Neutralizing MIP3α reduces renal immune cell infiltration and progressive renal injury in young obese Dahl salt-sensitive rats

Ubong S. Ekperikpe1, Bibek Poudel1, Corbin A. Shields1, Sautan Mandal1, Denise C. Cornelius1,2, and Jan M. Williams1

Departments of Pharmacology and Toxicology, and Emergency Medicine, University of Mississippi Medical Center, Jackson, MS
List of abbreviations: ACE, angiotensin-converting enzyme; AKI, acute kidney injury; APC, allophycocyanin; BV421, brilliant violet™ 421; CCL20, chemokine (c-c) ligand 20; CD, cluster of differentiation; CKD, chronic kidney disease; DC, dendritic cell; DNase, deoxyribonuclease; FACS, fluorescence activated and cell sorting; FITC, fluorescein isothiocyanate; FOXP3, forkhead box P3; FSC, forward scatter; GFR, glomerular filtration rate; IgG, immunoglobulin G; IL2, interleukin-2; IL4, interleukin-4; IL10, interleukin-10; IL17, interleukin-17; KIM-1, kidney injury molecule-1; MIP3α, macrophage inflammatory protein 3α; MNA, MIP3α neutralizing antibody; NGAL, neutrophil gelatinase-associated lipocalin; PAS, periodic acid Schiff; PE, phycoerythrin; PerCP, peridinin-chlorophyll protein; RAS, renin-angiotensin system; RORγt, retinoic acid-related orphan receptor-γt; RPMI, Roswell Park Memorial Institute (buffer); SS, Dahl salt-sensitive (rat); SSC, side scatter; SS⁻Leprmutant, Dahl salt-sensitive leptin receptor mutant (rat).
Abstract

Recently, we reported that the early progression of renal injury in obese Dahl salt-sensitive leptin receptor mutant (SS\textsuperscript{LepRmutant}) rats was associated with increased macrophage inflammatory protein alpha (MIP3a) expression prior to puberty. Therefore, this study tested the hypothesis that MIP3\textalpha{} plays a role in recruiting immune cells, thereby triggering renal inflammation and early progressive renal injury in SS\textsuperscript{LepRmutant} rats prior to puberty. Four-week-old SS and SS\textsuperscript{LepRmutant} rats either served as control (IgG; i.p., every other day) or received MIP3a neutralizing antibody (MNA; 100 \mu g/kg) for 4 weeks. MNA reduced circulating and renal MIP3a levels and pro-inflammatory immune cells by 50%. While MNA treatment did not affect blood glucose and plasma cholesterol levels, MNA markedly decreased insulin resistance and triglyceride levels in SS\textsuperscript{LepRmutant} rats. We observed no differences in MAP between SS and SS\textsuperscript{LepRmutant} rats, and MNA had no effect on MAP in either strain. Proteinuria was significantly increased in SS\textsuperscript{LepRmutant} rats versus SS rats over the course of the study. Treatment with MNA markedly decreased proteinuria in SS\textsuperscript{LepRmutant} rats while not affecting SS rats. Also, MNA decreased glomerular and tubular injury and renal fibrosis in SS\textsuperscript{LepRmutant} rats while not affecting SS rats. Overall, these data indicate MIP3a plays an important role in renal inflammation during the early progression of renal injury in obese SS\textsuperscript{LepRmutant} rats prior to puberty. These data also suggest that MIP3a may be a novel therapeutic target to inhibit insulin resistance and prevent progressive proteinuria in obese children.

\textbf{Keywords:} childhood obesity, SS, SS\textsuperscript{LepRmutant}, MIP3a, immune cells, renal injury
Significance Statement

Childhood obesity is increasing at an alarming rate and is now being associated with renal disease. While most studies have focused on the mechanisms of renal injury associated with adult obesity, few studies have examined the mechanisms of renal injury involved during childhood obesity. In the current study, we observed that the progression of renal injury in obese SS\textsuperscript{LepR} mutant rats was associated with an increase in MIP3a, a chemokine, before puberty, and inhibition of MIP3a markedly reduced renal injury.
Introduction

In recent decades, obesity has become an epidemic globally, and in the United States (Ogden et al., 2015; Ogden et al., 2016). The prevalence of obesity has risen, not only in adults, but also in children (Cattaneo et al., 2010). According to the WHO, more obese and overweight children have died than underweight children due to breathing difficulties, elevated risk of fractures, hypertension, and early markers of cardiovascular disease and insulin resistance. Obese patients have an increased risk to develop diabetes and hypertension, the two leading causes of kidney disease (Kramer et al., 2006; Srivastava, 2006). Childhood obesity is positively correlated with markers of renal injury such as elevated serum creatinine and microalbuminuria (Ferris et al., 2007; Kaneko et al., 2011; Önerli Salman et al., 2019; Savino et al., 2010). Interestingly, renal injury in obese children and adolescents starts long before the development of hypertension or diabetes. Therefore, it is necessary to identify novel targets to prevent early progressive renal disease in this unique population. Recently, we reported that the Dahl salt-sensitive leptin receptor mutant rat (SS\textsuperscript{Lepr}mutant rat) develops renal injury without hyperglycemia and elevations in arterial pressure, prior to puberty, and is therefore a novel model to study mechanisms of childhood obesity-induced renal disease (McPherson et al., 2019; McPherson et al., 2020; McPherson et al., 2016; Poudel et al., 2020; Poudel et al., 2022).

An early hallmark characteristic of obesity-induced renal disease is elevations in glomerular filtration rate (renal hyperfiltration) (Hostetter et al., 1982; Kasiske & Napier, 1985). We hypothesized that renal hyperfiltration damages various renal cells such as podocytes and tubular cells, which are a source of pro-inflammatory cytokines (Rayego-Mateos et al., 2020). In support of our hypothesis, studies from our laboratory have demonstrated that early progressive proteinuria in obese SS\textsuperscript{Lepr}mutant rats was associated with renal hyperfiltration and inflammatory
cytokines such as macrophage inflammatory protein 3-alpha (MIP3α) (Brown et al., 2021; McPherson et al., 2020). Moreover, prevention of renal hyperfiltration in SS\(^{\text{LepRmutant}}\) mutant rats prior to puberty slowed the progression of glomerular injury and decreased renal MIP3α levels (Brown et al., 2021). MIP3α is a low-molecular weight chemotactic cytokine which recruits dendritic cells (DCs) and lymphocytes (Th17s, Tregs, and B-cells) (Wiede et al., 2013), and is secreted by podocytes and tubular cells, as well as immune cells such as macrophages (Nandi et al., 2014). However, the role of MIP3α in the progression of obesity-induced renal injury has not been studied. Therefore, the goal of the current study was to test the hypothesis that MIP3α plays a role in recruiting immune cells, thereby triggering renal inflammation and early progressive renal injury in SS\(^{\text{LepRmutant}}\) mutant rats prior to puberty.

**Methods**

*General.* Experiments were performed on a total of 59 female and male Dahl salt-sensitive (SS) and Dahl salt-sensitive leptin-receptor mutant rats (SS\(^{\text{LepRmutant}}\) mutant rats). Both rat strains were generated from our in-house colony of heterozygous SS\(^{\text{LepRmutant}}\) mutant rats, which were originally created at the Medical College of Wisconsin with the zinc finger nuclease technology (McPherson et al., 2016). Genotyping was done by the Molecular and Genomics Facility of the University at Mississippi Medical Center. The rats were given free access to food and water for the entire study. Rats were fed a 1% NaCl diet (Envigo, Madison, WI). MIP3α neutralizing antibody (MNA) and IgG isotype control were purchased from R&D Systems (Minneapolis, MN). The rats were housed in the Laboratory Animal Facility of the University of Mississippi Medical Center, which is approved by the American Association for the Accreditation of Laboratory Animal Care. All
protocols were approved by the University of Mississippi Medical Center Institutional Animal Care and Use Committee.

**Protocol.** These experiments were carried out on 4-week-old female and male SS and SS<sup>Lep<sup>R</sup></sup>mutant rats. At baseline, rats were weighed and placed in metabolic cages overnight to collect urine for the determination of proteinuria using the Bradford method (Bio-Rad Laboratories, Hercules, CA). Blood was collected from the tail vein to measure non-fasting blood glucose levels (glucometer from Bayer HealthCare; Mishwaka, IN). After the collection of baseline data, SS and SS<sup>Lep<sup>R</sup></sup>mutant rats were randomly separated into four groups as follows: 1) SS and 2) SS<sup>Lep<sup>R</sup></sup>mutant rats serving as control (IgG; 100 μg/kg ip, every other day), and 3) SS and 4) SS<sup>Lep<sup>R</sup></sup>mutant rats receiving MNA 100 μg/kg ip, every other day, for 4 weeks. The doses of MNA and IgG were selected from a previous study (Hu et al., 2016). Every two weeks, non-fasting blood glucose and proteinuria were measured in the rats. Urine collected at the end of the protocol were used to measure the excretion rates of kidney injury molecule 1 (KIM-1; Abcam, Waltham, MA), neutrophil gelatinase-associated lipocalin (NGAL; Abcam, Waltham, MA), albumin (Abcam, Waltham, MA), and nephrin (NPB2-76751 Novus Biologicals, Littleton, CO) via ELISA according to the manufacturer’s recommendations. Glomerular filtration rate (GFR) was assessed via creatinine clearance (Cr<sub>Cl</sub>) (Bioassay Systems, Hayward, CA).

At the end of the study, rats were anesthetized, and a catheter was inserted into the carotid artery to measure mean arterial pressure (MAP). After a 24-hour recovery period, catheters were connected to pressure transducers (MLT0699, ADInstruments, Colorado Springs, CO) coupled to a computerized PowerLab data-acquisition system (ADInstruments). After a 30-minute equilibration period, MAP was recorded continuously for 30 minutes. Then, a final blood sample was drawn from the abdominal aorta for the measurement of plasma cholesterol (Cayman...
Chemical, Ann Arbor, MI), triglyceride (Cayman Chemical, Ann Arbor, MI), insulin (Mercodia rat insulin ELISA, Uppsala, Sweden), and MIP3a (Bio-Rad Laboratories, Hercules, CA) concentrations. Next, the kidneys were perfused with saline until they appeared visibly pale. The kidneys were collected and weighed. The right kidney was cut into two equal halves; one half was fixed in 10% neutral buffered formalin solution for histological analysis. The other half was snap-frozen in liquid nitrogen and stored at -80 °C. Renal cytokines were measured using the Bio-Plex Pro Rat Cytokine 5-Plex Assay Reagent Kit on a Bio-Rad Bio-Plex 200 system as described by manufacturer’s protocol (Bio-Rad Laboratories, Hercules, CA). The cytokines measured were MIP3a, IL-2, IL-4, IL-10, and IL-17. The left kidney was used to measure the renal infiltration of immune cells as described below.

Renal Immune Cell Isolation and Flow Cytometry. Immune cells from the left kidneys of control and MNA-treated SS and SS
t^mut^ mutant rats were isolated as previously described (Poudel et al., 2020). Briefly, the kidneys were minced in RPMI-1640 containing 0.1% collagenase and 10 μg/ml DNAse 1. These were homogenized, filtered through a 100 μm strainer, incubated at 37 °C for 30 minutes, and subsequently filtered through 70 μm, and 40 μm strainers. Mononuclear cells were separated by Percoll density gradient centrifugation at 1200 rpm for 30 minutes at 25 °C. The pellet obtained was washed and resuspended in 1 mL FACS buffer, after which immune cells were counted using an Automated Cell Counter and Image Cytometer (Nexcelom Bioscience, Lawrence, MA.). Mononuclear cells were stained with viability (Viobility™ 405/520 for macrophage and dendritic cells; 1:50, and 405/452 for lymphocytes; 1:50) fixable dyes (Miltenyi Biotec, Auburn, CA) for 20 minutes at 4 °C to identify live cells. The macrophage panel consisted of the following antibodies: anti-rat CD68-PE-Vio770 (1:10; Miltenyi Biotec, Auburn, CA), anti-rat CD86-PE (1:50; Miltenyi Biotec, Auburn, CA), and mouse anti-rat CD163-FITC (1:50; Bio-
Rad Laboratories). The DC panel consisted of the following antibodies: anti-rat CD103-APC (1:10; Miltenyi Biotec, Auburn, CA), anti-rat CD86-PE (1:50; Miltenyi Biotec, Auburn, CA), and mouse anti-rat CD80-BV421 (1:10; BD Biosciences, Haryana, India). The lymphocyte panel consisted of the following antibodies: anti-rat CD3-VioGreen (1:10; Miltenyi Biotec, Auburn, CA) for total T-cells, anti-rat CD4-FITC (1:10; Miltenyi Biotec, Auburn, CA) for total T-helper cells, anti-rat CD8a-APC-Vio770 (1:50; Miltenyi Biotec, Auburn, CA) for cytotoxic T-cells, mouse anti-rat CD25 PE (1:50; BD Biosciences, Haryana, India.) for Th17 cells, mouse anti-rat FOXP3-AlexaFluor-647 (1:2; R and D systems, Minneapolis, MN) for regulatory T-cells, and anti-rat CD45R-PE-Vio770 (1:10; Miltenyi Biotec, Auburn, CA) for total B-cells. Flow cytometry was carried out using the Miltenyi MACSQuant Analyzer 10 (Miltenyi Biotec, Auburn, CA), and data were analyzed using FlowLogic software (Miltenyi Biotec, Auburn, CA).

Renal Histology. Paraffin-embedded kidney sections were prepared from half of the right kidneys collected from SS and SSLeqRmutant rats. Kidney sections were cut into 5 μm sections and stained with Periodic acid-Schiff (PAS) and Picrosirius red. Thirty glomeruli per PAS section were scored blindly, on a scale of 0-4 to assess glomerular injury, where 0 represented a normal glomerulus, 1 represented a 25 % loss, 2 represented a 50 % loss, 3 represented a 75 % loss, and 4 represented a greater than 75 % loss of capillaries in the glomerular tuft (McPherson et al., 2016; Spires et al., 2018). To assess the extent of renal cortical and medullary fibrosis from Picrosirius red-stained sections, 10 representative images per kidney section per animal were taken with a SeBa microscope equipped with a colored camera (Laxco Inc., North Creek, WA). We analyzed for the percentage of each image stained red (collagen) by identifying the animal whose kidney had the most collagen. This was used to set a threshold for red staining in the sections using NIS-
Elements D 3.0 software (McPherson et al., 2016; Spires et al., 2018). Next, those same thresholding parameters were used for the red staining on each kidney image per rat in the study to measure renal fibrosis.

**Statistical Analysis.** The data are presented as mean values ± SD. Statistical analysis was performed using GraphPad Prism 8 (GraphPad Software, San Diego, CA). The significance of the difference in mean values for a single time point was determined by two-way ANOVA followed by Holm-Sidak’s multiple-comparisons test. Time course changes in protein excretion were compared from baseline, between and within strains using three-way ANOVA followed by Tukey’s multiple-comparisons test. *P* values of < 0.05 were considered significantly different.

**Results**

*Flow Cytometry.* The flow cytometry gating strategy for immune cell infiltration is provided in Figure 1. After gating for the mononuclear cell population using forward scatter (FSC) and side scatter (SSC), dead cells were excluded using viability staining and doublet exclusion. From this population, CD3⁺ cells were gated as T-lymphocytes, and CD4⁺ T-Helper cells and CD8⁺ cytotoxic T-cells were identified from the CD3⁺ cell population. From the CD4⁺ T-Helper cell population, CD25⁺FOXP3⁺ cells were identified as regulatory T-cells, while CD25⁻FOXP3⁻ cells were identified as Th17s. CD45R⁺ B-cells were identified from the CD3⁻ cell population. Also, from the CD3⁻ population, CD68⁺ and CD103⁺ cells were identified as macrophages and DCs, respectively. CD68⁺CD86⁺ and CD68⁺CD163⁺ cells were identified as M1 and M2 macrophages, respectively. CD103⁺CD80⁺ and CD103⁺CD86⁺ cells were identified as stimulatory DCs.

*Measurement of Circulating and Renal MIP3α.* The effects of chronic MNA administration on plasma and renal MIP3α are shown in Figure 2. Plasma and renal MIP3α were
markedly greater in control SS<sup>LepR</sup>mutant rats versus SS rats, and chronic MNA administration only decreased MIP3a in the plasma and kidneys of SS<sup>LepR</sup>mutant rats (Figure 2A and 2B).

**Metabolic Endpoints.** The effects of chronic MNA administration on metabolic endpoints are presented in Table 1. There was a marked increase in body weight in control SS<sup>LepR</sup>mutant rats in comparison to their SS counterparts, and MNA treatment did not affect body weight in either strain. We detected no differences in blood glucose levels in any of the groups. We observed an almost ten-fold increase in plasma insulin in control SS<sup>LepR</sup>mutant rats in comparison to SS rats, and the administration of MNA markedly reduced plasma insulin levels in SS<sup>LepR</sup>mutant rats by over 50 %, while not affecting SS rats. Plasma triglycerides were over six times higher in control SS<sup>LepR</sup>mutant rats compared to SS counterparts (467±91 mg/dL vs. 70±28 mg/dL, respectively), and the administration of MNA decreased plasma triglycerides by over 50 % (210±30 mg/dL), while not affecting SS rats (71±24 mg/dL). There was a marked increase in plasma total cholesterol in control SS<sup>LepR</sup>mutant rats in comparison to SS rats (252±46 vs 143±38 mg/dL, respectively), and the administration of MNA did not affect plasma total cholesterol in either strain.

**Measurement of Mean Arterial Pressure and Markers of Renal Injury.** The effects of MNA administration on MAP and markers of renal injury (proteinuria and albuminuria) are presented in Figure 3. There were no marked differences in MAP among control and MNA-treated SS and SS<sup>LepR</sup>mutant rats (Figure 3A). At baseline, we detected no differences in protein excretion between SS and SS<sup>LepR</sup>mutant rats. During the course of the study, proteinuria rose from 41±7 mg/day to 426±47 mg/day in control SS<sup>LepR</sup>mutant rats, compared to control SS rats, where proteinuria only rose from 10±3 mg/day to 51±16 mg/day. Chronic MNA administration decreased proteinuria by over 50 % in SS<sup>LepR</sup>mutant rats, while not affecting SS rats (Figure 3B). At the end of the study, we observed over a 30-fold increase in albumin excretion in control SS<sup>LepR</sup>mutant
rats in comparison to SS rats, and chronic administration of MNA resulted in a near 5-fold decrease in albumin excretion in \( \text{SS}^{\text{LepR}} \) mutant rats, while not affecting SS rats (Figure 3C).

Renal Function Assessment and Markers of Glomerular and Tubular Injury. The effects of MNA on renal function (CrCl), as well as markers of glomerular and tubular injury are presented in Figure 4. There was a marked increase in CrCl in control \( \text{SS}^{\text{LepR}} \) mutant rats in comparison to SS rats. Chronic MNA administration did not affect CrCl in \( \text{SS}^{\text{LepR}} \) mutant rats and SS rats (Figure 4A). We detected a 4-fold increase in nephrin excretion in control \( \text{SS}^{\text{LepR}} \) mutant rats in comparison to SS rats, and the administration of MNA decreased nephrin excretion by about 50% in \( \text{SS}^{\text{LepR}} \) mutant rats, while not affecting SS rats (Figure 4B). There was a marked increase in excretion of urinary markers of tubular injury (KIM-1 and NGAL) in control \( \text{SS}^{\text{LepR}} \) mutant rats in comparison to their SS counterparts (Figure 4C and 4D). The chronic administration of MNA markedly decreased KIM-1 and NGAL excretion in \( \text{SS}^{\text{LepR}} \) mutant rats, while not affecting the SS counterparts.

Renal Histology. Representative images and a corresponding analysis of renal histopathology in control and MNA-treated SS and \( \text{SS}^{\text{LepR}} \) mutant rats are shown in Figure 5. The kidneys from control \( \text{SS}^{\text{LepR}} \) mutant rats showed increased mesangial expansion and glomerular injury in comparison to their SS counterparts (Figure 5A and 5D), and chronic MNA administration significantly reduced glomerular injury in \( \text{SS}^{\text{LepR}} \) mutant rats. Greater fibrosis was seen in the renal cortex (Figure 5B and 5E) and renal medulla (Figure 5C and 5F) of \( \text{SS}^{\text{LepR}} \) mutant rats in comparison to their SS counterparts, and chronic MNA administration reduced cortical and medullary interstitial fibrosis.

Measurement of Renal Immune Cell Infiltration. The effects of MNA on renal immune cell infiltration are shown in Figures 6 and 7. There was a marked increase in renal CD103\(^+\) and
CD103+/CD80+ DCs in control SS^{LepR}_{mutant} rats in comparison to SS rats, with no noteworthy differences detected in CD103+/CD86+ DCs. Chronic administration of MNA markedly reduced the renal infiltration of these DCs without affecting SS rats (Figure 6A and 6B). Renal total macrophages were significantly increased in control SS^{LepR}_{mutant} rats versus SS rats. Similar results were observed with M1 and M2 macrophages (Figure 6E and 6F). The administration of MNA significantly decreased the infiltration of total and M1 macrophages, but not M2 in SS^{LepR}_{mutant} rats (Figure 6D and 6E).

The effect of the administration of MNA on the renal infiltration of various lymphocytes is presented in Figure 7. We detected a marked increase in the renal infiltration of total T-cells, Th17s, and cytotoxic T-cells in control SS^{LepR}_{mutant} rats in comparison to SS rats. The chronic administration of MNA markedly reduced the renal infiltration of Th17s and cytotoxic T-cells, but not total T-cells in SS^{LepR}_{mutant} rats (Figure 7A, 7B and 7E). No differences were detected in total T-Helper cells and regulatory T-cells across the groups (Figure 7D and 7F). Total B-cells were markedly increased in the kidneys of control SS^{LepR}_{mutant} rats in comparison to SS rats. The administration of MNA only reduced the renal infiltration of B-cells in SS^{LepR}_{mutant} rats (Figure 7C).

**Renal Cytokine Measurements.** The effects of chronic MNA administration on other renal cytokines are presented in Figure 8. There was a marked decrease in renal IL-10 in control SS^{LepR}_{mutant} rats in comparison to SS rats. Chronic MNA treatment did not affect renal IL-10 in SS^{LepR}_{mutant} rats (Figure 8A). Similar to IL-10, we noticed a 50% reduction in renal IL-4 in control SS^{LepR}_{mutant} rats versus SS rats, and chronic MNA treatment did not affect renal IL-4 in SS^{LepR}_{mutant} rats (Figure 8B). There were no differences in renal IL-2 and IL-17 expression for both control and MNA-treated SS and SS^{LepR}_{mutant} rats (Figure 8C and 8D).
Discussion

Obese adults and children are susceptible to renal disease independent of diabetes or hypertension (Kovesdy et al., 2017; Kramer et al., 2006; Srivastava, 2006). While studies have focused on mechanisms of renal disease in adult obesity, little effort has been put into studying renal disease associated with childhood/prepubertal obesity. A major characteristic of obese patients is renal hyperfiltration (Eirin et al., 2017; Ferris et al., 2007; van Bommel et al., 2020). Studies have demonstrated that obesity-induced hyperfiltration leads to injury of specialized cells of the glomerulus such as the podocytes, and tubular epithelial cells, which are a source of pro-inflammatory mediators (Brown et al., 2021). Recently, we reported that early progressive renal injury was associated with renal hyperfiltration, glomerular injury, renal macrophage infiltration, and increased renal MIP-3a expression in obese SS<sup>LepR</sup> mutant rats before puberty (Brown et al., 2021; McPherson et al., 2016; Poudel et al., 2020; Poudel et al., 2022). Moreover, preventing renal hyperfiltration reduced MIP-3a and renal disease during the prepubescent stage (Brown et al., 2021). Thus, the aim of the current study was to determine the role of MIP-3a during the early progression of renal injury in SS<sup>LepR</sup> mutant rats prior to puberty. The administration of MNA decreased circulating and renal levels of MIP-3a by over 50% in SS<sup>LepR</sup> mutant rats. Additionally, MNA administration reduced the renal infiltration of various immune cells (i.e. DCs, macrophages, Th17s, cytotoxic T-cells, and B-cells) in SS<sup>LepR</sup> mutant rats, and significantly decreased renal injury in SS<sup>LepR</sup> mutant rats. In addition, the administration of MNA improved metabolic parameters in SS<sup>LepR</sup> mutant rats by decreasing insulin resistance and plasma triglyceride levels. Taken together, these results show that the neutralizing MIP3a ameliorates metabolic endpoints, and decreases renal inflammation, and renal injury in SS<sup>LepR</sup> mutant rats before puberty.
Insulin is essential for the maintenance of physiologic levels of triglycerides, as it promotes triglyceride storage, while preventing its breakdown in adipose tissues (Czech et al., 2013). The SS\textsuperscript{LepR} mutant rats displayed features of metabolic syndrome, and MNA administration decreased hyperinsulinemia/insulin resistance and dyslipidemia, while not affecting their body weight and blood glucose levels. A plausible explanation for this is that the anti-inflammatory effects of MNA improved insulin resistance in SS\textsuperscript{LepR} mutant rats. Hypersinsulinemia is a known indicator of systemic insulin resistance (Czech, 2017; Petersen & Shulman, 2018). The chemokine system contributes to insulin resistance during obesity by regulating immune cell recruitment, thereby triggering inflammation, and impairing insulin sensitivity (Cancelllo et al., 2005; Huber et al., 2008; Kitade et al., 2012; Neels et al., 2009; Ota, 2013). This is supported by studies that show that inhibiting the chemokine signaling pathway, MCP1-CCR2, improved insulin sensitivity, while the overexpression of MCP1-CCR2 stimulated insulin resistance in mice. (Kamei et al., 2006; Kanda et al., 2006; Weisberg et al., 2006). While information on the role of MIP3a in metabolic syndrome are limited, a study reported that MIP3a is elevated in the serum of obese mice. (Burke et al., 2015). In the current study, plasma MIP3a was elevated in SS\textsuperscript{LepR} mutant rats, and the administration of MNA decreased plasma MIP3a and reduced hyperinsulinemia, suggesting a relationship between plasma MIP3a and obesity-related hyperinsulinemia and insulin resistance. Hyperinsulinemia and insulin resistance may, in part, contribute to dyslipidemia seen in SS\textsuperscript{LepR} mutant rats, and the reduction of plasma triglycerides in MNA-treated SS\textsuperscript{LepR} mutant rats may, in part, be due to decreased hyperinsulinemia and insulin resistance. These findings may be associated with the anti-inflammatory effects of MNA. While various studies have demonstrated that improvements in insulin sensitivity are associated with weight loss (Clamp et al., 2017; Ikeda et al., 1996), chronic MNA administration did not lead to weight loss in SS\textsuperscript{LepR} mutant rats despite
improvements in insulin sensitivity. The lack of effect of MNA on body weight in SS^{LepR} mutant rats may be due to the mutation in their leptin receptor which makes SS^{LepR} mutant rats hyperphagic leading to increased food intake and weight gain despite the effects of MNA on insulin resistance. These data suggest that MIP3a plays a role in insulin resistance and dyslipidemia during childhood obesity.

Despite overwhelming evidence to support the role of inflammation in the development of hypertension (Fehrenbach & Mattson, 2020; Kirabo et al., 2014; Mattson, 2019; Olsen, 1972; Van Beusecum et al., 2019), we did not observe a decrease in arterial pressure when inhibiting MIP3a in SS and SS^{LepR} mutant rats. Furthermore, the inhibition of other chemokines has been shown to reduce arterial pressure by decreasing immune cell recruitment in various animal models of hypertension (Alsheikh et al., 2020; Chung & Lan, 2011; Ruiz-Ortega et al., 2002). There are three potential reasons that could explain our observation. (1) The first possible reason is the choice of diet used during the experiment. Mattson and colleagues have fed their SS rats a high salt diet containing 4% NaCl or greater, which causes a rapid increase in arterial pressure within 3 weeks (Rudemiller et al., 2014). In the current study, both SS and SS^{LepR} mutant strains are fed a diet containing 1% NaCl, which minimized the development of hypertension in the SS strain. (2) Another potential reason is the age of the rats used in our study. Previous studies have used SS rats older than 9 weeks of age (Mattson et al., 2006). Since the current study focused on progressive renal injury prior to puberty, it is reasonable not to expect differences in arterial pressure in response to various drugs that may typically lower arterial pressure in older animals. This is supported by recent studies from our laboratory that demonstrate that anti-inflammatory or arterial pressure lowering drugs do not reduce arterial pressure in these rats during the prepubescent stage (Brown et al., 2021; Poudel et al., 2020). (3) The third reason is the
development of hypertension within SS rat genetic background is multifactorial and specifically inhibiting MIP3a is not enough to reduce arterial in SS\textsuperscript{LepR}\textsuperscript{R}mutant rats. These results suggest the lack of arterial pressure reduction in SS\textsuperscript{LepR}\textsuperscript{R}mutant rats’ response to MNA administration may due to the amount of NaCl content in the diet, age, and the genetic background of the SS rats.

A significant finding from this study was that the chronic administration of MNA markedly reduced early progressive renal injury in SS\textsuperscript{LepR}\textsuperscript{R}mutant rats before puberty. Clinical and experimental studies have shown that the progressive renal injury in certain severe forms of renal disease is associated with increased renal MIP3a expression (González-Guerrero et al., 2018; Lu et al., 2017; Turner et al., 2010). MIP3a is a strong chemotactic for immune cells such as DCs, T-cells, and B-cells (Nandi et al., 2014; Wiede et al., 2013; Woltman et al., 2005). During renal injury, stimulatory DCs activate T-cells via CD80/86 upregulation (Banchereau & Steinman, 1998; Kurts et al., 2020; Zhang et al., 2020), which elicit a pro-inflammatory response and macrophage recruitment leading to renal inflammation and progressive renal injury (Kurts et al., 2020; Zhang et al., 2020). This is supported by results of this study and preliminary reports from our laboratory demonstrating that inhibition of T-cell activation slows progressive renal injury in SS\textsuperscript{LepR}\textsuperscript{R}mutant rats before puberty (Ekperikpe et al., 2021). Therefore, we believe that chronic MIP3a neutralization with MNA reduces progressive renal injury in SS\textsuperscript{LepR}\textsuperscript{R}mutant rats by decreasing the recruitment of DCs and macrophages, thereby preventing T-cell activation. Furthermore, chronic MNA administration produces an overall anti-inflammatory effect by reducing renal MIP3a expression. Although treatment with MNA decreased renal Th17 infiltration, there were no noticeable differences in renal IL-17 expression. A plausible explanation for this observation is that there are multiple sources of IL-17 other than Th17s (Keijzers et al., 2014). Overall, these data
reveal that MIP3a contributes to progressive renal injury in SS\textsuperscript{L-epR}\textsuperscript{mutant} rats by mediating pro-inflammatory response during prepubertal obesity.

In conclusion, the data from this study suggest that during the early stages of renal injury in SS\textsuperscript{L-epR}\textsuperscript{mutant} rats, renal hyperfiltration damages glomerular and tubular epithelial cells. These cells secrete MIP3a leading to the recruitment of DCs, which activate T-cells causing a pro-inflammatory response and macrophage recruitment, stimulating renal inflammation and progressive renal injury associated with obesity prior to puberty. Moreover, chronic MIP3a inhibition ameliorated metabolic syndrome and renal injury in obese SS\textsuperscript{L-epR}\textsuperscript{mutant} rats by decreasing renal inflammation. While the current study specifically focused on the effects of MIP3a blockade on renal injury in young obese SS\textsuperscript{L-epR}\textsuperscript{mutant} rats, we would speculate that similar results would be observed in older SS\textsuperscript{L-epR}\textsuperscript{mutant} rats. Inflammation is known to contribute to renal injury in various adult animal models of obesity (Fernández-Sánchez et al., 2011; Lindfors et al., 2021; Wang et al., 2015). To the best of our knowledge, this is the first study to demonstrate a role for MIP3a in the early development of renal injury associated with obesity. Future studies from our laboratory will focus on downstream events after MIP3a signaling stimulation during the early progression of obesity-induced renal injury such as T-cell activation. These data indicate that MIP3a may be a pharmacological target for managing renal injury and metabolic disease associated with childhood obesity.
Acknowledgement

The authors are grateful to Tyler D. Johnson and Sarah M. Safir for maintaining the animal colony.

Authorship Contributions

Participated in research design: Ekperikpe U., Cornelius D., Williams J.

Conducted experiments: Ekperikpe U., Poudel B., Shields C., Cornelius D., Williams J.

Performed data analysis: Ekperikpe U., Cornelius D., Williams J.

Wrote or contributed to the writing of the manuscript: Ekperikpe U., Mandal S., Cornelius D., Williams J.
References.


Proteinuria In Obese Dahl Salt-sensitive Rats. *Hypertension*, 78(Suppl_1), A42-A42.

https://doi.org/doi:10.1161/hyp.78.suppl_1.42


https://doi.org/10.1152/ajprenal.00454.2019


Footnotes

This work was financially supported by the National Institutes of Diabetes and Digestive and Kidney Diseases of the National Institutes of Health (NIH/NIDDK, DK109133) awarded to JMW, the National Heart, Lung and Blood Institute of the National Institutes of Health (NIH/NHLBI, HL151407) awarded to DCC. The work performed through the UMMC Molecular and Genomics Facility is supported, in part, by funds from the NIGMS, including Mississippi INBRE (P20GM103476), Obesity, Cardiorenal and Metabolic Diseases-COBRE (P20GM104357), and Mississippi Center of Excellence in Perinatal Research (MS-CEPR)-COBRE (P20GM121334). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Financial Disclosures

No author has an actual or perceived conflict of interest with the contents of this article.

Citation of meeting abstract (Experimental Biology 2021).


All requests for reprints should be addressed to Dr. Jan Williams, Department of Pharmacology and Toxicology, University of Mississippi Medical Center, 2500 North State Street, Jackson, MS 39211. jmwilliams5@umc.edu
Figure Legends

Figure 1. Flow cytometry gating strategy: Flow cytometry was carried out for macrophage, dendritic cell and lymphocyte panels. After gating for mononuclear cells using forward scatter (FSC) and side scatter (SSC), dead cells were excluded using viability staining, and doublets were excluded. CD45+ staining was used to gate for total lymphocytes. From this population, gates were placed for CD3- cells and CD3+ T-cells. CD45R+ B-cells were identified in the CD3- cell population, and CD4+ T-helper and CD8+ cytotoxic T subsets were identified within the CD3+ T-cell population. From the CD4+ T-helper cell population, CD25+/FOXP3+ cells were identified as regulatory T-cells (Tregs), while CD25+/RORγT+ cells were identified as IL-17 producing T-helper cells (Th17s). Additionally, singlet CD45+/CD3- cells were gated. Within this population, CD68+ and CD103+ were identified as macrophages and dendritic cells (DCs), respectively. CD68+/CD86+ and CD68+/CD163+ cells were identified as M1 and M2 macrophages, respectively. CD103+/CD80+ and CD103+/CD86+ cells were identified as stimulatory DCs.

Figure 2. Effects of chronic treatment with a macrophage inflammatory protein 3 – alpha neutralizing antibody (MNA) on plasma and renal expression of MIP3a levels in Dahl salt-sensitive (SS) and obese SS leptin receptor mutant (SSLepRmutant) rats. Control SS and SSLepRmutant rats are represented by circles and squares outlined in black, respectively, and MNA-treated rats are represented by circles and squares outlined in red. Female and male rats in each group are represented by partially-filled and open symbols, respectively. (Panel A) plasma MIP3a levels and (Panel B) renal MIP3a levels. Values are means ± SD. The significance of the difference in mean values for a single time point was determined by a two-way ANOVA followed by Holm Sidak’s multiple comparisons test. A p-value <0.05 was considered significantly different. † indicates a significant difference from the corresponding value in SS rats within the same
treatment, and # indicates a significant difference from the corresponding value in control-treated rats within the same strain.

**Figure 3. Effects of chronic treatment with a macrophage inflammatory protein 3 – alpha neutralizing antibody (MNA) on mean arterial pressure (MAP) and markers of renal injury in Dahl salt-sensitive (SS) and obese SS leptin receptor mutant (SS^{LepRmutant}) rats.** Control SS and SS^{LepRmutant} mutant rats are represented by circles and squares outlined in black, respectively, and MNA-treated rats are represented by circles and squares outlined in red. Female and male rats in each group are represented by partially-filled and open symbols, respectively. (Panel A) mean arterial pressure (MAP), (Panel B) temporal changes in proteinuria, and (Panel C) albumin excretion. Values are means ± SD. The significance of the difference in mean values for a single time point was determined by a two-way ANOVA followed by Holm Sidak’s multiple comparisons test. Temporal changes in protein excretion were compared between and within groups using three-way ANOVA followed by a Tukey’s multiple-comparisons test. A p-value <0.05 was considered significantly different. * indicates a significant difference from the corresponding value within the same strain at baseline, † indicates a significant difference from the corresponding value in SS rats within the same treatment, and # indicates a significant difference from the corresponding value in control-treated rats within the same strain.

**Figure 4. Effects of chronic treatment with a macrophage inflammatory protein 3 – alpha neutralizing antibody (MNA) on creatinine clearance and markers of glomerular and tubular injury.** Control SS and SS^{LepR} mutant rats are represented by circles and squares outlined in black, respectively, and MNA-treated rats are represented by circles and squares outlined in red. Female and male rats in each group are represented by partially-filled and open symbols, respectively. (Panel A) Creatinine clearance, (Panel B) nephrin excretion, (Panel C) kidney injury.
molecule-1 (KIM-1), and (Panel D) neutrophil gelatinase-associated lipocalin (NGAL). Values are means ± SD. The significance of the difference in mean values for a single time point was determined by a two-way ANOVA followed by Holm Sidak’s multiple comparisons test. A \( p \)-value <0.05 was considered significantly different. † indicates a significant difference from the corresponding value in SS rats within the same treatment, and # indicates a significant difference from the corresponding value in control-treated rats within the same strain.

Figure 5. Representative images and analyses of renal histopathology in Dahl salt-sensitive (SS) and obese SS leptin receptor mutant (SS\(^{LepR\text{mutant}}\)) rats treated with either control or a macrophage inflammatory protein 3 – alpha neutralizing antibody (MNA). Control SS and SS\(^{LepR\text{mutant}}\) rats are represented by circles and squares outlined in black, respectively, and MNA-treated rats are represented by circles and squares outlined in red. Female and male rats in each group are represented by partially-filled and open symbols, respectively. (Panel A) Periodic acid-Schiff staining, (Panel B) Picrosirius red staining for renal cortical fibrosis, (Panel C) Picrosirius red staining for renal medullary fibrosis, (Panel D) glomerular injury score, (Panel E) renal cortical fibrosis (% red staining), and (Panel F) renal medullary fibrosis (% red staining). Values are means ± SD. The significance of the difference in mean values for a single time point was determined by a two-way ANOVA followed by Holm Sidak’s multiple comparisons test. A \( p \)-value <0.05 was considered significantly different. † indicates a significant difference from the corresponding value in SS rats within the same treatment, and # indicates a significant difference from the corresponding value in control-treated rats within the same strain.

Figure 6. Comparison of renal dendritic cell and macrophage infiltration in Dahl salt-sensitive (SS) and obese SS leptin receptor mutant (SS\(^{LepR\text{mutant}}\)) rats treated with either control or a macrophage inflammatory protein 3 – alpha neutralizing antibody (MNA).
Control SS and SS\textsuperscript{LepRmutant} rats are represented by circles and squares outlined in black, respectively, and MNA-treated rats are represented by circles and squares outlined in red. Female and male rats in each group are represented by partially-filled and open symbols, respectively. (Panel A) dendritic cells, (Panel B) stimulatory CD80\textsuperscript{+} dendritic cells, (Panel C) stimulatory CD86\textsuperscript{+} dendritic cells, (Panel D) total macrophages, (Panel E) M1 macrophages, and (Panel F) M2 macrophages. Values are means ± SD. The significance of the difference in mean values for a single time point was determined by a two-way ANOVA followed by Holm Sidak’s multiple comparisons test. A \textit{p}-value <0.05 was considered significantly different. † indicates a significant difference from the corresponding value in SS rats within the same treatment, and # indicates a significant difference from the corresponding value in control-treated rats within the same strain.

**Figure 7. Comparison of renal lymphocyte infiltration in Dahl salt-sensitive (SS) and obese SS leptin receptor mutant (SS\textsuperscript{LepRmutant}) rats treated with either control or a macrophage inflammatory protein 3 – alpha neutralizing antibody (MNA).** Control SS and SS\textsuperscript{LepRmutant} rats are represented by circles and squares outlined in black, respectively, and MNA-treated rats are represented by circles and squares outlined in red. Female and male rats in each group are represented by partially-filled and open symbols, respectively. (Panel A) total T-cells, (Panel B) cytotoxic T-cells, (Panel C) B-cells, (Panel D) T-helper cells, (Panel E) IL-17 producing T-cells (Th17s), and (Panel F) T-regulatory cells (Tregs). Values are means ± SD. The significance of the difference in mean values for a single time point was determined by a two-way ANOVA followed by Holm Sidak’s multiple comparisons test. A \textit{p}-value <0.05 was considered significantly different. † indicates a significant difference from the corresponding value in SS rats within the same treatment, and # indicates a significant difference from the corresponding value in control-treated rats within the same strain.
Figure 8. Effects of chronic treatment with a macrophage inflammatory protein 3 – alpha neutralizing antibody (MNA) on the expression of renal cytokines in Dahl salt-sensitive (SS) and obese SS leptin receptor mutant (SSLepRmutant) rats. Control SS and SSLepRmutant rats are represented by circles and squares outlined in black, respectively, and MNA-treated rats are represented by circles and squares outlined in red. Female and male rats in each group are represented by partially-filled and open symbols, respectively. (Panel A) IL-10, (Panel B) IL-4, (Panel C) IL-2, and (Panel D) IL-17. Values are means ± SD. The significance of the difference in mean values for a single time point was determined by a two-way ANOVA followed by Holm Sidak’s multiple comparisons test. A p-value <0.05 was considered significantly different. † indicates a significant difference from the corresponding value in SS rats within the same treatment, and # indicates a significant difference from the corresponding value in control-treated rats within the same strain.
Table 1. Effects of chronic macrophage inflammatory 3 – alpha inhibition (MNA) on metabolic parameters in Dahl salt-sensitive (SS) rats and obese SS leptin receptor mutant (SS\textsuperscript{LepR}mutant) rats

<table>
<thead>
<tr>
<th>Metabolic Parameters</th>
<th>Control Treatment</th>
<th>MNA Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SS</td>
<td>SS\textsuperscript{LepR}mutant</td>
</tr>
<tr>
<td>Total animals</td>
<td>12</td>
<td>10</td>
</tr>
<tr>
<td>(Female, Male)</td>
<td>(7, 5)</td>
<td>(4, 6)</td>
</tr>
<tr>
<td>Body Weight (g)</td>
<td>202±13</td>
<td>310±14†</td>
</tr>
<tr>
<td>Glucose (mg/dl)</td>
<td>98±3</td>
<td>97±4</td>
</tr>
<tr>
<td>Insulin (ng/ml)</td>
<td>1.0±0.3</td>
<td>11.0±2.0†</td>
</tr>
<tr>
<td>Triglycerides (mg/dl)</td>
<td>70±28</td>
<td>467±91†</td>
</tr>
<tr>
<td>Total Cholesterol (mg/dl)</td>
<td>143±38</td>
<td>252±46†</td>
</tr>
</tbody>
</table>

Values are means ± SD. † indicates a $p<0.05$ vs SS rats within the same treatment and # indicates a $p<0.05$ vs vehicle treated rats within the same strain.
Figure 2

A

B

MIP3a (pg/mL)

MIP3a (pg/mg protein)

SS  SS$^{\text{LepR}^+}$  mutant

SS  SS$^{\text{LepR}^+}$  mutant
Figure 7

A

Total T-cells (CD3^+ cells/kidney)  
6x10^6  
4x10^6  
2x10^6  
0  
SS  
SS_LepR^+ mutant

B

Cytotoxic T-cells (CD3^+ CD8^+ cells/kidney)  
2x10^5  
1.5x10^5  
1x10^5  
5x10^4  
0  
SS  
SS_LepR^+ mutant

C

Total B-cells (CD45R^+ cells/kidney)  
3x10^6  
2x10^5  
1x10^5  
0  
SS  
SS_LepR^+ mutant

D

Total T-helper cells (CD3^+ CD4^+ cells/kidney)  
6x10^6  
4x10^6  
2x10^6  
0  
SS  
SS_LepR^+ mutant

E

TH17s (ROD3^+ cells/kidney)  
8x10^5  
6x10^5  
4x10^5  
2x10^5  
0  
SS  
SS_LepR^+ mutant

F

Tregs (FoxP3^+ cells/kidney)  
8x10^5  
6x10^5  
4x10^5  
2x10^5  
0  
SS  
SS_LepR^+ mutant

* #