ELISA for plasma and postmortem frozen blood

Plasma samples were assayed for insulin (Mercordia, Winston Salem, NC Cat# 10-1113-01) and C-peptide (Mercodia, Cat# 10-1136-01) by ELISA (1). Proteins were precipitated from postmortem frozen blood samples by orderly mixing 1 ml of ice-cold acetone, 0.125 ml trichloroacetic acid (6.1 N), and 0.125 ml of thawed frozen blood and vortex vigorously for 1.5 min. After -20^{0}C overnight precipitation, the pellets were collected by centrifugation at 13,000 x g for 15 min at 4°C and washed 5 times by vigorous vortex and centrifugation at 8,000 x g for 5 min with 1 ml of acetone and air dry completely before dissolving in 0.5 ml phosphate-buffered saline (PBS) for C-peptide ELISA (2).

SRM-MS analysis

Brief:

The method includes: 1) the selection of potential tryptic peptides based on the alternative splicing sites, *i.e.*, pep-U1, -U2, and -U3 peptides are encoded by the exon-1U, and the pep-U4 and pep-U5 peptides are non-canonically encoded by intron-1, and pep-UF (frameshift) by intron-2 (Fig 1 and Fig 2A; Supplemental Table 4). Pep-US is encoded by spliced exon-1UB and exon-2 (Fig 2B and Supplemental Fig 1A-G). The pep-B (B-chain) is encoded by exon-2 and located after the signal peptide, while the pep-A (A-chain) is encoded by exon-3 (Supplemental Fig 1H,I) and is the same as the complete A-chain peptide. The 19-AA pep-Cα (Cα) and non-processed pep-CαK (Fig 2C, CαK, as a surrogate for the 74-AA proinsulin) are derived from INS3B that is alternatively spliced exon-2 and exon-3B (Supplemental Fig 1J,K); 2) the selected peptides were synthesized as isotope-labeled and unlabeled analogues (Supplemental Table 4) by Genemed Synthesis Inc. (San Antonio, TX), and after reconstitution each peptide concentration was determined by amino acid analysis (New England Peptide, Gardner, MA); 3) the optimal

charge state, declustering potential (DP), collision energy (CE), collision cell exit potential (CXP) were selected as detailed elsewhere (3). Three to six interference-free precursors and fragment ion masses (transitions) for a given peptide were constituted in the final multiple SRM assay. For further enhancement of SRM sensitivity, we scheduled the mass spectrometer to collect subsets of peaks based on the target analyte retention times (RT) on the column; 4) analytical validations for SRM assay performance in the study matrices are shown in Supplemental Tables 5 and 6.

Detail:

Under a stereo microscope, approximately 200 intact islets from individual donors were handpicked into a polypropylene tube containing 1 ml of ice-cold PBS. After washing twice with 1 ml PBS containing 1X protease inhibitor cocktails, the islets were resuspended in 100 µl of 0.1% RapiGest (w/v) (Waters Corp., Milford, MA) containing 100 mM Tris-HCl (pH 8.0) and 100 nM DTT. For choroid plexus postmortem frozen sections 100 µl of 0.1% RapiGest was added on each slide, and then tissue was scraped off into a clean 1.5 ml of tube. Then, islet and choroid plexus in 0.1% RapiGest were sonicated 3 x 3 seconds on/30 seconds off on ice. After centrifugation (16,000g, 20 min at 4°C), supernatants were collected and protein concentration was determined by BCA assay (Cat#: 23225, Thermo Fisher Scientific, Waltham, MA), and stored at -80°C until further analysis. Due to low amounts of islet lysate, the islet protein concentration was not determined, and thus relative quantification was used for quantification, *e.g.*, the ratio of CαK/Cα.

Brain MTG and MFG tissues were homogenized in 500 µl of 0.1%RapiGest with100 mM Tris-HCl (pH 8.0) and 100 nM DTT. The lysates were further solubilized by incubation for 1 hour at 4°C with continuous rotation. After centrifugation (16,000g, 20 min at 4°C), the

supernatant was collected, and the protein concentration was determined by protein BCA assay. All islet lysate and 150 µg of brain ChP, MTG and MFG lysates were used for the digestion procedure.

Fresh-frozen human plasma was thawed on the day of analysis. After centrifugation (16,000g, 15 min at 4°C), cleared fractions were transferred with a loading tip into a fresh 1.5 mL polypropylene tube, discarding insoluble aggregates and the upper layer of floating lipids. This procedure was enough to eliminate the confounding influence of lipids on downstream protein separation procedures. Then 5 µl of delipidated plasma was mixed with 95 µl of 0.1% RapiGest. Tryptic digestion was performed with an automated robotic procedure aimed at minimizing sample handling variability in a flow for SRM analysis(4). Briefly, sample lysate (100 µl) in 0.1% RapiGest were transferred into the reaction plate, incubated 1 hour at 55°C for denaturation and reduction, followed by 30 min alkylation with a fresh made 0.1 M solution of iodoacetamide (Sigma-Aldrich) to a final concentration of 50 mM at room temperature in the dark. After alkylation, trypsin/LysC mix (Promega, Madison, WI) was added at an enzyme-to-substrate ratio of 1:50. Digestion was carried out for 18 hours at 37°C and terminated with 10% MS-grade trifluoroacetic acid (Fisher Scientific, Hampton, NH) to a final concentration of 1%. Acidified tryptic digests were cleaned up with 96-well SPE plate (Phenomenex, Torrance, CA) according to manufacturer's instruction. A 96-well plate vacuum manifold (Waters Corp., Milford, MA) was used for all desalting procedures to provide uniform peptide wash, retention, and elution. The elution reagents were evaporated to dryness and stored at -80°C until SRM analysis. All internal standard peptides of the novel *INS* uORF isoforms (INSU1 and INSU2) were post-spiked into tryptic digests.

We used a Shimadzu LC-HPLC equipped with LC-20ADXR pumps (Shimadzu Corp., Columbia, MD) for solvent and sample delivery and a 2.1 mm X 100 mm, 130 Å pore size, 3.5 μm particle size C18 column (Waters Corp.) for the peptide separations using the following linear gradient: 0min 5%B; 10 min 36%B; 12 min 90%B; 13.5 min 90%B; 14 min 5%B at a flow rate of 0.2ml/min. The total run time was 18 min per sample. Triplicate injections of 10 μl of sample were carried out via the SIL-20AXR autosampler (Shimadzu Corp.). To eliminate possible carryover, the column was re-equilibrated at 50%B for 10 min and a blank run was performed prior to initiating the next sample injection. QTRAP 5500 mass spectrometer with electrospray ionization (ESI) source controlled by Analyst 1.6.3 software (AB Sciex, Framingham, MA) was used for all LC-MS/MS detection and analysis. Mass spectrometric analyses were performed in positive ion mode. ESI interface parameters were set as follows: capillary temperature 650°C and a curtain gas setting of 30 psi. By using scheduled SRM, a total of 148 SRM transitions from 15 peptides were monitored during an individual sample analysis with Q1 and Q3 set by declustering potential (DE) 10 V and peptide-specific tuned collision energy (CE), entrance potential (EP) and collision cell exit (CXP) voltages for each transition.

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