Impact of Obesity on Renal Structure and Function in the Presence and Absence of Hypertension: Evidence from Melanocortin-4 Receptor Deficient Mice

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ABSTRACT

The purpose of this study was to determine the long-term impact of obesity and related metabolic abnormalities, in the absence and presence of hypertension, on renal injury and salt-sensitivity of blood pressure. Markers of renal injury and blood pressure salt-sensitivity were assessed in 52-55 week-old normotensive melanocortin 4 receptor deficient mice (MC4R-/-) and lean C57BL/6J wild type (WT) mice and in 22-week-old MC4R-/- and WT mice made hypertensive by L-NAME in the drinking water for 8 weeks. Old MC4R-/- mice were 60% heavier, hyperinsulinemic and hyperleptinemic but had similar mean arterial pressure (MAP) as WT mice (115±2 and 117±2 mmHg) on normal salt diet (0.4% NaCl). High salt diet (4.0% NaCl) for 12 days did not raise MAP in obese or lean mice (ΔMAP: MC4R (-/-) 4±2 mmHg; WT, 2±1 mmHg). Obese MC4R-/- mice had 23 % greater glomerular tuft area and moderately increased GFR compared to WT mice. Bowman’s space, total glomerular area, mesangial matrix, urinary albumin excretion (UAE), renal TGF-β and collagen expression were not significantly different between old MC4R-/- and WT mice. Renal lipid content was greater but renal macrophage count was markedly lower in MC4R-/- than WT mice. Mild increases in MAP during L-NAME treatment (~16 mmHg) caused small, but greater, elevations in UAE, renal TGF-β content and macrophage infiltration in MC4R-/- compared to WT mice without significant changes in glomerular structure. Thus despite long-term obesity and multiple metabolic abnormalities, MC4R-/- mice have no evidence of renal injury or salt-sensitivity of blood pressure. These observations suggest that elevations in blood pressure may be necessary for obesity and related metabolic abnormalities to cause major renal injury, or that MC4R-/- mice are protected from renal injury by mechanisms that are still unclear.

Keywords: obesity, metabolic syndrome, renal injury, hypertension, salt-sensitivity.
INTRODUCTION

Obesity *per se* has not been considered a major contributor of renal disease until recent years, although diabetes mellitus and hypertension, two major consequences of obesity, account for more than 70% of end stage renal disease. Multiple observational studies suggest that the obesity epidemic is associated with a parallel increase in the incidence of kidney disease (11, 13, 21). Moreover, population studies demonstrate a strong correlation between obesity and renal disease even after adjustment for hypertension, diabetes, or preexisting renal disease (9, 16).

Obesity is associated with several functional and structural alterations in the kidney that resemble those seen in diabetic nephropathy. Previous studies, including those from our laboratory in obese dogs, demonstrate that even early in the course of obesity, there is significant glomerular hyperfiltration, glomerular basement membrane thickening, mesangial matrix expansion and enlargement of the Bowman’s capsule (15). These early changes may be precursors to more severe injury such as focal and segmental glomerulosclerosis, which often occur with prolonged obesity (14, 20).

Although the association between obesity and kidney disease has been previously established, the pathogenic mechanisms involved are still unclear. Multiple metabolic and hemodynamic factors have been proposed to contribute to obesity associated nephropathy. Some of these factors include insulin resistance, hyperinsulinemia, hyperglycemia, hyperleptinemia, abnormal lipid metabolism, oxidative stress, angiotensin II, inflammation, increased arterial pressure and glomerular hypertension (1, 2, 5, 7, 8, 10, 27, 41). However, the relative importance of the hemodynamic and the metabolic factors in causing renal injury in obesity has not been determined, mainly because it has been difficult to separate the hemodynamic and metabolic effects of obesity on the kidneys.
Melanocortin-4 receptor deficient (MC4R-/-) mice develops early onset obesity because MC4R activation normally suppresses appetite and increases energy expenditure (17). MC4R-/- mice are overweight compared to wild type (WT) mice as early as 5-6 weeks of age (35). We found that at 22 weeks of age, MC4R-/- mice exhibited many characteristics of the metabolic syndrome including visceral obesity, insulin resistance, hyperinsulinemia, hyperglycemia and hyperlipidemia. However, chronic monitoring of blood pressure using telemetry in these mice revealed that they were not hypertensive despite other characteristics of the metabolic syndrome (35). The lack of hypertension in the MC4R-/- mouse may be due to impaired sympathetic nervous system (SNS) activation that normally mediates obesity-induced hypertension (34, 35). Therefore, the MC4R-/- mouse provide an opportunity to study the impact of obesity and metabolic abnormalities per se on renal injury and salt-sensitivity of blood pressure in the absence of hypertension.

The objective of this study was to investigate the impact of prolonged obesity on kidney function and structure in the absence of hypertension. We also investigated whether obesity and metabolic abnormalities in MC4R-/- mice exacerbate the effects of hypertension on renal function and structure compared to lean WT mice. We therefore studied young (20-22 weeks) and old (52-55 weeks) MC4R-/- mice to allow prolonged exposure of the kidneys to the metabolic disturbances associated with obesity. Histopathological and molecular markers of renal injury and salt-sensitivity of blood pressure that normally occur with renal damage were assessed as measures of renal structure and function in the MC4R-/- and in control WT mice.

METHODS

All experimental procedures conform to the National Institute of Health Guide for the
Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee, University of Mississippi Medical Center. MC4R-/- mice were generated as previously described from MC4R deficient breeding pairs obtained from Dr. Roger Cone, Oregon Health Sciences University (34). The mice received food and water ad libitum throughout the study and were placed on a 12:12hr light-dark cycle.

**Experimental Protocol for Studies in 52-55 Week Old Mice**

52-55 week old MC4R-/- mice and age-matched C57BL/6J wild type mice (N=6 per group) were implanted with telemetric pressure transmitters as previously described (Model TA11PAC20, Data Sciences International, MN, USA) (35). Ten days after surgery we began monitoring mean arterial pressure (MAP) and heart rate (HR). C57BL/6J mice were used as controls since the MC4R-/- mice were bred into the C57Bl/6J background for more than 10 generations. After 5 days of stable control measurements on a normal salt diet (NSD, 0.4 % NaCl, Test Diet, IN, USA), mice were placed on a high salt diet (HSD, 4% NaCl diet, Test Diet) for 12 days followed by a return to 3 days of NSD for post-control measurements. 24-hr MAP, HR and food intake were recorded daily.

At the end of the study, mice were fasted for 6-7 hrs, anesthetized with isoflurane, and blood samples (200 μl) were collected by cardiac puncture to determine plasma glucose, insulin and leptin concentrations. Mice were then transcardially perfused with 0.1% PBS and kidneys and visceral fat (epididymal and retroperitoneal) were collected and weighed. One kidney was frozen in liquid nitrogen for renal transforming growth factor-β (TGF-β) and collagen measurements and the other kidney was fixed in 10% buffered formalin for histological assessment of glomerular morphology, mesangial matrix, renal lipids and macrophages. Urinary albumin excretion (UAE) rate and glomerular filtration rate (GFR) were measured in a separate
group of 52-55 week old mice.

**Experimental Protocol in 20-22 Week-Old MC4R-/- and WT Mice**

Since the duration of obesity is also a factor that could cause renal changes, we studied younger 20-22 week old obese MC4R-/- (n=7) and lean WT mice (n=8). We assessed several markers of renal injury including UAE, mesangial matrix and glomerular collagen in the younger mice. Kidney and visceral fat pad weights were also obtained in these mice.

**L-NAME Hypertension in 20-22 Week-Old MC4R-/- and WT mice**

To induce chronic hypertension, L-NAME (Sigma, ST Louis, MO) was administered to mice at the age of 20-22 weeks at a dose of 120 mg/kg/day in the drinking water for 8 consecutive weeks. MAP and HR were measured by telemetry for three consecutive days during the control period and every 2 weeks until the end of the 8\(^{\text{th}}\) week. Twenty-four hour urine samples were collected for three consecutive days during the control period and at the end of the 8\(^{\text{th}}\) week of L-NAME treatment for determination of urinary albumin excretion (UAE). At the end of the L-NAME treatment, mice were sacrificed and renal tissue was harvested to measure TGF-\(\beta\) in whole kidney and for morphological and histological studies.

**UAE and GFR Measurements**

Mice were acclimatized for several days in metabolic cages with *ad libitum* access to food and water. Twenty-four hour urine samples were collected on 3 consecutive days for UAE analysis using an ELISA kit (Exocell, PA, USA.). The older mice were then prepared for GFR measurements as described previously (N=6 per group) (34). Briefly, the femoral artery and vein were cannulated for blood collection and infusions, respectively. Four to five hours after surgery, mice were transferred to metabolic cages and maintained on a constant infusion of saline to maintain sodium intake at normal levels (~ 462 \(\mu\text{eq/day}\)) with *ad libitum* access to sodium.
deficient diet and water. The arterial catheter was flushed daily with heparin to prevent clotting. Approximately 7 days after surgery, GFR was measured using a 6-7 hour fasted plasma sample following a 24-hour IV infusion of $^{125}$I-iothalamate as previously described (34).

**Histological Methods**

A coronal cross section containing the hilus of the kidney was removed, fixed in formalin and embedded in paraffin. Sections (5 μm thick) were mounted on glass slides and stained with Hemotoxylin and Eosin (H & E), Methanamine Silver, Picrosirius red or Oil red O. All histological measurements were made an observer who was blinded to the experimental protocols. Only glomeruli containing visible afferent and efferent arteriolar stalks were used to ensure that all glomeruli were measured in the same plane. Glomerular images at 40X magnification were digitized using a color video camera attached to a Leica microscope. After digitization, Bowman’s space and the glomerular tuft areas in the H & E stained sections, mesangial matrix area stained with methanamine silver and glomerular collagen area stained with picrosirius red were traced and measured using Imagepro analysis software. Total area for each glomerulus was calculated as the sum of glomerular tuft and Bowman’s space areas. Thirty glomeruli were measured per kidney per animal for estimating glomerular morphology and twenty glomeruli were measured for mesangial matrix and renal collagen assessment. Mesangial matrix and glomerular collagen areas were normalized to the glomerular tuft area.

For estimating renal lipid accumulation, the area of Oil Red O stain on each image was measured using Imagepro (Universal Imaging Corporation, PA, USA). Forty fields including ten each of cortical and medullary and twenty glomerular areas per kidney per animal were analyzed. The total tissue area per field was approximately equal to the total area of the field.

For immunohistochemical analyses of macrophages, sections were deparaffinized,
rehydrated and antigen retrieval was performed by incubating sections in Trypsin at 37°C for 20 min. Immunohistochemistry for the macrophage antigen was performed using the Vectastain Elite ABC kit (Vector Laboratories, CA). Sections were incubated in primary antibody (anti-mouse macrophage F4/80 antigen, Serotec, Oxford, UK) at 1:10 dilution in normal rabbit serum overnight. Slides that were simultaneously processed without primary antibody served as negative controls. The area of stained macrophages was measured using Imagepro. Forty fields including ten each of cortical and medullary and twenty glomerular areas per kidney per animal were analyzed. Macrophage area was normalized to the total kidney area measured.

**Analytical Methods**

Plasma insulin and leptin were determined by ELISA (Linco Insulin ELISA, MO, USA; R&D Leptin ELISA, MN, USA). Plasma glucose was determined by the glucose oxidation method (Beckman glucose analyzer 2, CA, USA). Kidney collagen was assessed with the Sircoll assay (Biocolor, NY, USA). Kidney TGF-β was measured by ELISA (Pierce, IL, USA).

**Statistical Methods**

The data are expressed as mean ± SEM. Data obtained on a daily basis were analyzed using ANOVA with repeated measures. Comparisons between groups were performed using one-way ANOVA followed by the Bonferroni’s post-hoc test or Student’s t-test where appropriate. Statistical significance was accepted at a level of P<0.05.

**RESULTS**

**Anthropometric and Metabolic Data**

The average body weight of MC4R-/- mice at both age groups was 60% greater compared to age-matched WT mice (Table 1). Parallel to the increase in body weight, MC4R-/-
mice had several-fold greater visceral fat compared to WT mice. WT and MC4R-/- mice at 52-55 weeks of age were significantly heavier and had greater visceral fat accumulation than at 22 weeks of age (Table 1).

Much of the increase in body weight in the 52-55 week old MC4R-/- compared to WT mice may have been caused by increased food intake. Daily food intake was approximately 45% greater in 52-55 week old MC4R-/- compared to WT mice (Table 1) and it was not significantly altered by HSD (5.6 ± 0.1 and 3.6 ± 0.1 g, respectively). Plasma leptin and insulin levels were several-fold greater in the old MC4R-/- than in WT mice. However, plasma glucose levels were not significantly different between old MC4R-/- and WT mice at 22 and 52-55 weeks of age. Plasma glucose levels in WT mice at 52-55 weeks were significantly greater than at 22 weeks of age as observed in our previous studies, suggesting that aging contributed to insulin resistance even in WT mice (34).

**Arterial Pressure and Heart Rate in 52-55 week Old Mice**

*Normal Salt Diet:* Despite 60% greater body weight in the old MC4R-/- mice, 24-hr average MAP in MC4R-/- mice (115 ± 2 mm Hg) was not significantly different from that in WT mice (117 ± 2 mmHg) during NSD (Fig. 1A). Similarly, day and night time average MAP were not different between MC4R-/- (day: 111 ± 2; night: 121 ± 3 mm Hg) and WT mice (day: 113 ± 3; night: 121 ± 2 mm Hg). Basal 24-hr average HR and day and night time HR during NSD in MC4R-/- mice (24-hr: 568 ± 36; day: 559 ± 29; night: 576 ± 43 bpm) were significantly lower than in WT mice (24-hr: 635 ± 12; day: 623 ± 10; night: 648 ± 15 bpm, Fig. 1A).

*High Salt Diet:* HSD (4% NaCl) feeding for 12 days did not significantly alter 24-hr MAP or HR in MC4R-/- or WT mice (average ΔMAP: MC4R-/-, 4 ± 2 mmHg; WT, 2 ± 1 mmHg, Fig. 1A). Consistent with this observation, HSD also did not significantly alter day or night time MAP and
HR in either group of mice.

Renal function, Molecular and Histological Data in 52-55 week Old Mice

UAE and GFR:

Twenty-four-hour UAE, an early marker of glomerular injury was not significantly different between the 52-55 week old MC4R-/- and WT mice (Fig. 2A). Another major functional change that usually accompanies obesity is glomerular hyperfiltration. GFR was only moderately elevated in the normotensive obese MC4R-/- group compared to WT mice (Figure 3A) and the changes were not statistically significant. Even when expressed per g kidney weight, GFR was not significantly different in MC4R-/- compared to WT mice (Figure 3B).

Kidney Morphology, Histological and Molecular Data

Obese MC4R-/- mice had significantly greater kidney weights compared to lean WT mice (Table 1) and also exhibited slight glomerular hypertrophy. Glomerular tuft area was 23 % greater in MC4R-/- than in WT mice. Bowman’s space and total glomerular areas, however, were not significantly different between the two groups (Fig. 4A).

Renal mesangial matrix accumulation, a marker of glomerulosclerosis, was assessed by methanamine silver staining. After analyzing 20 glomeruli per kidney per animal we found no significant differences in mesangial matrix accumulation between MC4R-/- and WT mice (Fig. 5A). Consistent with this observation, there were no significant differences in total collagen extracted from the kidneys of both groups (Fig. 5B).

To determine whether prolonged obesity in MC4R-/- mice increased renal lipid accumulation we performed Oil Red O staining of kidney sections. Histological analysis of these sections revealed increased accumulation of neutral lipids in the glomeruli and cortical tubules in MC4R-/- mice compared to WT mice. However, we found no significant differences in lipid
accumulation in the medullary tubules between groups (Fig. 5C).

Furthermore, whole kidney expression of TGF-β protein, which is implicated in renal fibrosis by increasing extracellular matrix deposition, was not significantly different between obese MC4R-/- and control WT mice (Fig. 2C). We also evaluated renal macrophage infiltration which has been proposed to play a major role in lipid-induced renal injury and diabetic nephropathy. Surprisingly, MC4R-/- mice had reduced macrophage infiltration, compared to WT mice, in all measured areas of the kidneys including cortical and medullary tubules and the glomeruli (Fig. 6A).

**Renal Histological and Molecular Data in 20-22 week old Mice**

Observations in the 20-22 week old mice were similar to that in the older 52-55 week old mice (Table 1). Kidney weights were significantly greater in the MC4R-/- compared to WT mice. We found no significant differences in UAE (17 ± 2 vs. 15 ± 2 μg/24-hr) mesangial matrix expansion (33 ± 2 vs. 30 ± 1 %) or glomerular collagen expression (35 ± 4 vs. 31 ± 4 %) between MC4R-/- and WT mice. A noteworthy finding from our previous studies in 20-22 week old MC4R-/- mice is that they also had no salt-sensitivity of blood pressure despite obesity and associated metabolic abnormalities (35).

**Antropometric Data and Tissue Weight in 20-22 week old WT and MC4R-/- Mice Treated with L-NAME.**

The average body weight of MC4R-/- mice, at the end of L-NAME treatment, was 54 % greater compared to age-matched WT mice (Table 2). Food intake was 58% higher in MC4R-/- compared to WT mice, and MC4R-/- mice had 3-fold greater epididymal fat compared to WT mice. Heart and kidney weights were also higher in MC4R-/- compared to WT mice (Table 2).
L-NAME.

Twenty-four-hour average baseline MAP before beginning L-NAME treatment was significantly lower in MC4R-/- (98 ± 7 mmHg) than in and WT mice (107 ± 2 mmHg, Fig. 1A). L-NAME treatment caused similar increases in MAP (~16 mmHg) in both groups. Baseline 24-hr average HR in MC4R-/- mice (532 ± 24 bpm) was not different from HR in WT mice (554 ± 20 bpm). L-NAME treatment caused a small but significant reduction in HR in WT mice while HR in MC4R-/- mice remained unchanged (Fig. 1A).

Renal Histological and Molecular Data in 20-22 week old WT and MC4R-/- Mice Treated with L-NAME

Baseline 24-hr UAE was not significantly different between MC4R-/- and WT mice (Fig. 2B). L-NAME treatment caused a significant elevation in UAE in both groups, however UAE increased to a greater extent in MC4R-/- . Despite the increase in UAE caused by L-NAME, TGF-β protein levels in whole kidney lysates were only slightly, but not significantly, increased in MC4R-/- compared to WT mice (Fig. 2C). No significant differences were observed in renal macrophage infiltration in MC4R-/- compared to WT mice for all measured areas of the kidney, including cortical and medullary tubules and glomeruli (Fig. 5B).

Renal Histological in 20-22 week old WT and MC4R-/- mice Treated with L-NAME

Kidney weight was 33% greater in the MC4R -/- compared to WT mice (Table 2), and also exhibited slight glomerular hypertrophy. Total glomerular area and glomerular tuft area were 29% and 37% larger, respectively, in MC4R-/- compared to WT mice. Bowman’s space area, however, was not different between the groups (Fig. 4B).

DISCUSSION

The key finding of this study is that normotensive 52-55 week old MC4R-/- mice did not
develop significant renal injury despite obesity and prolonged exposure to metabolic disturbances such as insulin resistance, hyperinsulinemia, hyperglycemia, hyperlipidemia and hyperleptinemia, factors that are considered by many investigators to play a major role in the etiology of obesity associated nephropathy (1, 2, 7, 8, 10, 41). Our observations suggest that obesity and associated metabolic abnormalities may not be a major cause of severe renal disease in the absence of hypertension. Elevations in arterial pressure may be necessary for obesity and related metabolic abnormalities to cause major renal injury.

Although a variety of animal models have been used to investigate obesity-induced renal injury, few studies have attempted to assess the importance of hemodynamic versus metabolic factors. The obese MC4R-/- mouse is an attractive model to study this issue for several reasons. First, the 52-55 week old MC4R-/- mouse is a late middle aged mouse (i.e. the average life span of a mouse is 1.5 to 2 years) that has been obese for its entire life; previous studies, including those from our lab, indicated that the MC4R-/- mouse has early-onset obesity (17, 35). Second, 52-55 week old obese MC4R-/- mice have many of the metabolic abnormalities, such as insulin resistance, hyperinsulinemia, hyperleptinemia and dyslipidemia, which have been suggested to cause renal injury (1, 7, 8, 10, 40). Third, despite metabolic abnormalities and obesity, MC4R-/- mice have normal arterial pressures as assessed by telemetry. Our previous studies suggest that this normotensive state despite severe obesity may be due to an impaired SNS activation response to obesity (14, 35). Visceral obesity usually increases adipocyte secretion of leptin which, in turn, activates hypothalamic POMC neurons causing release of α-MSH, activation of MC4R, increased SNS activity and increased blood pressure (14, 34). Thus, the MC4R may be a key mechanism linking visceral obesity and hypertension (14).

A comparison of the metabolic characteristics of MC4R-/- mice at 52-55 weeks of age
with those of younger MC4R-/- mice in our previous studies clearly depicts a progression of several features of the metabolic syndrome with age (34). Most of the metabolic changes in the MC4R-/- mice start as early as 8-9 weeks of age (17, 34, 35). MC4R-/- mice at 52-55 weeks of age are significantly heavier (~30-50%) compared to 20-22 week old MC4R-/- mice (34, Table 1). The weight gain in 52-55 week old mice was reflected in a doubling of circulating levels of leptin compared to younger MC4R-/- mice (35). Insulin resistance also worsened with age as depicted by significantly greater plasma insulin levels in the 52-55 week old MC4R-/- mice compared to younger MC4R-/- mice (34). Old WT mice also exhibited metabolic changes such as increases in body weight, plasma glucose, insulin and leptin levels compared to younger WT mice suggesting the development of insulin resistance. However, these metabolic changes were much milder than those observed in the MC4-/- mice.

Apart from systemic metabolic changes, the kidneys in old MC4R-/- mice were also exposed to local metabolic changes such as increased lipid accumulation as assessed by Oil-red O staining. Previous studies have provided evidence that accumulation of lipids in tissues such as the heart, pancreas, and liver, is associated with organ dysfunction (36, 37). The results from the present study suggest that lipid accumulation in the kidneys of MC4R-/- mice in the absence of increased blood pressure does not appear to cause major organ dysfunction. Whether renal lipid accumulation may exacerbate the detrimental effects of more severe, prolonged increases in blood pressure is unclear and is an important area for further investigation.

We estimated the impact of obesity and associated metabolic abnormalities on renal function, by measuring salt-sensitivity of blood pressure, GFR and UAE rate in conscious mice. In addition, we evaluated glomerular morphology, renal collagen, renal TGF-β expression and mesangial matrix expansion as structural markers of renal injury. Since the kidney is important
in sodium homeostasis and long-term regulation of blood pressure, salt-sensitivity of blood pressure provides an assessment of renal dysfunction. Blood pressure in patients with chronic kidney disease is usually very salt-sensitive with substantial increases in blood pressure occur with high salt-intake (39). 52-55 week old MC4R-/- mice, however, did not develop a significant increase in arterial pressure during 12 days of a 10-fold increase in salt-intake.

It is possible that compensatory activation of melanocortin-3 receptors (MC3R) may contribute to lack of salt-sensitivity of blood pressure in MC4R-/- mice. MC3R activation has been demonstrated to exert natriuresis and lack of MC3R or its endogenous agonist, δ-MSH, result in salt-sensitive hypertension (18). However, the role of MC3R in protecting against salt-sensitivity of blood pressure in MC4R deficient mice is unclear and was not the major objective of the present study.

Multiple lines of evidence, including lack of salt-sensitivity of blood pressure and lack of major structural changes indicate that obesity and its associated metabolic abnormalities, in the absence of hypertension, did not cause major renal injury in MC4R-/- mice. Furthermore, 24-hour UAE, which increases early in the course of renal injury due to a selective increase in glomerular permeability and glomerular hyperfiltration, was also not increased in MC4R-/- compared to WT mice. Similarly, fibrotic markers of renal injury, namely renal collagen, TGF-β and mesangial matrix were not significantly different between old MC4R-/- and WT mice.

These findings were closely recapitulated in the 20-22 week-old MC4R-/- mice which, despite obesity, had no significant increases in UAE, glomerular collagen or mesangial matrix compared to WT mice and had no salt-sensitivity of blood pressure (35). Similar observations in both young and old MC4R-/- mice strongly suggest that exposure to obesity and its related metabolic abnormalities over a prolonged period of time failed to cause significant renal injury in
normotensive MC4R-/- mice. The only changes observed in the old MC4R-/- mice were moderate increases in kidney weight, GFR and glomerular tuft size in comparison to old WT mice. Increases in glomerular tuft size may have contributed to increases in GFR.

The absence of major renal injury in 50-52 week old obese MC4R-/- mice suggests either that MC4R-/- mice are somehow protected from renal injury or that the metabolic disturbances may not cause significant kidney damage in the absence of hypertension, Reduced renal macrophage infiltration in the MC4R-/- mice compared to WT mice suggests that obesity in MC4R-/- mice was not associated with a renal inflammatory response. Whether the reduced accumulation of macrophages in kidneys of MC4R-/- mice explains the protection of these mice from obesity-induced renal injury, however, is unclear. Although previous studies suggest that inflammation may participate in renal disease progression, this may not be required for initiating obesity-induced renal disease (22, 29). Signs of glomerulosclerosis were observed in obese Zucker rats even before significant inflammation occurred in the kidneys (22).

Another possible explanation for the absence of renal injury in the MC4R-/- mice is that the metabolic disturbances were not severe enough. However, previous studies in other mouse models show that obesity and metabolic abnormalities similar to those seen in the MC4R-/- mice were associated with significant renal injury even after a short period of time. For instance, Levine et al demonstrated a 5-fold increase in UAE, an early marker of renal injury, in only 10-11 week old db/db mice with plasma glucose levels of ~14 mM and a body weight of ~50 g, which is lower than that of the MC4R-/- mice in the present study (23). Similarly, Cohen et al found albuminuria in db/db mice as early as 8 weeks of age and increased urinary type IV collagen excretion at 12 weeks of age suggesting renal injury (6). Furthermore, recent studies show that C57Bl/6J mice (which is the background of the MC4R-/- mice used in the present
study) developed glomerulosclerosis with significant increases in mesangial matrix, renal collagen, fibronectin and proteinuria despite developing only moderate obesity (~ 48 g) and hyperglycemia (~12 mM) after only 12 weeks on a high fat diet (19).

Thus, metabolic disturbances even milder than those observed in the 52-55 week old MC4R-/- can be associated with severe nephropathy when accompanied by other abnormalities such as increased blood pressure. Indeed, previous studies using telemetry for blood pressure measurements demonstrate that C57Bl/6J mice after 12-14 weeks of high fat diet not only developed obesity but also increased arterial pressure (31, 40). Reduced renal vascular resistance in obesity may facilitate a greater transmission of the increase in arterial pressure to the glomerular capillaries making them more susceptible to glomerulosclerosis. Whether db/db mice also have elevated arterial pressure is unclear due to conflicting evidence but they appear to have increased glomerular capillary pressure. Levine et al showed using micropuncture techniques that db/db mice have increased single-nephron GFR and impaired tubuloglomerular feedback in response to renal hyperperfusion (23). Similar single-nephron GFR changes were attributed to increased glomerular capillary pressures in the obese Zucker rats, which like db/db mice have a mutant leptin receptor (28).

A role for hypertensive hemodynamic changes in contributing to renal injury associated with metabolic syndrome is supported by several studies. Previous studies in animals and in humans suggest that hypertension can accelerate and anti-hypertensive therapy can retard diabetic nephropathy (3, 12, 24, 25). Mauer et al using the two-kidney one-clip rat model in streptozotocin treated diabetic rats showed that glomerular hypertension greatly aggravates glomerulosclerosis in diabetes (24). Absence of such hypertensive hemodynamic changes in the MC4R-/- mice may have protected them from developing significant renal injury. To test this
possibility we induced mild hypertension in MC4R-/− and WT with L-NAME for 8 weeks. We found that L-NAME treatment increased UAE, renal TGF-β expression and macrophage infiltration to a greater extent in obese MC4R-/− compared to lean WT. Although no significant differences were observed for renal macrophage infiltration between WT and MC4R-/− treated with L-NAME, the degree of macrophage infiltration in the old normotensive MC4R-/− mice was much less than in young mild hypertensive MC4R-/− mice whereas no differences were observed between old normotensive WT and young mild hypertensive WT mice. These observations indicate that obesity enhances the impact of arterial pressure on renal injury. The L-NAME treatment in our study caused only mild hypertension and small increases in the markers of renal injury investigated in this study. One explanation for lack of more severe renal injury in mice treated with L-NAME may be the fact that we induced only mild increases in blood pressure to more closely mimic the physiological elevations in arterial pressure one observes during weight gain (33). Despite the fact we induced only mild hypertension for 8 weeks, our data support the hypothesis that hypertension may be necessary for obesity and related metabolic abnormalities to cause major renal injury.

The importance of hypertensive changes in initiating renal injury in obesity is also supported by other observations. Bidani et al reported that spontaneously reduced ambient blood pressure load may be the reason for the slow development of glomerulosclerosis in the streptozotocin treated diabetic rat despite being associated with several putative non-hemodynamic mediators of nephropathy for 36-40 weeks (4). Also, Nielsen and Jensen found normal UAE in obese normotensive glucose tolerant subjects suggesting lack of renal injury in the absence of hypertension despite obesity (26). In contrast, the PREVEND study reported that body mass index is associated with microalbuminuria independent of other factors such as
hypertension and diabetes (38). However, there are no reports, to our knowledge, demonstrating significant renal injury or glomerulosclerosis in the absence of hypertension in normotensive obese humans.

Although our data support the view that obesity-associated metabolic disturbances alone may not initiate major renal injury in the absence of hypertension, our findings do not exclude the possible contributions of metabolic disturbances and obesity in contributing to nephropathy. It is possible that strain-specific mechanisms in MC4R-/- mice may confer lesser susceptibility for the development of obesity-related renal dysfunction, independent of the fact that these mice are not hypertensive. As discussed previously, MC4R-/- mice have reduced macrophage infiltration which could be related to lower blood pressure or to other mechanisms that are still unclear.

Previous studies suggest that obesity accelerates pre-existing renal injury. For instance, Praga et al reported in a cross-sectional study of 73 obese patients that obesity increased the risk for developing proteinuria and chronic renal failure after unilateral nephrectomy (30). Also, Ribstein et al showed that obesity magnified the effect of hypertension on albuminuria in obese patients, thus raising the possibility of an increased susceptibility of obese hypertensives to renal damage (32).

Thus, the present study demonstrates that obesity and metabolic disturbances such as hyperglycemia, hyperinsulinemia, hyperleptinemia and dyslipidemia, even when present together for a prolonged period of time, do not contribute to renal injury in the absence of hypertension in MC4R-/-mice which could be due to lower macrophage infiltration in the kidney.
Perspectives and Significance

Although, experimental and clinical research suggest that obesity and associated metabolic abnormalities may be risk factors for chronic renal disease, it has been difficult to separate the impact of obesity and hemodynamic changes (e.g. hypertension) in causing renal injury. Our studies indicate that MC4R-/- mice, despite having severe early-onset obesity and other metabolic abnormalities, are not hypertensive and are protected against renal injury as well as the salt-sensitivity of blood pressure that often accompanies chronic renal disease. Although it is possible that more severe or more prolonged obesity and metabolic abnormalities could cause renal disease in the presence or absence of hypertension, further studies are needed to test this idea. Also, obese MC4R-/- mice may have additional characteristics besides lower blood pressure that could protect them from renal injury. For example, they do not display the usual accumulation of renal macrophages and inflammation associated with obesity. Further studies are needed to determine if MC4R-/- deficiency per se prevents the usual inflammatory response associated with obesity or whether this is due to lower blood pressure. It is also important to determine if the results observed in MC4R-/- mice translate to humans with MC4R-/- deficiency, and whether these individuals are protected against renal disease despite severe obesity.

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REFERENCES:


35. Tallam LS, Stec DE, Willis MA, da Silva AA, Hall JE. Melanocortin-4 receptor-deficient
mice are not hypertensive or salt-sensitive despite obesity, hyperinsulinemia, and hyperleptinemia. Hypertension 46: 326-332, 2005.


Legends to Figures

Figure 1: (A) 24-hr telemetry mean arterial pressure (MAP) and heart rate (HR) in 52-55 week-old wild type (WT) and MC4R-/- mice fed normal or high salt diet, n=5-6 per group. R1-R3 indicates days 1 and 3, respectively, of the recovery period. (B) 24-hr telemetry mean arterial pressure (MAP) and heart rate (HR) in 20-22 week-old WT and MC4R-/- mice during control period and on week 8 of L-NAME treatment. * p<0.05 compared to WT mice. # p<0.05 compared to control values.

Figure 2: (A) 24-hr urinary albumin excretion in conscious 52-55 week old wild type (WT) and MC4R-/- mice. (B) 24-hr urinary albumin excretion in 20-22 week-old WT and MC4R-/- mice during control period and after 8 weeks of L-NAME treatment. (C) Transforming grown factor – beta (TGF-β) in whole kidney lysates of 52-55 week-old WT and MC4R-/- and in 20-22 week-old WT and MC4R-/- mice after 8 weeks of L-NAME treatment. # p<0.05 compared to control values. * p<0.05 compared to 52-55 week-old MC4R-/-. † p<0.05 compared to WT mice treated with L-NAME.

Figure 3: (A) Glomerular filtration rate (GFR) in conscious 52-55 week-old wild type (WT) and MC4R-/- mice. (B) GFR in 52-55 week-old wild type (WT) and MC4R-/- mice normalized by kidney weight.

Figure 4: (A) Glomerular morphology in normotensive 52-55 week-old wild type (WT) and MC4R-/- mice and (B) in hypertensive 20-22 week-old WT and MC4R-/- mice after 8 weeks of L-NAME treatment. * p<0.05 compared to WT mice.
Figure 5: (A) Mesangial matrix, (B) whole kidney collagen, and (C) Renal Oil red O staining (marker of lipid accumulation) in 52-55 week-old wild type (WT) and MC4R-/- mice. * p<0.05 compared to WT mice.

Figure 6: (A) Renal macrophage accumulation in 52-55 week-old wild type (WT) and MC4R-/- mice and (B) in 20-22 week-old WT and MC4R-/- mice after 8 weeks of L-NAME treatment. * p<0.05 compared to WT mice.
Table 1

Anthropometric and renal characteristics of 20-22 and 52-55 week-old lean wild type (WT) and obese MC4R-/- mice

<table>
<thead>
<tr>
<th>Parameters</th>
<th>20-22 week-old</th>
<th>52-55 week-old</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT</td>
<td>MC4R</td>
</tr>
<tr>
<td>Food Intake (g)</td>
<td>3.5 ± 0.4</td>
<td>5.0 ± 0.3*</td>
</tr>
<tr>
<td>Body Weight</td>
<td>29 ± 1</td>
<td>48 ± 2 *</td>
</tr>
<tr>
<td>Plasma Glucose (mM)</td>
<td>9 ± 0.3</td>
<td>11 ± 0.2</td>
</tr>
<tr>
<td>Plasma Leptin (ng/ml)</td>
<td>3 ± 1</td>
<td>47 ± 13*</td>
</tr>
<tr>
<td>Plasma Insulin (ng/ml)</td>
<td>1.33 ± 0.58</td>
<td>4.80 ± 1.88*</td>
</tr>
<tr>
<td>Total Kidney Weight</td>
<td>0.33 ± 0.02</td>
<td>0.39 ± 0.02 *</td>
</tr>
<tr>
<td>Visceral Pad Fat</td>
<td>0.4 ± 0.1</td>
<td>3.7 ± 0.2</td>
</tr>
</tbody>
</table>

* p<0.05 compared to WT mice, n=6-7 per group. Values are Means ± SE.
Table 2

Body weight, food intake, heart, kidney, and epididymal fat weights in 20-22 week-old wild type (WT) and MC4R-/- mice treated with L-NAME

<table>
<thead>
<tr>
<th>Parameters</th>
<th>WT</th>
<th>MC4R-/-</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>29 ± 1</td>
<td>45 ± 3*</td>
</tr>
<tr>
<td>Food Intake (g)</td>
<td>3.8 ± 0.2</td>
<td>6.0 ± 0.2*</td>
</tr>
<tr>
<td>Heart (g)</td>
<td>0.16 ± 0.01</td>
<td>0.23 ± 0.01*</td>
</tr>
<tr>
<td>Total Kidney weight (g)</td>
<td>0.36 ± 0.01</td>
<td>0.48 ± 0.02*</td>
</tr>
<tr>
<td>Epididymal fat (g)</td>
<td>0.38 ± 0.06</td>
<td>1.27 ± 0.21*</td>
</tr>
</tbody>
</table>

* p<0.05 compared to WT mice, n=6-7. per group. Values are Means ± SE.
Figure 2

A

B

C

24 - Urinary Albumin (μg)

30

25

20

15

10

5

0

Old WT, n=6
Old MC4R-/-, n=5

24 - Urinary Albumin (μg)

300

250

200

150

100

50

0

Control
L-NAME

WT, n=8
MC4R-/-, n=7

24 - Urinary Albumin (μg)

100

80

60

40

20

0

Old WT
Old MC4R-/-
WT + L-NAME
MC4R-/- + L-NAME

TGF β (pg/mg protein)

100

80

60

40

20

0

*
Figure 3

A

GFR (ml/min)

Old WT, n=6
Old MC4R-/-, n=7

B

GFR (ml/min/Kidney Weight)

Old WT, n=6
Old MC4R-/-, n=7
Figure 5

A

Mesangial Matrix Area/Total Glomerular Area (%)

Old WT, n=6
Old MC4R−/−, n=5

B

Collagen (µg/g protein)

C

Oil Red O staining (µm²)

Cortical tubules Glomeruli Medullary tubules

*
Figure 6

A

Old WT, n=6
Old MC4R-/-, n=6

Macrophage staining (mm²/100mm² kidney)

Cortical tubules Glomeruli Medullary tubules

WT + L-NAME, n=6
MC4R-/- + L-NAME, n=7

B

WT + L-NAME, n=6
MC4R-/- + L-NAME, n=7

Macrophage staining (mm²/100mm² kidney)

Cortical tubules Glomeruli Medullary tubules