Ben-Jonathan Laboratory - Material and Methods

Description of the test articles used, study material evaluations (diet, drinking water, cage and bedding leachates), general study design, animal treatments, and animal allocations to the 2 year toxicology study conducted at NCTR and the grantee studies can be found in Heindel et al. (1).

Animal. Male and female NCTR Sprague Dawley (SD) rats, continuously treated from gestation day 6 through postnatal (PND) 90 or 180, as described (1), were used in this study. Rats were treated by a daily gavage of five log-spaced doses of BPA: 2.5, 25, 250, 2,500, and 25,000 μg/kg bw/day, two doses of ethinyl estradiol (EE2): 0.05 and 0.5 μg/kg bw/day, or vehicle control (0.3%carboxymethylcelullose). Prior to sacrifice, retro-orbital blood was collected. Serum was prepared, frozen, stored at a -80 freezer, and then shipped on dry ice from NCTR to the investigators at University of Cincinnati.

Tissue removal and processing. Following sacrifice, adipose tissue samples from two sites: visceral (Vis) and subcutaneous (SC), were collected from each rat at PND 90 or 180. Vis adipose tissue in males was represented by the epididymal fat pads, and in females by the ovarian/parametrial fad pads. SC adipose tissue was represented by the inguinal fat pads in either sex. Each fat pad was divided in approximately two equal halves; one half was fixed in 10% neutral-buffered formalin for 72h and transferred to 70% ethanol, while the other half was snap frozen and stored at a -80 freezer. Samples were shipped at room temperature (fixed samples) or on dry ice (frozen samples) from NCTR to the investigators at University of Cincinnati.

Hormone analyses. Four serum hormones were analyzed: prolactin (PRL), leptin, adiponectin and interleukin 6 (IL-6). PRL was determined by the very sensitive Nb2 bioassay, as previously described (2). Leptin, adiponectin and IL-6 were determined by sandwich ELISA. Rat adiponectin (ACRP30) was quantified using a commercial ELISA kit (Assaypro ERA 2500-1, Assaypro, St. Charles, MO). Rat leptin was quantified with an in-house fluorometric sandwich ELISA using paired detection antibodies: LS-C104375 and LS-C104376 (LSBIO, Seattle, WA). Rat IL-6 was assayed with an in-house fluorometric sandwich ELISA using a paired antibody kit: DY-506 (R&D Systems, Minneapolis, MN). All standards were purified recombinant rat proteins (Prospec Bio, New Brunswick, NJ).

Adipose tissue gene expression. Using custom-designed PCR array from Qiagen (Hilden, Germany), five categories of genes were analyzed: 1) adipokines/cytokines (leptin, adiponectin, IL-6, Ccl2 and TNFα); 2) metabolic factors (adipose triglyceride lipase, lipoprotein lipase, fatty acid synthase, Glucose transporter 4, hormone sensitive lipase, and sterol regulatory binding protein); 3) transcription factors (PPARγ and C/EBPα); 4) Receptors/aromatase (ERα, ERβ, GPR30, aromatase, insulin receptor, and prolactin receptor; 5) house-keeping genes (β2-microglobulin and hypoxanthine phosphoribosyl transferase). Briefly, RNA was extracted by adding Tri-reagent (MRC, Cincinnati, OH) to frozen samples. Following homogenization and centrifugation, RNA was isolated using Illustra RNAspin Mini kit from GE Healthcare (Buckinghamshire, UK), and its concentration and purity were determined by UV-Vis

spectrophotometry using a NanoDrop (ThermoFisher, Waltham, MA). Total RNA was reverse transcribed using the RT^2 First Strand kit from Qiagen. Quantitative real-time PCR (qPCR) was performed in triplicate/sample, using 20ng of cDNA/well. SYBR Green PCR products were fluorometrically detected using Applied Biosystem StepOnePlus (Foster City, CA). Product purity was confirmed by DNA melting curve analysis. PCR efficiency was determined using the LinRegPCR program. The relative gene expression was determined using an Excel matrix provided by the array manufacturer (Qiagen). Additional expression analysis was performed on selected genes using the REST program and significance was tested by a Pair Wise Fixed Reallocation Randomisation Test and Taylor series (3).

Adipose tissue cellularity. Two parameters were analyzed: adipocyte size by immuno-histochemistry (IHC), and macrophage infiltration by immunofluorescence. Sections (8μm) of formalin-fixed, paraffin-embedded tissue were used for both adipocyte size and macrophage infiltration. For adipocyte size, sections were stained with hematoxylin and eosin (H&E) for histologic examination by a Zeiss (Thornwood, NY) microscope equipped with an Axiovert digital camera. Images (3-5 per sample) were taken and analyzed using the Adiposoft plugin software in ImageJ (4).

For macrophage infiltration, antigen retrieval was done by boiling in citrate buffer (10 mM, pH 6.0) for 30 minutes. Slides were blocked in 10% Donkey serum in IF buffer for one hour followed by overnight incubation at 4^0 C with 1:4000 anti-F4/80 Rabbit anti-Rat antibody from Abcam (Cambridge, UK), followed by 1:2000 Donkey anti-Rabbit Alexa-Fluor 594 and 1:1000 DAPI (Invitrogen, Carlsbad, CA). Images were obtained using a 10X objective on 2-3 images per sample were analyzed by ImageJ.

Statistical analysis. Statistical analysis of serum hormones and cytokines was performed using GraphPad Prism 5. One-way ANOVA with Dunnett's Multiple Comparison Test (posthoc) was performed on all data sets. Gene expression data was compared for significance after normalization to two housekeeping genes using the Qiagen-provided Excel matrix. After normalization and calculation of relative gene expression by the $2^{-\Delta\Delta C_t}$ method, followed by analysis with Student's t-test all calculated using the Qiagen Matrix. Additional analysis of the gene expression data was performed using the REST program (3). Adipocyte size, cellularity, and macrophage infiltration data were analyzed for significance, using Microsoft Excel Student's t-test.

References

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4. Galarraga M, Campion J, Munoz-Barrutia A, Boque N, Moreno H, Martinez JA, Milagro F, and Ortiz-de-Solorzano C. Adiposoft: automated software for the analysis of white adipose tissue cellularity in histological sections. J Lipid Res. 2012; **53** (12): 2791-2796.