Maternal exposure to high-fat and high-fructose diet evokes hypoadiponectinemia and kidney injury in rat offspring

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Abstract

Background. Maternal exposure to overnutrition during fetal development contributes to metabolic and renal damage in offspring. Adiponectin plays a protective role against obesity-related renal injury. However, role of adiponectin in renal injury of offspring exposed to maternal overnutrition remains unknown. We addressed the issue.

Methods. Female Sprague-Dawley rats were fed either a standard (N) or a high fat- and high fructose (HFF)-diet for 6 weeks before mating, and kept each diet during the gestation and lactation period. After 4 weeks postpartum, all the offspring were fed N diet, and followed by 12 weeks. Kidney weight, urinary albumin excretion, blood pressure, and blood chemistry including adiponectin and malondialdehyde, a marker of oxidative stress, were evaluated in the offspring.

Results. Compared with N-offspring, serum adiponectin levels of 1-day- and 4-week-old HFF-offspring were significantly lower, the latter of which was inversely associated with malondialdehyde. Kidney weight was significantly decreased in 1-day-old HFF-offspring, whereas increased in 4-week-old HFF-offspring. Urinary albumin excretion levels of HFF-offspring at 8, 12 and 16-week old were significantly higher than those of N-offspring at the same age, whose levels at 16-week old were inversely correlated with plasma adiponectin. Compared with N-offspring, HFF-offspring at 16-week old exhibited glomerulosclerosis, hyperglycemia, and high mean blood pressure associated with reduced podocin and increased transforming growth factor-β1 expression in the kidneys.

Conclusions. Our present study suggests that exposure to maternal HFF diet during fetal and early postnatal development induces hypoadiponectinemia in offspring, which might cause renal injury and metabolic derangements later in life.

Introduction

According to the report of World Health Organization, there has been a worldwide increase in the prevalence of obesity over the last 3 decades [1, 2]. Metabolic syndrome has become a leading public health problem, which could cause the development and progression of chronic kidney disease (CKD) via various mechanisms, such as central obesity, insulin resistance, hypertension, altered adipocytokine profiles, and glomerular hyperfiltration [3-5]. Indeed, circulating levels of adiponectin, an adipocytokine with insulin-sensitizing and anti-inflammatory properties, were decreased in patients with the metabolic syndrome, and hypoadiponectinemia was associated with insulin resistance and renal dysfunction in these subjects [6, 7].

Gestational weight gains due to overnutrition have emerged as one of the causes of obesity pandemic among all the population, including infants and children [8, 9]. It is postulated that perinatal overnutrition could play a role in obesity-related organ damages, including renal injury later in life [10-15]. Several studies have reported that maternal overnutrition might elicit the development and progression of the metabolic syndrome and cardiorenal disorders in the offspring [11-13]. Further, a postnatal adverse environment during the lactation period has also contributed to organ damage in adulthood [15, 14]. These observations have suggested that maternal exposure to

overnutrition during fetal and postnatal development could be involved in metabolic and renal damage in the offspring. However, role of adiponectin in renal injury of offspring exposed to maternal overnutrition remains unknown. In this study, we addressed the issue.

Methods

Experimental design

12-week-old female Sprague-Dawley (SD) rats were fed either a standard (N) diet (CRF-1: 14% of total calories from fat, Charles River Japan Inc.) or a high fat- and high fructose (HFF)-diet [HFD32 (56.7% of total calories from fat, CLEA Japan Inc.) with 0.15 g/ml fructose in a tap water] for 6 weeks before mating. The rats were then bred with normal male SD rats fed N diet. Each diet was maintained throughout the pregnancy and lactation periods.

After delivery, litters were culled to the same number for control of equal access to nourishment during lactation. In the process of culling, the ratio of male to female offspring was maintained equal in all the litters. Pups were weaned at 4-week old. After weaning, all male offspring were selected and fed N diet. There were two groups designated: N-offspring (male, n=11), offspring from mothers fed N diet during pregnancy and lactation; HFF-offspring (male, n=11), offspring from mothers fed HFF

during pregnancy and lactation. Offspring were placed into metabolic cages for 24 hours for urinalysis during overnight fast, and then blood pressure was evaluated by tail-cuff sphygmomanometer using an automated system with a photoelectric sensor (BP-98A; Softron, Tokyo, Japan) at 16 weeks of old. Offspring were anesthetized with 99% diethyl ether (Nakarai Tesque, Kyoto, Japan) and sacrificed at 1 day, 4 weeks, and 16 weeks of old with a decapitator after overnight fast. Serum samples were obtained, and the kidneys were removed, measured the weight, and stored immediately prior to analyses at −80°C for western blots or immunofluorescence, or were fixed with 10% buffered formalin for histology. Perirenal fat was collected and stored immediately at −80°C for subsequent real-time PCR analysis. All experimental procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the ethical committee of Kurume University School of Medicine.

Biochemical analysis

Serum adiponectin (Otsuka Pharmaceuticals, Tokyo, Japan) and malondialdehyde (MDA) (Cell Biolabs, Inc., San Diego, CA, USA) levels were measured by commercially available kits. Serum levels of blood creatinine (Cr) were measured by an auto-analyzer (Nihondenshi Co., Tokyo, Japan). Blood glucose was determined by a

glucose oxidase method (Shionotest Co., Tokyo, Japan). Urinary albumin excretion (UAE) was measured with a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Exocell, Philadelphia, USA). Creatinine clearance (Ccr) was calculated by the following formula: Ccr (μl/min/g) = (urinary creatinine x urinary volume (μl/day) / serum Cr x 24 x 60) / body weight [16].

Western blotting

The whole kidney tissues were homogenized and lysed with 25 mmol/l Tris-HCl (pH7.4) containing 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), 2 mmol/l ethylenediaminetetraacetic acid, and 1% protease inhibitor cocktail (Nakarai Tesque, Tokyo, Japan). Then the supernatant was separated by SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA, USA). The aliquot of tissue homogenate was subjected to immunoblotting using primary antibodies raised against rabbit transforming growth factor-beta1 (TGF-β1) (1:200) (Santa Cruz Biotechnology, Inc., TX, USA), rabbit synaptopodin (1:500) (Abcam plc, Cambridge, UK), rabbit Wilms' tumor-1 (WT1) (1:500) (Abcam plc, Cambridge, UK), mouse β-actin (1:4000) (Sigma-Aldrich, CO., MO), and a peroxidase-conjugated anti-rabbit secondary antibody (1:2000 dilution) (GE Healthcare, UK Ltd).

The immune complexes were visualized with an enhanced chemiluminescence detection system (Amersham Bioscience, Buckinghamshire, UK).

Real-time quantitative RT-PCR

Total RNA was extracted from perirenal adipose tissue using Trizol regent (Invitrogen, Carlsbad, CA, USA) according to the supplier's instruction, and then cDNA was synthesized with the Superscript First Strand synthesis system for RT-PCR (Invitrogen, Carlsbad, CA). Quantitative real-time RT-PCR was performed using Assay-on-Demand and TaqMan 5 fluorogenic nuclease chemistry (Applied Biosystems, Foster city, CA) according to the supplier's recommendation. Identification of primers and probe for rat adiponectin gene was Rn00595250_m1 (Applied Biosystems). TaqMan Ribosomal RNA Control Reagents (18S) was used as an endogenous control (Applied Biosystems).

Immunofluorescence

Frozen tissues were sectioned at 2-μm intervals, fixed with acetone for 5 minutes, and mounted on glass slides. The sections were incubated with blocking reagent (Dako, Glostrup, Denmark) for 1 hour and with polyclonal rabbit anti-podocin antiserum (kindly given from Dr. Asanuma, Kyoto University) (1:100 dilution) for overnight at 4°C. The

sections were then incubated with Alexa-Flour 488 goat anti-rabbit antibody (life technologies, Carlsbad, CA) (1:750 dilution) for 2 hours at room temperature. Podocin-positive glomeruli were evaluated by fluorescence intensity using an imaging analysis software, Image J (National Institute of Mental Health, Bethesda, Maryland, USA). One-hundred glomeruli per each offspring of 16-week old (N-offspring, n=5; HFF-offspring, n=5) were counted.

Renal histological analysis

The kidneys were obtained from each rats, cut transversally, fixed in Bouin's solution, followed by 10% buffered formalin, and embedded in paraffin. Four-micrometer paraffin sections were stained with Masson's trichrome for analyzing the accumulation of extracellular matrix (ECM) in renal interstitium and glomeruli. We evaluated renal interstitial fibrosis in 5 fields per each offspring of 16-week old (N-offspring, n=5; HFF-offspring, n=5) at the low magnification (x100). One-hundred glomeruli per each offspring of 16-week old (N-offspring, n=5; HFF-offspring, n=5) were evaluated at the high magnification (x600). The intensity of Masson's trichrome staining in the glomeruli was quantitatively analyzed by Image J.

Intravenous glucose tolerance test (IVGTT)

IVGTT was performed in 16-week-old offspring (N-offspring, n=5; HFF-offspring, n=5). After an overnight fast, a baseline blood sample was taken via a tail vein for determination of baseline fasting blood glucose. Then glucose (0.2 g/kg body weight) was infused via a tail vein. Blood samples were collected at baseline and 15, 30, 60, and 120 minutes after administration of glucose, and blood glucose levels were measured.

Statistical analysis

All data were expressed as means ± standard error. Unpaired *t*-test was performed for statistical comparisons between the groups. Linear regression analysis was performed to determine the association between serum levels of adiponectin and MDA in 4-week-old offspring and between adiponectin and UAE in 16-week-old offspring, respectively. All statistical analyses were performed with statistical software (StatView 5, SAS Institute, Cary, NC, USA). $p<0.05$ was considered a statistically significant.

Results

Effects of maternal HFF-diet on serum levels of adiponectin and its association with MDA

We first compared the kinetics of serum adiponectin levels in 1-day- and 4-week-old offspring exposed to maternal HFF diet with those in the same aged N-offspring. Serum adiponecin levels in 1-day- and 4-week-old HFF-offspring were significantly lower than those in N-offspring at the same age (1.2 ± 0.2 vs. 3.3 ± 0.8 μg/ml for 1-day old and 2.4 ± 0.4 vs. 10.5 ± 0.7 μg/ml for 4-week old, respectively) (Fig. 1A). Furthermore, there was a significant and inverse correlation between serum adiponectin levels and MDA values in 4-week-old HFF- and N-offspring ($r=-0.61$, $p<0.05$) (Fig. 1B). Moreover, kidney weight was significantly lighter in HFF-offspring at 1 day of old, while heavier in 4-week-old HFF-offspring compared with N-offspring of the same age (0.075 ± 0.004 vs. 0.099 ± 0.006g for 1-day old and 0.927 ± 0.004 vs. 0.838 ± 0.024g for 4-week old, respectively) (Table 1). There was no difference of kidney weight per body weight ratio between N- and HFF-offspring in each old, suggesting that renal hypertrophy might not occur in the offspring by exposure of HFF diet to dam (Table 1).

Effects of maternal HFF diet on UAE and podocyte loss in 16-week-old offspring and the association of UAE with serum adiponectin levels

Although there was no difference of UAE levels between N- and HFF-offspring at 4-week old (29.6 ± 5.3 vs. 31.5 ± 3.5 μg/day), UAE levels were increased as HFF-

offspring grew older and significantly higher than those of N-offspring at 8, 12, and 16 weeks of old (151.6 ± 17.7 vs. 56.2 ± 6.8, 204.7 ± 27.1 vs. 89.6 ± 17.6 and 211.3 ± 42.2 vs. 91.0 ± 9.5 μg/day, respectively) (Fig. 2A). UAE values in HFF- and N-offspring were inversely associated with serum adiponectin levels at 16-week old ($r=-0.47$, $p<0.05$) (Fig. 2B).

We further examined the effects of exposure to maternal HFF diet on podocyte loss in offspring, which was evaluated by immunofluorescence staining for podocin and western blot analysis for synaptopodin and WT1. As shown in Figs. 2C and 2D, intensity of podocin expression was significantly reduced in the glomeruli of 16-week-old HFF-offspring compared with that of N-offspring at the same age (21.1 ± 1.0 vs. 30.1 ± 1.2 %, $p<0.01$). Further, synaptopodin and WT1 protein expression in the kidney cortex was significantly decreased in HFF-offspring compared with N-offspring at 16-week old (0.55 ± 0.03 vs. 1.00 ± 0.15, $p<0.01$, 0.58 ± 0.03 vs. 1.00 ± 0.11, $p<0.01$, respectively) (Figs. 2E and 2F). Therefore, less podocin staining might be explained partly by podocyte loss and decreased podocyte biosynthesis.

Effects of exposure to maternal HFF diet on TGF-β1 expression and ECM accumulation in the interstitium and glomeruli of 16-week-old offspring

As shown in Fig. 3A, expression of TGF-β1 was dramatically increased in the kidney of 16-week-old HFF-offspring compared with N-offspring. Masson's trichrome staining revealed that interstitial ECM accumulation was not enhanced in HFF-offspring (4.27 ± 0.45 vs. 3.31 ± 0.45 %, p=0.13) (Fig. 3B). However, maternal exposure to HFF-diet significantly increased ECM accumulation in the glomeruli and decreased Ccr levels in offspring (Figs. 3C and 3D).

Effects of maternal HFF diet on blood glucose and blood pressure levels in 16-week old offspring

HFF-offspring at 16 weeks of old exhibited higher blood glucose levels, impaired glucose tolerance at 30 min after IVGTT, and higher mean blood pressure compared with those of N-offspring at the same age (6.9 ± 0.4 vs. 4.6 ± 0.2 mmol/L, 20.1 ± 1.5 vs. 13.8 ± 0.9 mmol/L, and 104.0 ± 1.2 vs. 94.8 ± 2.2 mmHg, respectively) (Figs. 4A, 4B, and 4C).

Discussion

In this study, we demonstrated that 1) adiponectin levels were significantly decreased in rat HFF-offspring at 1-day and 4-week old compared with N-offspring, and

inversely associated with serum MDA values, 2) kidney weight was significantly lower at birth, whereas it was heavier in HFF-offspring at weaning than N-offspring, 3) exposure to maternal HFF significantly increased UAE values, which were inversely associated with serum adiponectin levels in offspring at 16 weeks of old, 4) HFF-offspring significantly exhibited decreased podocin, synaptopodin, and WT1 protein expression and reduced Ccr levels in association with enhanced TGF-β1 expression and ECM accumulation in the kidney, and 5) high glucose levels, impaired glucose tolerance, and high mean blood pressure were observed in 16-week-old HFF-offspring compared with N-offspring at the same age.

Adiponectin is one of the adipokines secreted by adipose tissue, which has possessed various biological actions, such as anti-inflammatory, anti-atherosclerotic, and insulin-sensitizing properties in both animal model and humans, thereby protecting against obesity-related metabolic derangements and cardiorenal damage [17-20]. Indeed, adiponectin-deleted mice have exhibited increased albuminuria, fusion of podocyte foot process, and oxidative stress generation, which were abolished by the administration of adiponectin [20]. In contrast to the case of adiponectin deficiency, administration of adiponectin has been reported to improve podocyte permeability to albumin through the inhibition of NADPH oxidase activity [20]. Intraperitoneal infusion of adiponectin has

also been shown to decrease renal TGF-β expression and improve mesangial expansion in the kidneys of diabetic rats [21]. Furthermore, in an obese African-American population, there was a significant and inverse correlation between plasma adiponectin concentration and UAE values [20]. In this study, serum adiponectin levels were significantly reduced in HFF-offspring at 4-week old, and inversely associated with serum MDA, a marker of oxidative stress in the offspring at 4-week old and UAE levels at 16-week old, respectively. Since maternal exposure to HFF-diet significantly induced renal dysfunction with loss of podocyte, ECM accumulation, and increased TGF-β expression in the kidney in the offspring, our present findings suggest that hypoadiponectinemia might induce podocyte injury and subsequently cause glomerular sclerosis and renal damage in HFF-offspring via oxidative stress generation [22, 23]. Adiponectin-deleted mice with 5/6 nephrectomy have exhibited exacerbation of albuminuria and renal fibrosis [24, 20]. Moreover, podocyte-specific activation of TGF-β signaling has been shown to exacerbate podocyte damage and renal dysfunction in adriamycin-administered mice, an animal model of chronic kidney disease, thus suggesting that decreased adiponectin levels might be a therapeutic target for preventing renal damage in HFF-offspring.

In our study, although glomerular ECM accumulation was significantly

enhanced in HFF-offspring compared with N-offspring, interstitial ECM accumulation was not. Therefore, upregulation of TGF-β expression in the renal cortex of HFF-offspring might play a role for developing glomerular ECM accumulation, thereby leading to the progression of albuminuria and renal dysfunction.

Circulating adiponectin levels elevate with increasing age by adolescence in healthy and diabetic subjects [25]. Consistent with the finding, in our study, serum adiponectin levels were increased along with the age in N-offspring, however, the increase in adiponectin levels were significantly suppressed in HFF-offspring. Because serum adiponectin cannot pass through the placenta due to its higher molecular weight [26], it is unlikely that serum adiponectin levels in the offspring might be affected by dam's adiponectin values. In our study, body weight was significantly decreased at birth in the HFF-offspring compared with N-offspring. Since serum adiponectin levels in neonates are positively associated with fat volume [27], lower fat amount at birth might be responsible for decreased serum adiponectin levels at 1-day old. However, although there was no significant difference of body weight between N- and HFF-offspring at 4 weeks of old, serum adiponectin levels in HFF-offspring remained suppressed compared with N-offspring. Therefore, maternal exposure to HFF diet might impair adipogenesis and adiponectin secretion in the offspring. Given that decreased adiponectin levels play a

central role in the pathogenesis of insulin resistance and the metabolic syndrome as well, hyperglycemia at random and post glucose load and elevated mean blood pressure in HFF-offspring could be ascribed partly to hypoadiponectinemia.

It should be noted that although serum adiponectin levels tended to be decreased in 16-week old HFF-offspring compared with N-offspring, adiponectin gene expression in adipose tissue was significantly elevated (5.9 ± 0.9 vs. 8.3 ± 1.1μg/ml, p=0.11, 2.73 ± 0.21 vs. 1.00 ± 0.59, $p<0.05$, respectively), indicating that adiponectin gene expression in adipose tissue did not necessarily reflect adiponectin production in our animal models. These findings suggest that hypoadiponectinemia during the early postnatal development periods, which is caused by maternal exposure to HFF-diet might play a role in renal damage, high blood pressure, and metabolic derangements later in life. Hypoadiponectinemia early in life might partly explain the phenomenon of so-called "metabolic memory" in HFF-offspring, which is also observed in organ damage and death in patients with diabetes [28, 29].

We had several limitations in this study. First, although milk's adiponectin in mothers might affect serum adiponectin levels in infants [30]. We did not measure adiponectin values in the dam's milk. Second, our study was a cross-sectional one and therefore, did not elucidate the causal relationships between serum levels of adiponectin

during the early postnatal development periods in HFF-offspring and renal injury later in life. Therefore, further longitudinal interventional study is needed to clarify the clinical utility of measuring adiponectin levels and restoring its values for predicting and preventing renal damage in humans, respectively.

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Figure legends

Fig. 1. Effects of maternal HFF-diet on serum adiponectin levels and its association with MDA (A and B) in 1-day- and 4-week-old offspring. (A) Serum adiponectin levels in 1-day- and 4-week-old offspring. (n=5 for 1-day old and n=6 for 4-week old, respectively). **(B)** Inverse correlation between serum adiponectin and MDA levels in 4-week-old offspring ($r=-0.61$, $p<0.05$) (n=6 for N-offspring and n=6 for HFF-offspring, respectively). N, N-offspring; HFF, HFF-offspring; MDA, malondialdehyde.

Fig. 2. Effects of maternal HFF-diet on UAE values (A), its relation with adiponectin (B), and podocin, synaptopodin, and WT1 expression (C-F) in 16-week-old offspring. (A) UAE levels in 4-week-, 8-week-, 12-week-, and 16-week-old offspring. (n=4-12 per group). **(B)** Inverse correlation between UAE levels and serum adiponectin at 16-week old (n=11 for N-offspring and n=11 for HFF-offspring, respectively). **(C)** Panel shows the representative photographs of podocin expression in the glomeruli of 16-week-old offspring (x600). **(D)** Quantitative analysis of immunofluorescence staining for podocin in the glomeruli of 16-week-old offspring. **(E)** Western bolt and quantitative analysis for synaptopodin protein expression in the renal cortex of 16-week-old offspring. **(F)** Western bolt and quantitative analysis for WT1 protein expression in the renal cortex of 16-week-old offspring. (n=5 for N-offspring and n=5 for HFF-offspring, respectively). N, N-offspring; HFF, HFF-offspring; UAE, urinary albumin excretion; WT1, Wilms' tumor-1.

Fig. 3. Effects of exposure to maternal HFF-diet on TGF-β1 protein expression (A) and ECM accumulation in the interstitium (B) and glomeruli (C) of 16-week-old offspring and Ccr levels (D). (A) Upper panel shows the representative immunoblots of TGF-β1 protein expression. Lower panel shows the quantitation data. Data were normalized by the intensity of β-actin-derived signals and related to the value of N (n=11 for N-offspring and n=11 for HFF-offspring, respectively). **(B)** Upper panel shows the representative photographs of ECM accumulation in the renal interstitium of 16-week-old offspring evaluated by Masson's trichrome staining (x100) (n=5 for N-offspring and n=5 for HFF-offspring, respectively). Lower panel shows the quantitative analysis. **(C)** Upper panel shows the representative photographs of ECM accumulation in the glomeruli of 16-week-old offspring evaluated by Masson's trichrome staining (x600) (n=5 for N-offspring and n=5 for HFF-offspring, respectively). Lower panel shows the quantitative data. **(D)** Ccr levels in 16-week-old offspring (n=11 for N-offspring and n=11 for HFF-offspring, respectively). N, N-offspring; HFF, HFF-offspring; TGF-β, transforming

growth factor-β; ECM, extracellular cell matrix; Ccr, creatinine clearance

Fig. 4. Effects of maternal HFF-diet on blood glucose, IVGTT, and mean blood pressure in 16-week-old offspring. (A) Blood glucose levels in 16-week-old offspring (n=11 for N-offspring and n=11 for HFF-offspring, respectively). **(B)** Glucose tolerance test in 16-week-old offspring (n=5 for N-offspring and n=5 for HFF-offspring, respectively). **(C)** Mean blood pressure levels in 16-week-old offspring (n=5 for N-offspring and n=5 for HFF-offspring, respectively). N, N-offspring; HFF, HFF-offspring; IVGTT, intravenous glucose tolerance test.