mice were humanely euthanized by carbon dioxide asphyxiation and cervical dislocation following above described metabolic conditions. Livers were extracted immediately following exposure to described metabolic conditions, snap frozen and stored at −80 °C prior to proteomic analysis.

2.3. Hepatic protein isolation

Approximately 40 mg of tissue was excised from a lobe of mouse liver for tissue lysis. Tissue was submerged in cold lysis buffer (100 mM Tris-HCl, 100 mM DTT, 4% SDS and 1× HALT protease inhibitor) and homogenized using a Qiagen TissueRupter. Cell lysate was then heated at 95 °C for five minutes followed by brief sonication. The tissue lysate was cleared by centrifugation at 16,000× *g* for 5 min and the supernatant was collected and stored at −80 °C prior to further analysis. The same procedure for protein purification was performed on the "heavy" labeled ($^{13}C_6$ L-Lysine) murine male liver (MT-LYSC6-ML-PK, Cambridge Isotope Laboratories, Inc.).

2.4. Sample digestion, desalt and SCX fractionation

Protein concentration was quantified using the Pierce 660 nm protein assay kit supplemented with the provided ionic detergent compatibility reagent (IDCR). Equal mass of labeled and unlabeled protein or "light" and "heavy" protein respectively, were combined and digested using the filter-aided sample preparation (FASP) method (Expedeon). Briefly, 30 μL of the protein mixture (~12.5 mg/ml) was added to the spin column. The lysate buffer was exchanged to 8 M urea using centrifugation prior to alkylation by iodoacetamide. The solution was then exchanged to 50 mM ammonium bicarbonate for trypsin/Lys-C digestion at a ratio of 1:40 (w/w). Digestion was carried out overnight in a humidified incubator at 37 °C. Peptides were eluted off the column by addition of 0.5 M NaCl followed by centrifugation.

Samples were desalted using solid phase enrichment C18 columns (The Nest Group, Inc.). Briefly, columns were activated using 100% acetonitrile followed by equilibration with 0.1% formic acid in water. Samples were loaded onto the columns and washed three times using equal volumes of 0.1% formic acid in water. Samples were eluted using 90% acetonitrile/0.1% formic acid in water and then concentrated in a vacuum concentrator (Thermo) prior to resuspension with 5 mM ammonium formate and 25% acetonitrile. Peptides were fractionated by strong cation exchange chromatography as previously described (Bell-Temin et al., 2015).

2.5. LC-MS/MS and pathway analysis

Fractions were separated on a 10 cm × 75 μm I.D. reversed phase column packed with 5 μm C18 material with 300 Å pore size (New Objective) using 120 min gradients of 2–40% ACN in 0.1% formic acid. Inline mass spectrometric analysis was performed on a hydrid linear ion trap-Orbitrap mass spectrometer (Orbitrap XL, Thermo Fisher Scientific). Full MS survey scans were performed at a resolving power of 60,000, and the top 10 most abundant peaks were selected for subsequent MS/MS analysis in the linear ion trap. Raw files were processed in MaxQuant 1.2.2.5 employing the Andromeda search algorithm and searched against the UniprotKB reference database for *Mus musculus*, concatenated with reversed protein sequences. A second database of known contaminants provided with the MaxQuant suite was also employed. All fractions for each biological sample were combined for analysis. Constant modification of carbamidomethylation of cysteine and variable modifications of oxidized methionine and acetylated protein N-termini were used. Additionally, Lys-6 for the spike-in internal standard was set as a label in the group-specific parameter section. A false discovery rate of 1% was used for peptides and proteins. A minimum peptide length of 6 amino acids was used. Razor and unique peptides were used for identification and quantification. Protein ratio values were reconstructed using median peptide ratio values across all three biological replicates for each experimental group where the final ratio for each protein was calculated by determining the ratio-of-ratio (PANTG/Internal Standard/(WT/Internal Standard)). Final ratios were input into the Perseus processing suite (Perseus version 1.2.0.13). Statistical analysis was performed using the Significance A outlier test where statistical