Kynurenine-Induced Aryl Hydrocarbon Receptor Signaling in Mice Causes Body Mass Gain, Liver Steatosis, and Hyperglycemia

METHODS AND MATERIALS

Histology

At the conclusion of the 20-week diet regimen, liver and adipose tissue were harvested at sacrifice following perfusion with phosphate-buffered saline. The tissues were then fixed in 4% paraformaldehyde (Sigma Aldrich, St. Louis, MO). Formalin-fixed paraffin embedded block and slide preparations, hematoxylin and eosin staining, and immunohistochemistry were carried out by the Dartmouth Pathology Shared Resource. Antibody staining for CYP1B1 (1:250; Abcam, Cambridge, MA) was carried out using Leica Bond Rx (Leica Biosystems, Buffalo Grove, IL). Four representative sections from each tissue sample were stained with each section taken at least 30 µm apart. The stained tissue was examined at 40X magnification using an Olympus BX51 microscope (Waltham, MA). Images were generated using identical settings with a QImaging Micro Publisher 5.0 RTV camera (Surrey, British Columbia, Canada). The ImageJ (FIJI) open source software Adiposoft was used to quantify adipose tissue cellularity.

Western blotting

Proteins from liver were isolated in radioimmunoprecipitation assay (RIPA) buffer on ice and pelleted by centrifugation. Lysate protein concentration was determined, and the proteins resolved by SDS-PAGE under reduced conditions and transferred to polyvinylidene difluoride membrane (EMD Millipore, Burlington, MA). Primary rabbit antibodies to CYP1B1 (Abcam, Cambridge, MA) and vinculin (Cell Signaling Technology, Danvers, MA) were incubated overnight with the membrane. Horseradish peroxidase-conjugated secondary antibodies (Cell

Signaling Technology, Danvers, MA) were added and signal detected using electrochemiluminescence (ECL, ThermoFisher, Waltham, MA).

Enzyme-linked immunosorbent assays for SPP1 and insulin

Blood was obtained from mice at sacrifice via cardiac puncture. Plasma was separated via centrifugation and stored at -80^{o}C. Plasma was purified by a Ficoll (GE Life Sciences, Pittsburgh, PA) gradient and assayed using an ELISA for SPP1 (osteopontin, R&D Systems, Bio-Techne, Minneapolis, MN). Plasma insulin was assayed similarly using the Rat/Mouse Insulin ELISA (EMD Millipore, Burlington, MA) according to the manufacturer's instructions. Samples were undiluted. Standard curve and unknown values were interpolated using Prism 8 GraphPad (San Diego, CA).

Determination of Kyn, Trp, and arachidonic acid plasma concentrations

Plasma was obtained by centrifugation from collected blood samples and stored at -80^{o} C. High-performance liquid chromatography tandem mass spectrometry (LC-MS/MS) was carried out by the Dartmouth Clinical Pharmacology Shared Resource. HPLC-grade acetonitrile, methanol, and ammonium acetate were purchased from MilliporeSigma (Burlington, MA). Formic acid was purchased from Fisher Chemical (Leicestershire, England). Ultrapure water was provided using a Barnstead purification system. Ammonium hydroxide, L-tryptophand (Trp), Kynurenine (Kyn), and arachidonic acid were purchased from Sigma Aldrich (St. Louis, MO), L-tryptophan-d5 (Trp-d5) from Santa Cruz Biotech (Dallas, TX), and arachidonic acid-d8 from Caymen Chemical (Ann Arbor, MI). Human albumin from Alfa Aesar (Haverhill, MA), PBS from National Diagnostics (Atlanta, GA), and saline from Baxter (Deerfield, IL).

For Trp and Kyn, Trp-d5 was used as the internal standard. The Trp and Kyn powders were dissolved in deionized water and stored at -40^{o}C. Working dilutions were made in deionized water. Calibrators and quality controls were made using 45 mg/ml human albumin in 0.9% saline and

processed as plasma samples. Plasma (50 μl) protein was precipitated with 150 μl ice-cold methanol containing 0.1 μg/ml Trp-d5. Samples were vortexed 2 min and incubated on ice for 10 min prior to centrifugation at 15,000 rpm (21,130xg, max speed) for 10 min at 4°C. A 160-μl supernatant sample was collected and dried under nitrogen at room temperature. Samples were suspended in 50 μl of 5% methanol, 5% acetonitrile, and 90% water for injection onto the LC-MS/MS system. HPLC separation was achieved on a Dionex Ultimate 3000 HPLC system with isocratic conditions of 5% methanol, 5% acetonitrile, 90% water, and 0.1% formic acid over 6 min at a flow rate of 0.3 ml/min on a Phenomenex Luna C18 50 x 2.1 mm, 1.6 μm column fitted with a 10 x 2.1 mm C18 guard (Phenomenex, Torrance, CA). A TSQ Vantage mass spectrometer was operated in positive ion mode with a collision pressure of 1.8 mTorr to measure Trp (205.062→188.040 m/z), Kyn (209.062→192.040 m/z), and Trp-d5 (210.100→122.060 m/z) with collision energies of 8, 5, and 27, respectively. The ESI source was operated with a spray voltage of 500 V, vaporizer temperature of 37°C, capillary temperature of 20°C, and sheath, ion sweep, and auxiliary gases at 30, 0.5, and 5 arbitrary units, respectively. The quantitative range of Trp was 0.5-40 μg/ml with inter- and intraday accuracies of 89-92% with confidence values of 4-15% across three quality control levels. The quantitative range of Kyn was 0.05-4 μg/ml with inter- and intraday accuracies of 85-113% with confidence values of 4-9% across three quality control levels.

For arachidonic acid, arachidonic acid-d8 was used as an internal standard. Arachidonic acid and arachidonic acid-d8 stocks were dissolved in DMSO and stored at -4°C. Working dilutions were made daily in 70% acetonitrile. Calibrators and quality control solutions were made in 45 mg/ml human albumin in phosphate-buffered saline (PBS) and processed as plasma samples. Plasma samples (50 μl) were processed by adding 2.5 μl of a 100 μg/ml internal standard and 100 μl of 3% ammonium hydroxide and briefly vortexed and centrifuged for 30 sec at 6,000xg. Oasis MAX SPE cartridges were used to purify samples according to manufacturer instructions (Waters, Milford, MA), and eluent was collected in 600 μl 3% formic acid in acetonitrile. Samples were dried under nitrogen

at 45°C and suspended in 50 μl 70% acetonitrile for injection onto the LC-MS/MS system. HPLC separation was achieved on a Dionex Ultimate 3000 HPLC system with a Phenomenex Luna Omega C18 2.1x50 mm, 1.6 μm column with 2.1x10mm C18 guard at 40°C. Isocratic separation utilized 30% 5mM ammonium acetate and 70% acetonitrile with a flow rate of 0.3 μl/min. A TSQ Vantage mass spectrometer was operated in negative ion mode with a collision pressure of 1.4 mTorr to measure arachidonic acid (303.206→259.26 m/z) and arachidonic acid-d8 (311.255→267.29 m/z) with collision energies of 16 and 15, respectively. The ESI source was operated with a spray voltage of 500 V, vaporizer temperature of 409°C, capillary temperature of 257°C, and sheath and auxiliary gases at 30 and 5 arbitrary units, respectively. The quantitative range was 0.2-50 μg/ml with inter- and intraday accuracies of 90-100% and 84-105%, respectively, across three quality control levels.

Microarrays

Four biological replicates per experimental condition were carried out for the microarray studies. Total RNA was isolated and purified from mouse liver (sliced into small pieces) and homogenized in Tri-Reagent (Sigma-Aldrich, St. Louis, MO). RNA purity, quantity, and quality were determined using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Waltham, MA) and an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). The mRNA gene expression microarray experiments were carried out by the Dartmouth Genomics & Molecular Biology Shared Resource using the Mouse 430A 2.0 Arrays (Affymetrix, Santa Clara, CA). Approximately 100 ng of total RNA per sample was labeled for each array using the GeneChip 3´ IVT Reagent Kit (Applied Biosystems, Foster City, CA). Labeled RNA was incubated with the arrays and stained using the corresponding kit on the GeneChip Fluidics Station. The arrays were scanned using the Affymetrix GeneChip Scanner 3000.

Human studies

To determine whether Kyn may be associated with obesity in humans, a companion blood collection prospective study was carried out. Samples were analyzed from 60 female patients requiring routine breast or abdominal surgery for benign or malignant conditions. All participants provided written informed consent. Subjects were recruited through surgeon referrals. All potential subjects were screened and only those with a body mass index (BMI) greater than 19 who were appropriate surgical candidates were included. Subjects were between 18 and 75 years old and able to provide consent. Each subject had height, weight, and age recorded; and each patient provided a fasting blood sample pre-operatively. The plasma was processed immediately and stored at -80°C. Human plasma Trp and Kyn levels were assayed by HPLC in the Dartmouth Clinical Pharmacology Shared Resource as described above. The study protocol was reviewed and approved by the Committee for Protection of Human Safety at Dartmouth College.

Data analysis

Microarray analyses were performed using BRB-Array Tools (1) Version 4.5 (Biometric Research Branch of the Division of Cancer Treatment & Diagnosis of the National Cancer Institute under the direction of Dr. Richard Simon). Probe set summaries from the imported CEL files were computed using the RMA method, which applies a background correction on the PM (Perfect Match) data and a quantile normalization, and summarizes the probe set information using Tukey's median polish algorithm (2). Differentially expressed genes were identified using a random-variance t-test (3) and multiple testing correction methods (4).

For the clinical trial data, univariate linear regression was used to assess relationships between continuous variables in the study population (e.g., patient age, BMI, Kyn plasma levels). Regression lines and the slope estimates and associated *p*-values for regression models are provided on scatter plots. For analyses stratifying the population-based obesity status, BMI values of 19 to <30 and ≥30 were considered lean/overweight and obese,

respectively. Such analyses were conducted separately within strata. All statistical analyses were performed using the R statistical software environment (v3.6.1).

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