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Maternal fructose-intake-induced renal programming in adult male offspring

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Abstract

Nutrition in pregnancy can elicit long-term effects on the health of offspring. Although fructose consumption has increased globally and is linked to metabolic syndrome, little is known about the long-term effects of maternal high-fructose (HF) exposure during gestation and lactation, especially on renal programming. We examined potential key genes and pathways that are associated with HF-induced renal programming using whole-genome RNA next-generation sequencing (NGS) to quantify the abundance of RNA transcripts in kidneys from 1-day-, 3-week-, and 3-month-old male offspring. Pregnant Sprague–Dawley rats received regular chow or chow supplemented with HF (60% diet by weight) during the entire period of pregnancy and lactation. Male offspring exhibited programmed hypertension at 3 months of age. Maternal HF intake modified over 200 renal transcripts from nephrogenesis stage to adulthood. We observed that 20 differentially expressed genes identified in 1-day-old kidney are related to regulation of blood pressure. Among them, *Hmox1, Bdkrb2, Adra2b, Ptgs2, Col1a2* and *Tbxa2r* are associated with endothelium-derived hyperpolarizing factor (EDHF). NGS also identified genes in arachidonic acid metabolism (*Cyp2c23, Hpgds, Ptgds* and *Ptges*) that may be potential key genes/pathways contributing to renal programming and hypertension. Collectively, our NGS data suggest that maternal HF intake elicits a defective adaptation of interrelated EDHFs during nephrogenesis which may lead to renal programming and hypertension in later life. Moreover, our results highlight genes and pathways involved in renal programming as potential targets for therapeutic approaches to prevent metabolic-syndrome-related comorbidities in children with HF exposure in early life.

Keywords: Arachidonic acid; Developmental programming; Endothelium-derived hyperpolarizing factor; Fructose; Hypertension; Next-generation sequencing

1. Introduction

Widespread consumption of fructose-containing foods and beverages has increased dramatically over the last century, which is linked to global burden of metabolic syndrome and associated comorbidities, including kidney disease and hypertension [1]. Early-life environmental insults during critical periods of kidney development can elicit epigenetic alterations, morphological changes and functional adaptations, leading to metabolic syndrome and related disorders in adult life [2–4]. Despite the rising incidence of maternal obesity, little attention has been paid to maternal fructose-intake-induced permanent changes in renal structure and function in the offspring, referred to as renal programming [5].

The kidney can be programmed by a variety of pre-, peri- and postnatal factors [2–4]. We recently observed that renal programming and hypertension developed in the adult male offspring of mother rats exposed to caloric restriction [6,7], diabetes [8], dexamethasone treatment [9,10] and high-fructose (HF) diet [11]. A number of mechanisms, including glucocorticoid effects, oxidative stress, epigenetic regulation, alterations of renin–angiotensin system (RAS), impaired tubular sodium handling and reduction in nephron numbers, have been put forward to interpret renal programming [2–4]. However, currently available knowledge gathered from different programming models is unable to identify the common underlying mechanism that drives the programming processes.

Given that renal programming has been linked to increased vulnerability of kidney disease and hypertension in adulthood, it is clinically relevant to investigate the effects of maternal HF intake on the renal programming in offspring at different developmental stages in order to differentiate primary (*i.e.*, exist at birth) and secondary programmed changes (*i.e.*, in hypertensive stage). Nephrogenesis occurs predominantly from late gestation to weaning period by postnatal weeks 1–2 in rodents. Our previous report demonstrated that programmed hypertension developed in the 3-month-old adult male offspring exposed to maternal HF diet [11]. Therefore, we employed the whole-genome RNA next-generation sequencing (NGS) to quantify the abundance of RNA transcripts in the offspring kidneys to capture commonality in transcriptional regulatory gene networks from 1-day- (*i.e.*, nephrogenesis stage), 3-week- (completion of nephrogenesis stage) and 3-month-old (hypertensive stage) offspring in response to maternal HF exposure. These identified genes and pathways in renal programming may provide new information to design therapeutic approaches for the prevention of metabolic-

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syndrome-related comorbidities in children with HF exposure in the early life.

2. Material and methods

2.1. Animals and experimental design

This study was carried out in strict accordance with the recommendations in the *Guide for the Care and Use of Laboratory Animals* of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee of the Kaohsiung Chang Gung Memorial Hospital (IACUC approval number: 2012101601). Virgin Sprague–Dawley (SD) rats (12–16 weeks old) were obtained (BioLASCO Taiwan Co., Ltd., Taipei, Taiwan), and housed and maintained in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. Rats were exposed to a 12-h light/12-h dark photoperiod. Male SD rats were caged with individual females until mating was confirmed by observation of a vaginal plug.

Pregnant SD rats (n=8) were randomly assigned to receive regular chow (n=4) or chow supplemented with fructose (60% diet by weight, n=4) during the whole period of pregnancy and lactation [11]. Since cardiovascular events occur at a higher rate and at an earlier age in males than females [12,13], only male offspring was selected from each litter and used in subsequent experiments. After birth, the subjects that came from litters were culled to eight pups to standardize the received quantity of milk and maternal pup care. Body weight (BW) and food consumption were monitored weekly. One third of the male offspring were killed at 1 day, one third at 3 weeks and one third at 3 months (n=6/group) after birth. Heparinized blood samples were collected at each time point, and the kidneys were subsequently collected. Kidneys were harvested after perfusion with phosphate-buffered saline, divided into cortex and medulla regions, and snap frozen. Blood pressure (BP) was measured in conscious rats on postnatal weeks 3, 4, 8 and 12 by an indirect tail-cuff method (BP-2000; Visitech Systems, Inc., Apex, NC, USA) described previously [11]. Renal level of 14,15-dihydroxyeicosatrienoic acids (DHET), lipid mediators synthesized from arachidonic acid [14], was measured using a commercially available enzyme-linked immunosorbent assay kit (Detroit R&D Inc., Detroit, MI, USA) following manufacturer's protocol.

2.2. Nephron numbers counting

The total number of nephrons was assessed by two independent investigators on a blinded fashion to calculate the glomeruli in the 3-week-old offspring kidney, as previously described [6]. Briefly, left kidney was cut into six pieces and incubated in 15 ml of 5 N HCl at 37°C for 90 min. The HCl was removed, and the renal tissues were washed and then incubated in 50 ml of H_2O overnight at 4°C. Samples were then gently macerated with a pestle and brought up to exactly 9 ml with H_2O. An even suspension was created by gently swirling the mixture, and 10×90-μl aliquots were placed on slides. Total glomeruli were counted under a 40× magnification power.

2.3. High-performance liquid chromatography (HLPC)

The levels of L-arginine, L-citrulline and asymmetric dimethylarginine (ADMA, an endogenous inhibitor of nitric oxide synthase) were measured using HPLC (HP series 1100; Agilent Technologies Inc., Santa Clara, CA, USA) with the OPA-3MPA derivatization reagent following the procedures reported previously [6]. Homoarginine (Sigma) was used as the internal standard. The standards contained L-arginine, L-citrulline and ADMA at concentrations in the range of 1–100, 1–100 and 0.5–5 mM, respectively. The recovery rate was approximately 90%–105%. The tissue concentration was factored for protein concentration, which was represented as micromolars per milligram of protein.

2.4. NGS and analysis

Kidneys were isolated and snap frozen for whole-genome RNA NGS (RNA-Seq) performed by Welgene Biotech Co., Ltd. (Taipei, Taiwan). Purified RNA was quantified at 260 nm (OD_{600}) by using a ND-1000 spectrophotometer (Nanodrop Technology, Wilmington, DE, USA) and analyzed using a Bioanalyzer 2100 (Agilent Technologies Inc.) with RNA 6000 LabChip kit (Agilent Technologies Inc.). All procedures were performed according to the Illumina (San Diego, CA, USA) protocol. For all samples, library construction was performed using the TruSeq RNA Sample Prep Kit v2 for ~160-bp (single-end) sequencing and the Solexa platform. The sequence was directly determined by sequencing-by-synthesis technology using the TruSeq SBS Kit. Raw sequences were obtained using the Illumina GA Pipeline software CASAVA v1.8, which was expected to generate 30 million reads per sample. Quantification for gene expression was calculated as reads per kilobase of exon per million mapped reads (RPKM). The cuffdiff tool from the cufflinks package was run to calculate expression changes and associated q values (false-discovery rate adjusted P values) for each gene between control and HF. The output files of cuffdiff were further annotated by adding gene functional descriptions and Gene Ontology (GO) classifications. The reference genome and gene annotations were retrieved from the Ensembl database (http://asia.ensembl.org/index.html). GO term enrichment and fold enrichment or depletion for gene lists of significantly up- and down-regulated genes in kidney were determined. GO analysis for significant genes was performed using KEGG (http://www.genome.jp/kegg/) and NIH DAVID Bioinformatics Resources 6.7 [15] to identify regulated biological themes.

2.5. Quantitative real-time polymerase chain reaction (qPCR) analysis

RNA was extracted according to the previously described procedures [11]. Two-step qPCR was conducted using Quantitect SYBR Green PCR Reagents (Qiagen, Valencia, CA, USA) on an iCycler iQ Multi-color Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). Several components of RAS analyzed in this study included renin (*Ren*), angiotensinogen (*Agt*), angiotensin-converting enzyme-1 (*Ace*), *Ace2*, angiotensin II type 1 & 2 receptor (*Agtr1a* & *Agtr1b*) and angiotensin (1–7) receptor *Mas1*. R18S was used as a reference in all analyses. Primers were designed using GeneTool Software (Biotools, Edmonton, Alberta, Canada) [11]. All samples were run in duplicate. For the relative quantification of gene expression, the comparative threshold cycle (C_T) method was employed. The averaged C_T was subtracted from the corresponding averaged r18S value for each sample, resulting in ΔC_T. $\Delta\Delta C_T$ was achieved by subtracting the average control ΔC_T value from the average experimental ΔC_T. The fold-change was established by calculating $2^{-\Delta\Delta CT}$ for experimental vs. reference samples.

2.6. Western blot

Western blot analysis was performed using the methods published previously [11]. The primary antibody used was a rabbit anti-rat soluble epoxide hydrolase (sEH) antibody (1:1000, 2 h; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Bands of interest were visualized using SuperSignal West Pico reagent (Pierce; Rockford, IL, USA) and quantified by densitometry as integrated optical density (IOD), factored for Ponceau S red (PonS) staining to correct for any variations in total protein loading and for an internal standard. The protein abundance was represented as IOD/PonS.

2.7. Statistics

The Shapiro–Wilk normality test was used first to determine which data were normally distributed. Normally distributed data are given as mean±S.E.M. Statistical analysis was done using unpaired t test. A P value <.05 was considered statistically significant. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) 15.0 statistics software (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Animal BW and BP

Litter sizes were not significantly affected by maternal HF (pups per litter: control=11.3±0.7; HF=11.6±0.8). Male pup mortality rate and BW did not differ between the two groups, nor did kidney weight or kidney-weight-to-BW ratios at 3 weeks and 3 months of age (Table 1). The numbers of nephron were not significantly different in the control and HF group at 3 weeks of age. As shown in Fig. 1, the systolic and diastolic BPs were significantly higher in the HF group compared to those in the control group starting at 3 weeks of age and remained elevated at 12 weeks of age.

3.2. ADMA and oxidative stress

Oxidative stress has been reported to play a role in renal programming of the offspring [2–4]. We also reported that ADMA-induced NO/reactive oxygen species (ROS) imbalance is involved in the development of hypertension in several programming models [6–10]. As shown in Table 2, plasma levels of L-citrulline, L-arginine and ADMA and L-arginine-to-ADMA ratio were not different between

Table 1
Body and kidney weights and numbers of nephron in male offspring of maternal HF vs. control group.

	3 weeks		3 months	
	Control	HF	Control	HF
Mortality (%)	0%	0%	0%	0%
BW (g)	82±2	75±3	405±13	418±3
LKW (g)	0.43±0.02	0.37±0.02	2.21±0.09	2.22±0.08
LKW/BW (g)	0.62±0.02	0.65±0.01	0.62±0.02	0.65±0.01
Numbers of nephron	5628±690	5750±655	ND	ND

LKW=left kidney weight; ND=not done; n=6/group.