Megalin-Mediated Tubuloglomerular Alterations in High-Fat Diet–Induced Kidney Disease

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ABSTRACT

Obesity, an important risk factor for metabolic syndrome (MetS) and cardiovascular disease, is often complicated by CKD, which further increases cardiovascular risk and causes ESRD. To elucidate the mechanism underlying this relationship, we investigated the role of the endocytic receptor megalin in proximal tubule epithelial cells (PTECs). We studied a high-fat diet (HFD)–induced obesity/MetS model using kidney-specific mosaic megalin knockout (KO) mice. Compared with control littermates fed a normal-fat diet, control littermates fed an HFD for 12 weeks showed autolysosomal dysfunction with autophagy impairment and increased expression of hypertrophy, lipid peroxidation, and senescence markers in PTECs of the S2 segment, peritubular capillary rarefaction with localized interstitial fibrosis, and glomerular hypertrophy with mesangial expansion. These were ameliorated in HFD-fed megalin KO mice, even though these mice had the same levels of obesity, dyslipidemia, and hyperglycemia as HFD-fed control mice. Intravital renal imaging of HFD-fed wild-type mice also demonstrated the accumulation of autofluorescent lipofuscin-like substances in PTECs of the S2 segment, accompanied by focal narrowing of tubular lumens and peritubular capillaries. In cultured PTECs, fatty acid–rich albumin induced the increased expression of genes encoding PDGF-B and monocyte chemoattractant protein-1 via megalin, with large (auto)lysosome formation, compared with fatty acid–depleted albumin. Collectively, the megalin-mediated endocytic handling of glomerular-filtered (lipid)toxic substances appears to be involved primarily in hypertrophic and senescent PTEC injury with autophagy impairment, causing peritubular capillary damage and retrograde glomerular alterations in HFD-induced kidney disease. Megalin could be a therapeutic target for obesity/MetS-related CKD, independently of weight, dyslipidemia, and hyperglycemia modification.
causing glomerular hypertrophy is likely to be associated with the development of glomerulosclerosis, which eventually worsens renal outcome.5

Megalin is a large (approximately 600 kDa) glycoprotein member of the low-density lipoprotein receptor family6 that is expressed primarily at clathrin-coated pits and partly at the microvilli of proximal tubule epithelial cells (PTECs).7 Megalin mediates intracellular signal transduction8,9 and plays a pivotal role in the reabsorption of diverse glomerular-filtered substances, including albumin and low molecular weight proteins.10 Kidney-specific megalin knockout (KO) mice established using the Cre/lox system,11,12 as well as with patients with Donnai–Barrow and facio-oculo-acoustico-renal syndromes caused by mutations in the megalin gene,13 show albuminuria and low molecular weight proteinuria without apparent impairment of renal function. Albumin is an efficient carrier of fatty acids and advanced glycation end products, which mediate cellular lipotoxicity14 and glyotoxicity,15 respectively. Therefore, megalin is a candidate molecule for mediating renal lipi-glycotoxicity; however, its pathologic role in kidney disease remains to be determined.

In this study, we investigated the role of megalin in kidney alterations in a well-characterized high-fat diet (HFD)–induced obesity/MetS model16 using kidney-specific megalin KO mice.11

RESULTS

Megalin KO and Control Mice Fed an HFD Show the Same Levels of MetS-Like Systemic Characteristics

In kidney-specific megalin KO (hereafter designated as megalin KO) mice (apoE cre, megalin lox/lox),11 megalin gene deletion occurs in a mosaic pattern in approximately 60% of PTECs, thereby allowing a direct comparison between megalin KO and intact PTECs in the same mice. To enhance the manifestation of kidney disease and specify primarily pathogenic lesions, megalin KO and control littermate mice were left-uninephrectomized at 10 weeks of age and then subjected to feeding with an HFD or normal-fat diet (NFD) for 12 weeks. As shown in Table 1, two-way ANOVA of body weight, fasting blood glucose, and total cholesterol levels showed a significant association with diet (HFD versus NFD), but not with genotype (megalin KO versus control), and revealed no two-factor (diet-genotype) interaction. These results indicate that obesity/MetS-related systemic features16 were manifested equally in megalin KO and control mice by feeding them an HFD. Kidney weight also showed a significant association only with diet and revealed no two-factor interaction.

Megalin Mediates Dysfunctional Autolysosome Accumulation in PTECs of the S2 Segment in HFD-Fed Mice, which Is Associated with Autophagy Impairment

The most prominent histologic feature in kidney sections of HFD-fed control mice was cytosolic vacuolar formation in PTECs that were immunostained with organic anion transporter 1 (OAT1), a marker for the S2 segment (Figure 1A). Electron microscopy showed that the vacuoles contained OsO4-stained multilamellar whirling structures (Figure 1B). The vacuolar membranes were immunostained with both lysosomal-associated membrane protein 1 (LAMP1), a lysosome marker, and microtubule-associated protein 1A/1B-light chain 3 (LC3), an autophagosome marker (Figure 1C), indicating that they were derived from dysfunctional autolysosomes particularly in the apical side of the cells.

Dysfunction of autolysosomes, the final sites of the autophagic pathway, is likely to be associated with impairment of this pathway in PTECs. To confirm this impairment, we used a transgenic mouse model that expresses green fluorescent protein (GFP)-LC3 systemically under the same experimental protocol as shown above (Supplemental Figure 1). p62, a marker of autophagy impairment, also accumulated in vacuolized PTECs and, to a lesser extent, in nonvacuolized PTECs of HFD-fed control mice (Figure 1D). These results indicate that HFD-induced autophagy impairment in PTECs is associated with autolysosomal dysfunction. HFD-induced autophagy impairment was reportedly caused by mammalian target of rapamycin complex 1 (mTORC1) activation.18 However, we found that S6Ser235/236 phosphorylation, a marker for mTORC1 activation, was increased by HFD feeding in only distal nephron segments and not in PTECs (Supplemental Figure 2).

In HFD-fed mosaic megalin KO mice, autolysosome-derived vacuoles were found exclusively in megalin-expressing PTECs, but scarcely in megalin KO PTECs, even in the OAT1-positive S2 segment (Figure 1E). A significant statistical interaction was

| Table 1. Comparison of general and renal parameters in control and megalin KO mice fed an HFD or NFD |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| KO-HFD | Cont-HFD | KO-NFD | Cont-NFD |
| Body wt (g) | 47.2 ± 4.9 | 48.0 ± 3.5 | 30.7 ± 2.9 | 32.5 ± 5.6 |
| KW (g) | 0.28 ± 0.03 | 0.26 ± 0.03 | 0.25 ± 0.04 | 0.23 ± 0.03 |
| BG (mmol/L) | 6.11 ± 1.45 | 5.83 ± 1.32 | 3.33 ± 0.65 | 3.88 ± 0.81 |
| TC (mmol/L) | 3.78 ± 0.56 | 3.60 ± 0.31 | 2.97 ± 0.56 | 2.96 ± 0.54 |
| FFA (μmol/L) | 648.0 ± 145.8 | 696.3 ± 465.5 | 655.2 ± 109.5 | 879.7 ± 246.9 |
| UAE (μg/day) | 293.6 ± 192.7 | 147.5 ± 75.8 | 126.3 ± 45.2 | 44.3 ± 34.9 |

KW, kidney weight; BG, blood glucose; TC, total cholesterol; FFA, free fatty acids; Cont, control. n=10 in each group. Values are means ± SD.
found between diet and genotype in vacuolar formation by two-way ANOVA (Figure 1F), indicating that the HFD-induced vacuolization in PTECs was significantly inhibited in megalin KO mice compared with control mice. These results suggest that HFD-induced autolysosomal dysfunction and autophagy impairment in PTECs were caused primarily by megalin-mediated endocytosis, which is presumably involved in the uptake of toxic glomerular-filtered substances, affecting the function of the endosome/lysosome system of the S2 segment.

**p27Kip1 Is Upregulated in PTECs of HFD-Fed Control Mice, but not in those of HFD-Fed Megalin KO Mice**

In the present study, the nuclear expression of p27Kip1, a marker of cell cycle inhibition and cellular hypertrophy, was more detectable in PTECs of HFD-fed control mice than in those of NFD-fed mice (Figure 2A). However, the number of nuclei immunostained for p27Kip1 in PTECs was significantly lower in HFD-fed megalin KO mice (Figure 2A), showing that p27Kip1 expression was increased in megalin-expressing PTECs with a hypertrophic phenotype, but not in megalin KO PTECs in the same kidney sections (Figure 2B). Hypertrophic tubules are likely to influence both intratubular and interstitial spaces, which should affect the structure and function of glomeruli in a retrograde manner.

**Vacuolated PTECs in HFD-Fed Mice Show Evidence for Lipid Peroxidation and Cellular Senescence**

The increased expression of p27Kip1 is also known as a marker of cellular senescence. In intravital imaging in kidneys of HFD-fed wild-type mice using two-photon laser scanning microscopy, abnormal autofluorescence was found in PTECs (Figure 2C) that were located in the S2 segment (Supplemental Figure 3). The intracellular distribution and

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**Figure 1.** Megalin mediates dysfunctional autolysosome accumulation in PTECs of the S2 segment in HFD-fed mice, which is associated with autophagy impairment. (A) Giant vacuolizations were seen in OAT1-positive S2 segments of proximal tubules (closed arrowheads), but not in OAT1-negative ones (open arrowheads) in PAS-stained kidney sections of control mice fed an HFD for 12 weeks. Bar = 50 µm. G, glomerulus. (B) Electron-dense multilamellar whirl structures (closed arrowheads) were detected in the cytoplasm of PTECs of HFD-fed control mice by electron microscopy. Bar = 5 nm. (C) Immunostaining for LAMP1 and LC3 (closed arrowheads) in the vacuolar membranes of PTECs of HFD-fed control mice. Bar = 20 µm. (D) p62 was heavily immunostained in vacuolized PTECs (closed arrowheads), but less so in nonvacuolized PTECs (open arrowheads) of HFD-fed control mice. Bar = 20 µm. G, glomerulus. (E) In mosaic megalin KO mice fed an HFD, vacuolization was only seen in megalin (stained purple)-expressing PTECs (closed arrowheads), but not in megalin-KO PTECs in OAT1 (stained brown)-positive S2 segments (open arrowheads). Bar = 20 µm. (F) Two-factor (diet-genotype) interaction plot by two-way ANOVA for mean number of vacuoles (≥ 5 µm in diameter) counted per ×400 magnification field in five randomly selected cortical regions on PAS-stained kidney sections of mosaic megalin KO (○, solid line) and control mice (+, dashed line) fed an HFD or NFD (n = 8 in each group). *P* < 0.001.
tubular localization patterns of the autofluorescent substances indicated that they were contained in dysfunctional autolysosomes of PTECs. Such autofluorescence was not detected in the vacuoles of PTECs in frozen kidney sections of HFD-fed LC3-GFP transgenic mice (Supplemental Figure 1, A and B), probably because the lysosomal contents were leaky from weakly fixed tissue sections.20 As shown previously,21 the contents of dysfunctional autolysosomes were stained with Luxol Fast Blue, but not with Oil Red O, indicating that they contain phospholipids and not neutral triglycerides (Supplemental Figure 4, A and B). Furthermore, the vacuolar contents were stained intensely with toluidine blue in kidney tissues fixed with glutaraldehyde and OsO₄ (Figure 2D), indicating that they have a lipofuscin-like senescent character.22,23 The vacuolar contents, as well as the cytoplasm around the vacuoles, were also immunostained for 4-hydroxy-2-nonenal (4HNE) (Supplemental Figure 4C), a marker for lipid peroxidation of unsaturated fatty acids24 and a potential source of fluorescent chromophores.25 In HFD-fed control mice, unsaturated fatty acids used for lipid peroxidation in the vacuoles or cytoplasm may be derived from increased glomerular-filtered, fatty acid–bound albumin or other megalin-mediated carrier proteins, including

Figure 2. Megalin induces hypertrophic and senescent phenotypes in PTECs of mice fed an HFD. (A) Two-factor (diet-genotype) interaction plot for mean number of p27Kip1–immunostained nuclei in five randomly selected PTEC regions (×800 magnification) on kidney sections of mosaic megalin KO (○, solid line) and control mice (+, dashed line) fed an HFD or NFD (n=10 in each group). The numbers are expressed per 10⁴ μm² of PTEC area. P<0.001. (B) Double immunostaining for p27Kip1 (brown) and megalin (purple) demonstrated that megalin-expressing PTECs showed nuclear p27Kip1 immunostaining (closed arrowheads) with hypertrophic changes that were barely seen in megalin-KO PTECs (open arrowheads) of megalin KO mice fed an HFD. Bar =20 μm. (C) Vacuolized PTECs showed strong autofluorescence (green-yellow) in intravital imaging in the kidneys of wild-type mice fed an HFD for 12 weeks. (D) In kidney sections fixed with glutaraldehyde and OsO₄, the contents of vacuoles (compatible to autolysosomes) were stained intensely with toluidine blue (closed arrowheads) in PTECs of HFD-fed control mice, whereas there were distinct, mostly smaller vesicles in the basal side, which were stained with OsO₄, but not with toluidine blue and also seen in some PTECs of NFD-fed control mice (open arrowheads, putative “lipid droplets”). Bar =20 μm.
apolipoproteins, even though free fatty acid concentrations in the serum were not significantly different between NFD- and HFD-fed mice (Table 1).

Intravital Imaging in the Kidney of HFD-Fed Mice Shows Narrowing of Tubular Lumens and Peritubular Capillaries Adjacent to PTECs Containing Autofluorescent Lipofuscin-Like Substances in the S2 Segment

In intravital imaging in kidneys of HFD-fed mice, excitation by a 720-nm laser, which can reveal cell shapes by visualization of cytosolic and mitochondrial NADH, showed that the tubular lumens were narrowed at the regions where autofluorescent lipofuscin-like substances accumulated in the PTECs (Figure 3A). Rhodamine B-conjugated 70-kDa dextran was then administered intravenously to visualize plasma. As blood cells are not excited by the laser, they are seen as shadows in the magenta plasma flow. Peritubular capillaries between vacuolized PTECs were narrowed (Figure 3B) and blood cell flow was disturbed (Figure 3C), features that were observed rarely in NFD-fed mice.

Peritubular Capillary Rarefaction Is Evident in HFD-Fed Control Mice, but not in HFD-Fed Megalin KO Mice

Peritubular capillary narrowing or obstruction was also found by electron microscopy in HFD-fed control mice, but scarcely in NFD-fed control mice (Figure 4A). This finding was accompanied by localized interstitial fibrosis (Figure 4, A and B), which was likely to be induced by activated fibroblasts/pericytes. Hence, we evaluated damage in peritubular capillaries via immunohistochemistry for CD31, an endothelial marker, in both HFD- and NFD-fed mice. Peritubular capillary rarefaction was more pronounced in HFD-fed control mice than in those fed an NFD (Figure 4C). In contrast, rarefaction was not evident in HFD-fed megalin KO mice (Figure 4, C and D). These results indicate that the HFD-induced, megalin-mediated phenotype changes in PTECs are likely to be involved in peritubular capillary damage.

Glomerular Hypertrophy and Mesangial Matrix Expansion Are Observed in HFD-Fed Control Mice, but Are Ameliorated in HFD-Fed Megalin KO Mice

By univariate (correlation matrix) and multivariate (principal component analysis) statistical analyses, urinary albumin excretion (UAE) was found to be associated with kidney weight rather than body weight. Hence, kidney weight appeared to be a confounding factor for UAE for the diet-genotype factors. Thus, we carried out two-way analysis of covariance, adjusted using kidney weight as a covariate, for UAE as a dependent variable and the diet-genotype factors as fixed effects, showing a significant diet-genotype interaction for UAE (Table 1). Namely, although UAE levels were higher in megalin KO mice, even in those fed an NFD, as megalin mediates albumin uptake in PTECs, UAE was less enhanced by diet (HFD) in megalin KO mice than in control mice. Histologic analysis also showed that HFD-induced glomerular hypertrophy with
mesangial matrix expansion was less evident in both the superficial and juxtamedullary cortical regions in megalin KO mice compared with control mice (Figure 5). Collectively, HFD-induced tubuloglomerular alterations are likely to be caused by a megalin-mediated mechanism.

Giant (Auto)lysosomes Accumulate More in Cultured PTECs following Incubation with Fatty Acid–Rich Albumin than with Fatty Acid–Depleted Albumin

Hyperglycemic factor(s) per se may not be apparently involved in megalin-mediated autolysosomal vacuolization in PTECs, because there is scarcely such a finding in a streptozotocin-induced type 1 diabetes model (data not shown). To examine the megalin-mediated mechanism associated with dyslipidemia, we performed in vitro experiments using megalin-expressing immortalized rat proximal tubule cells (IRPTCs) or IRPTCs stably expressing enhanced green fluorescent protein (EGFP)-LC3, an autophagosome marker. The cells were incubated with albumin, an endocytic ligand of megalin that had been either enriched in or depleted of fatty acids as a lipotoxic model. Compared with fatty acid–depleted albumin, incubation of IRPTCs with fatty acid–rich albumin induced an increase in intracellular giant vacuolar formation, as detected using the lysosomal marker LysoTracker (Figure 6A). These giant vacuoles were sometimes surrounded by an EGFP-LC3 signal (Figure 6B) in IRPTCs expressing EGFP-LC3, indicating that they were derived from lysosomes or autolysosomes. These results suggest that the cellular uptake of lipotoxic proteins may be involved in giant (auto)lysosome formation in PTECs. Autophagy flux in IRPTCs was also inhibited by using fatty acid–rich albumin.
albumin compared with fatty acid–depleted albumin (Supplemental Figure 5).

Megalin Mediates the Upregulation of PDGF-B and Monocyte Chemoattractant Protein-1 Expression in Cultured PTECs Incubated with Fatty Acid–Rich Albumin

The transition of interstitial fibroblasts/pericytes to myofibroblasts is activated via signaling mediated by α and β receptors for PDGF (PDGFRα and PDGFRβ).30,31 A major ligand of both PDGFRα and PDGFRβ, PDGF-B, is known to induce tubulointerstitial fibrosis,33 and the tubular expression of PDGF-B is upregulated in a unilateral ureteral obstruction model.30 We found that PDGF-B was upregulated in IRPTCs incubated with fatty acid–rich albumin rather than with fatty acid–depleted albumin (Figure 6C). Knockdown of megalin expression in the cells using specific small interfering RNA (siRNA) (approximately 46% suppression of megalin protein expression, Supplemental Figure 6) suppressed the fatty acid–rich albumin mediated upregulation of PDGF-B (approximately 44% suppression of the mRNA expression, Figure 6C). These results indicated that the megalin-mediated handling of lipotoxic proteins may be involved directly in the increased expression of PDGF-B in PTECs. Similar data were also obtained for monocyte chemoattractant protein-1 (MCP-1), a chemokine involved in inflammation (Figure 6C).

Collectively, the pathways of the megalin-mediated mechanism of tubuloglomerular alterations in HFD-induced kidney disease are illustrated in Figure 7.

DISCUSSION

Using kidney–specific megalin KO mice crossbred with an experimental nephrotic model of inducible podocyte injury for nonselective glomerular protein filtration, megalin was found to initiate PTEC damage by reabsorbing massive proteins into the cells.34 However, there is a conflicting report using a glomerular injury model with antiglomerular basement membrane serum in the KO mice, which indicated that megalin-mediated endocytosis may not be associated primarily with the development of tubulointerstitial damage.35 Because both analyses were performed using acute nephrotic models with overwhelming glomerular damage, further studies are required to clarify the role of megalin in slowly progressing CKD models.

In the current study, using an HFD-induced kidney disease model with low-grade proteinuria, we determined that autolysosomal dysfunction was induced by the megalin-mediated endocytic handling of qualitatively toxic substances in PTECs. We found that lipotoxicity by fatty acid–bound albumin is a candidate for megalin–mediated pathogenesis. However, apolipoproteins26 and/or obesity/MetS–associated molecules, such as adipokines released from visceral adipocytes, may also be involved in the megalin–mediated mechanism. Further studies are needed to elucidate how these toxic substances trigger intracellular pathogenic pathways via megalin.
Additionally, we found that HFD-induced, megalin-mediated autolysosomal dysfunction in PTECs is also associated with autophagy impairment. Autophagy protects PTECs in ischemia-perfusion, drug toxicity, and protein-overload nephropathy models. Autophagy impairment is also likely to affect PTEC functions in HFD-induced kidney disease. Megalin-mediated autolysosomal dysfunction may also be associated with the generation of profibrotic and/or inflammatory signals in PTECs via the NLRP3 (NACHT, LRR, and PYD domain-containing protein 3) inflammasome pathway.

The reason why PTECs in the S2 segment are susceptible to HFD-induced autolysosomal injury also remains unknown. The S1 segment is the major site for physiologic protein reabsorption. However, under proteinuric conditions, pathologic ligands of megalin may overflow to the S2 segment where megalin is expressed more prominently in microvilli than in the S1 and S3 segments. Subsequently, lysosomal enzyme function in the S2 segment may be impaired by toxic protein degradation load, resulting in the accumulation of lipofuscin-like substances via lipid peroxidation of unsaturated fatty acids, which is similar to the findings in aging kidneys.

On the basis of our data, megalin could be a novel pharmacologic target for preventing or treating obesity/MetS-related CKD, such as by competing with megalin binding to toxic ligands or by suppressing megalin expression to reduce the uptake of these ligands into PTECs. Bardoxolone methyl, an Nrf2 activator, may be such a candidate drug, because it reportedly suppresses renal megalin expression in monkeys and improves eGFR in CKD patients with type 2 diabetes. However, megalin-mediated endocytosis in PTECs plays an important physiologic role in some processes such as vitamin homeostasis. Therefore, complete megalin suppression may not be desirable. However, the megalin KO mice used in this study were deficient for megalin expression in approximately 60% of PTECs, but histologic tubuloglomerular alterations were significantly ameliorated. Thus, obesity/MetS-related CKD could be prevented effectively or treated by just partial pharmacologic megalin blocking, with vitamin supplementation if necessary.

In conclusion, the megalin-mediated endocytic handling of glomerular-filtered (lipo) toxic substances is involved primarily in hypertrophic and senescent PTEC injury with autophagy impairment, causing peritubular capillary damage and retrograde glomerular alterations in HFD-induced kidney disease. Blocking or suppressing the function of megalin would be a novel prophylactic or therapeutic strategy for obesity/MetS-related CKD, independently of the modification of weight and metabolic parameters such as dyslipidemia and hyperglycemia.

**CONCISE METHODS**

**Induction of HFD-Induced Kidney Disease in Mice**

Male kidney-specific megalin KO mice (apoE cre, megalin lox/lox) established by Professor Thomas Willnow (Max Delbrück Center...
Figure 7. The megalin-mediated mechanism appears to be involved primarily in HFD-induced tubuloglomerular injury. In the presence of glomerular hyperperfusion that should occur in HFD-fed hyperglycemic mice, the megalin-mediated uptake of glomerular-filtered (lipo)toxic substances may cause autolysosomal dysfunction via lipid peroxidation associated with autophagy impairment, leading to hypertrophic and senescent changes of PTECs. In addition, these processes may be associated with the increased production of profibrotic and inflammatory mediators, such as PDGF-B and MCP-1, which activate interstitial fibrocytes/pericytes to induce localized interstitial fibrosis. Peritubular capillaries would thereby become damaged and rarefactual by these complex mechanisms. These factors for glomerular hyperperfusion and increased tubular and peritubular capillary resistance should contribute to the development of glomerular hypertrophy with mesangial matrix expansion.

Blood and Urine Analyses
Blood glucose concentrations were measured by using FreeStyle Freedom (Nipro). Total cholesterol and free fatty acids were assayed by enzymatic methods. Urinary albumin was measured by a quantitative turbidimetric immunoassay method (Wako Pure Chemicals).

Preparation of Kidney Sections for Light Microscopy
From horizontal cross-sections containing the hila of kidneys, 3-mm thick tissues were obtained and fixed in a 4% paraformaldehyde phosphate buffer for 72 hours at room temperature. After fixation, the tissues were dehydrated in a graded ethanol series from 70% to 100%, cleared in xylene, and embedded in paraffin. From each tissue sample, 2-μm thick sections were cut using a microtome (RE-710; Yamato Kohki Industrial Co., Ltd.) and used for staining with periodic acid–Schiff (PAS) or Masson trichrome. In addition, 4-μm thick sections were used for Luxol Fast Blue staining. Kidney tissue samples were snap-frozen in liquid nitrogen and prepared for Oil Red O staining. Additionally, the HFD-fed mice were perfused transcardially with a 0.9% saline and 4% paraformaldehyde phosphate buffer under ip anesthesia with pentobarbital sodium (50 mg/kg) and kidney sections were fixed in a 4% paraformaldehyde phosphate buffer overnight and embedded in OCT compound (Sakura Finetek). Subsequently, 4-μm thick frozen sections were cut with a cryostat (CM1850; Leica) for immunohistochemical analysis of 4HNE.

Immunohistochemistry
Kidney tissue sections were deparaffinized by immersion in xylene and rehydrated through a graded ethanol series. Antigen retrieval was accomplished by heating the slides in a microwave in 10 mM citrate buffer, pH 6.0. To eliminate endogenous peroxidase activity, the sections were incubated in 3% H2O2. Following incubation with 5% goat serum to block nonspecific binding, the sections were incubated for 30 minutes at room temperature with a primary antibody against OAT1 (1:40; KE038; Trans Genic, Inc.), LAMP1 (1:200; ab24170; Abcam, Inc.), LC3 (1:1,000; PM036; MBL), or p62/SQSTM1 (1:3,000; PM045; MBL), and overnight with phospho-S6 ribosomal protein XP rabbit monoclonal antibody (1:200; Vector Laboratories) and avidin-biotin-peroxidase complex (ELITE ABC kit; Vector Laboratories) and avidin-biotin-peroxidase complex (Elite ABC kit; Vector Laboratories). Finally, peroxidase activity was visualized with the 3,3'-Diaminobenzidine substrate-chromogen system (Dako), counterstained with Mayer hematoxylin or PAS, and examined under a light microscope. Frozen sections were also stained with an anti-4HNE antibody (1:200; ab46545; Abcam, Inc.) and the avidin-biotin-peroxidase complex method described above. Negative control staining was always performed in parallel by incubating sections with PBS instead of the primary antibodies.

For immunohistochemical staining with an anti-CD31 antibody, antigen retrieval was performed using proteinase K. Endogenous biotin activity was blocked with an avidin/biotin blocking kit (Vector Laboratories) prior to incubation with a rat anti-mouse CD31 antibody (1:100; ab56299; Abcam) at 4°C overnight. A biotinylated mouse-absorbed, rabbit anti-rat IgG antibody (1:500; Vector Laboratories) was used as a secondary antibody. Double immunostaining was also performed with paraffin sections of mouse kidney specimens. The sections were incubated sequentially with either an anti-OAT1 or anti-p27

Ckip1 (phospho S10) antibody (1:50; ab62364; Abcam, Inc.) and horseradish peroxidase-labeled polymer anti-rabbit antibody (EnVision+ System; Dako).
Immunoreactivity was developed with the 3,3′-Diaminobenzidine substrate-chromogen system (Dako). Then, the sections were incubated with an anti-megalin antibody (1:3,000) and detected with the avidin-biotin-peroxidase complex method described above, except that a VIP substrate kit (Vector Laboratories) was used. Countertaining was performed with Mayer hematoxylin.

**Electron Microscopy**

For electron microscopy examination, the tissues (1-mm cubes) were fixed in a 2.5% glutaraldehyde solution in 0.1 M phosphate buffer, pH 7.4, and then in a 2.0% OsO4 solution in the same buffer. They were dehydrated with an ethanol series and embedded in Poly/Bed812 (Polysciences, Inc.). Ultrathin sections (75–80 nm) were obtained on a Porter-Blum MT-1 ultramicrotome (Sorvall, Inc.), placed on 100-mesh grids, and stained with a tannic acid and lead solution.

**Staining of Renal Sections with Toluidine Blue**

After fixing kidney tissues with glutaraldehyde and OsO4 and embedding them in Poly/Bed812, as described above, semithin sections mounted on glass slides were stained with a 1% toluidine blue dye solution including sodium borate on a slide warmer at 80°C for 5 seconds.

**Preparation of Kidney Tissues from LC3-GFP Transgenic Mice**

After the left kidneys were removed at 10 weeks of age, male GFP-LC3 #53 transgenic mice were fed an HFD or NFD for 12 weeks. Five mice fed an HFD or NFD were starved for 24 hours before euthanization and another five mice fed an HFD or NFD were euthanized under ad libitum conditions.

**Evaluation of Autophagosomes in PTECs of LC3-GFP Transgenic Mice**

Cryosections were examined using a fluorescence microscope (BZ-9000; Keyence) with a ×60 oil-immersion objective lens. Images captured for GFP-LC3 signals (autophagosomes) were detected specifically in PTECs by using a GFP filter, and not by using a tetramethylrhodamine isothiocyanate filter, in five randomly selected cortical regions of each sample (n=5 in each group). The images were analyzed using image processing and measuring software (Image-Pro Plus v.7.0; Media Cybernetics) to count the number of GFP-LC3 dot signals and measure the area of the proximal tubules. The results were expressed as the mean number of signals per 100 μm² area of the proximal tubules and the mean values of each mouse were evaluated statistically. All morphometric analyses in this study were performed by an investigator masked to sample identity.

**Counting the Number of Dysfunctional Autolysosome-Derived Intracellular Vacuoles in PTECs and p27kip1 Immunostained Nuclei**

Images of five cortical regions (×400 magnification) containing no glomeruli were taken randomly from PAS-stained kidney sections of megalin KO and control mice fed an HFD or NFD (n=8 in each group) using a BZ-8000 microscope (Keyence). The images were used to count the number of intracellular vacuoles (dysfunctional autolysosomes) with a diameter of 5 μm or more per field using Image-Pro Plus v.7.0. Similarly, images of five cortical regions (×800 magnification) were taken randomly from p27kip1-immunostained kidney sections of these mice (n=10 in each group) using a BZ-8000 microscope to count the number of positively p27kip1-immunostained nuclei, expressed as per 10⁴ μm² of PTEC area. The mean values of each mouse were evaluated statistically.

**In Vivo Multiphoton Imaging**

**Evaluation of Peritubular Capillary Rarefaction**

After immunostaining of CD31, images of ten cortical regions (×400 magnification) containing no glomeruli were selected randomly from megalin KO and control mice fed an HFD or NFD (n=6 in each group) using a BZ-8000 microscope and used to evaluate CD31-positive areas in the renal cortex with Image-Pro Plus v.7.0. The CD31-positive areas were expressed as percentages of the field and the mean values of each mouse were evaluated statistically.

**Evaluation of Glomerular Sizes and Mesangial Matrix Areas**

For morphometric analysis, the superficial and juxtamedullary cortical regions were chosen randomly (×200 magnification) in megalin KO and control mice fed an HFD or NFD (n=10 in each group) and all glomeruli in the regions were assessed. We eliminated...
glomeruli that were inappropriate for the evaluation of glomerular sizes and/or mesangial matrix areas if they were sectioned at their vascular poles or artificially compressed or distorted. Finally, 15 images (×800 magnification) of glomeruli were obtained randomly from each superficial and juxtamedullary cortical region of individual mice. The decision for selecting glomeruli for the analysis was made blindly by an experienced clinicopathologist. Glomerular areas and PAS-positive, nucleus-free areas in the mesangium (defined as mesangial matrix areas) were assessed using Image-Pro Plus v.7.0. The mesangial matrix areas were expressed as percentages of the glomerular areas. The mean values of each mouse were evaluated statistically.

Preparation of IRPTCs Stably Expressing EGFP-LC3

IRPTCs or IRPTCs expressing EGFP-LC3 were cultured in the medium described above, except for fatty acid–rich BSA or fatty acid–depleted BSA (Calbiochem, EMD Millipore) at a concentration of 1 mg/ml for 24 hours on gelatin-coated coverslips (Thermo Fisher Scientific). Then, LysoTracker Deep Red (Life Technologies, Molecular Probes), a lysosomal marker, and NucBlue Live Cell Stain (Thermo Fisher Scientific) were added to the culture media and incubated for 20 minutes. The cells were then fixed with a 4% paraformaldehyde phosphate buffer solution (Wako) and examined using a BZ-9000 microscope. LysoTracker-positive vacuoles and cell surface area were evaluated by using Image-Pro Plus v.7.0 in 20 cells selected at random.

Autophagy Flux Analysis

IRPTCs were cultured in the same condition described above and used for this analysis (see Supplemental Figure 5 for the detailed method).

RNA Interference of Megalin Expression in IRPTCs

Megalin expression was knocked down by transfecting IRPTCs with specific Silencer Select siRNAs (2.5 nM each of s130935 and s139036, Cat# 4390771; Life Technologies, Ambion) for 24 hours in medium without puromycin using the Lipofectamine RNAiMAX Reagent (Life Technologies, Invitrogen) according to the manufacturer’s instructions. Nonspecific Silencer Negative Control #5 siRNA (Cat#AM4642; Life Technologies, Ambion) was used for negative control experiments (see Supplemental Figure 6 for the detailed method).

Real-Time RT-PCR of PDGF-B and MCP-1

IRPTCs that were cultured in DMEM, low glucose, pyruvate, 25 mM HEPES, and 0.1 mM MEM nonessential amino acids were used for megalin RNAi for 24 hours and incubated with fatty acid–rich BSA or fatty acid–depleted BSA at a concentration of 1 mg/ml for 3 hours. The cells underwent RNA purification using a GenElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich). The expression of rat PDGF-B and MCP-1 was analyzed by real-time RT-PCR using specific primers (forward: GTCGAGTCGAAAAGCTCATG; reverse: ACACCTCGTACGGTCTCTTG; and forward: TCTTCTCCTCAC-CACGAGC; reverse: CTTGTAGTTCCAGCCGACTCA, respectively). The expression of β-actin mRNA was also measured as an internal control using the following primers (forward: GCAGTACAACCC-TCTCTGGACG; reverse: CACACCCACATCACACCC). Quantitative PCR was performed using the Thermal Cycler Dice Real Time System II (Takara) and a One Step SYBR PrimeScript PLUS RT-PCR Kit (Takara). The PCR reactions were initiated with reverse transcription at 42°C for 5 minutes, heat inactivation of reverse transcription at 95°C for 10 seconds, denaturation at 95°C for 10 seconds, followed by amplification with 45 cycles at 95°C for 5 seconds, annealing at 55°C for 30 seconds, and dissociation. Data were evaluated with Multiplate RQ software.

Statistical Analyses

The results of animal experiments were analyzed for two-factor (diet-genotype) interactions by two-way ANOVA or two-way analysis of covariance and comparisons between two groups were made by t test (two-tailed) using SAS Studio 3.1 (SAS Institute, Inc.), Predictive Analytics SoftWare Statistics 18 (SPSS, Inc.) and R version 3.1.2 (R Foundation for Statistical Computing). The level of significance was P < 0.05.

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DISCLOSURES

None.

REFERENCES


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