Skeletal muscle O-GlcNAc transferase is important for muscle energy homeostasis and whole-body insulin sensitivity

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Supplementary Methods

Metabolomics Analysis

Chemicals and reagents

All chemicals and reagents used were of liquid chromatography-mass spectrometry (LC-MS) grade unless otherwise stated. D5-tryptophan, methanol, water, acetonitrile, 2-propanol, formic acid, ammonium hydroxide were purchased from Sigma Aldrich (Denmark) and hexakis(2,2-difluoroethoxy)phosphazene from Apollo Scientific (UK).

Metabolites extraction

Samples were randomized for processing and blanks (empty microcentrifuge tubes) were included in the preparation. 20 µL of 0.2 mg/ml D5-tryptophan was added as an internal standard to each sample and blanks. 500 µL of cold 50% methanol solution was added and muscles underwent homogenization (TissueLyser II, Qiagen, USA) for 1 min at frequency 25/sec. Lysed tissues were shortly vortexed and shaken for 20 min at 1°C and 1400 rpm (Thermomixer Comfort, Eppendorf, Germany). Following the centrifugation at 0°C, 4000 rpm for 10 min (Centrifuge 5424R, Eppendorf, Germany) supernatant was collected into pre-chilled microcentrifuge tube. 80% cold methanol solution was added to the remaining pellet, shortly vortexed and shaken for 20 min using the same settings as previously described. After second centrifugation, again the supernatant was collected and combined with the previous extract. The same procedure was repeated using 100% methanol and the remaining pellet was disregarded. Combined extracts were dried using speed vacuum (EZ-2 Personal Evaporator, GeneVac, USA) without heating (~3h). Extracts were re-suspended in 100 µL of 5% acetonitrile with 0.1% formic acid cold solution via and shaken at 1°C for 20min at 1400 rpm. After centrifugation at 12 000 *g* at 0°C for 10 min supernatant was collected in new pre-chilled microcentrtifuge tubes and after short vortexing 5 µL of each sample was collected to one pre-chilled microcentrifuge tube, creating a Quality Control sample (QC). Finally, remaining extracts were stored at -80°C until LC-MS analysis.

LC-MS metabolic profiling

Muscle extracts, QC sample and blanks were defrosted on ice, vortexed and 10 µL of the sample was dissolved in in 90 µL of cold 5% acetonitrile with 0.1% formic acid solution in a pre-chilled LC-MS vial (Verex Vial, µVial i3 Qsert, Phenomenex) with a screw-cap (Verex Cert+ MSQ Cap, Phenomenex). Leftover samples were stored at -80°C.

Metabolic profiling was conducted using LC-MS system: Samples were maintained at 4°C throughout the analysis. QC samples and blanks were injected after each 5^{th} sample. Chromatographic separation was performed using UHPLC Dionex Ultimate 3000 (Thermo Scientific, Germany) with Luna Polar C18 column (1.6 µm, 2.1x100 mm, Phenomenex, USA) with EVO C18 guard column (sub-2 µm, 2.1 mm, Phenomenex, USA) kept at 40°C. Solvent A and B were 0.1% formic acid in acetonitrile and 0.1% formic acid with 5mM ammonium hydroxide in LC-MS grade water, respectively. A flow rate of 0.3 mL/min was applied with a gradient elution profile: 95% B 0-1min, 95%-5% B 1.0-10.0 min, 5% B 10.0-12.0 min, 5-95%

B 12.0-12.5 min, 95% B 12.5-14-5 min (equilibration step). LC was coupled with QToF Impact II mass spectrometer (Bruker Daltonics, Germany) operating in electrospray ionization. Samples were analyzed in positive and negative mode. 5 µL of the extract was injected in positive mode and 10 µL in the negative mode. Line and profile MS spectra were acquired in the mass range 50-1000 mass to charge ratio (m/z) at 2.00 Hz spectra rate using the source settings for positive mode: absolute threshold 50 cts per 1000 sum, End Plate Offset 500 V, Capillary 4500 V, Nebulizer 2.0 Bar, Dry Gas 10.0 L/min, Dry Temperature 220°C; Transfer: Funnel 1 RF 150.0 Vpp, Funnel 2FR 200.0 Vpp, isCID Eergy 0.0 eV, Hexapole RF 50.0 Vpp; Quadrupole: Ion Enegry 4.0 eV, Low Mass 100.0 m/z; Collision Cell: Collision Energy 7.0 eV, Transfer Time 65.0µs, Collision RF 650.0 Vpp, Pre Pulse Storage 5.0 µs. In negative mode Capillary voltage was set to 3000 and other parameters were identical as described above for both modes. MS spectra were divided into 3 segments: pre-analysis 0-0.1 min, calibration 0.1-0.5 min, analysis 0.5-14.5 min. External and internal calibration was based on sodium formate clusters in 2-propanol with Zoom of 1.0% and HPC mode. Additionally, lock-mass calibration based on hexakis(2,2-difluoroethoxy)phosphazene in 2-propanol (0.1 mg/mL) throughout the whole scan was applied. Targeted MSMS analysis was performed at the same LC-MS settings as the MS scans with additional collision energy set to 20 and scan width 1.0 m/z for both negative and positive mode.

LC-MS data analysis

Raw data from the positive and negative mode were automatically calibrated according to the sodium-clusters and lock-mass shifts throughout the analysis, using the Compass Data Analysis 4.3 (Bruker Daltonics, Germany). Files were converted to NetCDF format through the Bruker software and metabolic features were extracted using R-based[1] XCMS[2], following CAMERA[3] analysis in search for isotopes and adducts. Data were normalized according to the internal standard abundance and samples weight. Statistical analysis was performed using online analytical tools within MetaboAnalyst 3.0[4]. CAMERA-generated buckets were log-transformed and Pareto-scaled. Non-informative variables were removed based on their standard deviation. Initially Principal Component Analysis was made to visualize the clustering of WT, KO, QC and Blanks in search for potential contaminations and machine drifts. Student t-test with False Discovery Rate (FDR) analysis was used to select significantly different metabolic features between KO and WT. Targeted MSMS analysis was performed on the significantly different metabolic features (FDR<0.1, P<0.05) and matched with metabolites in Human Metabolome Database[5], Metlin Database[6] and/or Lipid Maps database[7] according to the mass to charge ratio, MSMS profile and evaluated according to their structure in relation to the retention time in the current study. However, with the LC-MS technique used we were not able to distinguish between carbon-6-sugars and positioning of the phosphorylation. Heatmap analysis was performed on the normalized and autoscaled intensities of the identified metabolites, using Euclidean distance measure and Ward clustering algorithm with samples recognition[4].

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