

Obesity-Mediated Autophagy Insufficiency Exacerbates Proteinuria-induced Tubulointerstitial Lesions

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ABSTRACT

Obesity is an independent risk factor for renal dysfunction in patients with CKDs, including diabetic nephropathy, but the mechanism underlying this connection remains unclear. Autophagy is an intracellular degradation system that maintains intracellular homeostasis by removing damaged proteins and organelles, and autophagy insufficiency is associated with the pathogenesis of obesity-related diseases. We therefore examined the role of autophagy in obesity-mediated exacerbation of proteinuria-induced proximal tubular epithelial cell damage in mice and in human renal biopsy specimens. In nonobese mice, overt proteinuria, induced by intraperitoneal free fatty acid–albumin overload, led to mild tubular damage and apoptosis, and activated autophagy in proximal tubules reabsorbing urinary albumin. In contrast, diet-induced obesity suppressed proteinuria-induced autophagy and exacerbated proteinuria-induced tubular cell damage. Proximal tubule-specific autophagy-deficient mice, resulting from an *Atg5* gene deletion, subjected to intraperitoneal free fatty acid–albumin overload developed severe proteinuria-induced tubular damage, suggesting that proteinuria-induced autophagy is renoprotective. Mammalian target of rapamycin (mTOR), a potent suppressor of autophagy, was activated in proximal tubules of obese mice, and treatment with an mTOR inhibitor ameliorated obesity-mediated autophagy insufficiency. Furthermore, both mTOR hyperactivation and autophagy suppression were observed in tubular cells of specimens obtained from obese patients with proteinuria. Thus, in addition to enhancing the understanding of obesity-related cell vulnerability in the kidneys, these results suggest that restoring the renoprotective action of autophagy in proximal tubules may improve renal outcomes in obese patients.

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In glomerular diseases, proteinuria is a leading cause of tubulointerstitial lesions and subsequent renal dysfunction, as well as a marker of glomerular lesions. Reducing proteinuria is therefore considered as a principal therapeutic target to improve renal outcomes in glomerular diseases.^{1–3} Clinical evidence has shown that treatment with some immunosuppressors or strict BP control could successfully reduce or abrogate proteinuria, improving renal prognosis in patients with glomerular diseases, including diabetic nephropathy.^{4,5} Unfortunately, however, some patients develop treatment-resistant proteinuria, resulting in ESRD. In these patients, protecting proximal tubular

epithelial cells (PTECs) against proteinuria may be the next therapeutic target to improve renal outcomes.

Obesity is strongly associated with the development of metabolic diseases, and its increased incidence has

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made obesity a major health problem worldwide. Obesity is also a cause of treatment-resistant proteinuria, as well as an independent risk factor for a rapid decline in renal function in patients with glomerular diseases.^{6–8} However, the mechanisms underlying the effects of obesity on renal function remain unclear. Because the severity of proteinuria-induced tubulointerstitial lesions is strongly correlated with renal outcomes,^{9,10} obesity may exacerbate proteinuria-induced tubulointerstitial lesions. If so, identifying the molecular mechanisms underlying obesity-mediated PTEC vulnerability may lead to new therapies that improve renal outcomes in obese and type II diabetes patients with persistent proteinuria.

Autophagy is an intracellular catabolic process that degrades proteins and organelles *via* lysosomes to maintain intracellular homeostasis during stress conditions, including starvation, hypoxia, and endoplasmic reticulum (ER) stress.^{11–14} Autophagosome formation is regulated by some autophagy-related (Atg) proteins.^{13,14} The initiation of starvation-induced autophagy is regulated by the phosphorylation of Atg1 and Atg13 *via* intracellular nutrient-sensing signals, and these steps are inhibited by phosphatidylinositol 3-kinase inhibitors such as 3-methyladenine (3-MA).¹⁵ The conjugation of LC3I, the mammalian homolog of Atg8, to phosphatidylethanolamine by Atg7 and Atg3 to form LC3II is a critical step in autophagosome formation, with LC3II remaining on the lysosomal membrane even after the lysosome fuses with the autophagosome. Moreover, this LC3 conjugation reaction is positively regulated by the Atg12-Atg5-Atg16 complex. Thus, monitoring LC3II formation can be used to detect autophagy activity, and knock-down of *Atg* genes has been used for a loss-of-function study.¹⁵

In addition to studies designed to reveal the molecular mechanisms of autophagy, many studies have evaluated autophagy in physiologic and disease states. Autophagy activity in some metabolic organs has been found to decline with obesity, and its insufficiency is involved in the pathogenesis of obesity-related metabolic diseases.^{16–18} In the kidneys, autophagy has been reported to play a renoprotective role against both normal aging and AKI.^{19–22} However, the involvement of autophagy in CKDs, including obesity-mediated exacerbation of tubulointerstitial lesions in proteinuric kidney diseases, has not been elucidated.

We hypothesized that obesity alters autophagy activity in PTECs, leading to cell vulnerability and subsequent exacerbation of proteinuria-induced PTEC damage. To test this hypothesis, we examined the effects of proteinuria and obesity on autophagy activity in PTECs of the intraperitoneal free fatty acid (FFA)-albumin-overload model of high-fat diet (HFD)-induced obese mice and in human renal biopsy specimens. We also assessed the renoprotective role of autophagy in proteinuria-induced tubulointerstitial lesions in PTEC-specific *Atg5*-deficient mice.

RESULTS

HFD-Induced Obese Mice Developed Severe Proteinuria-Induced Tubulointerstitial Lesions

Relative to nonobese control mice fed with a normal diet (ND), HFD-induced obese mice showed significant increases in body

weight, fasting blood glucose concentrations, and glucose intolerance, as determined by intraperitoneal glucose tolerance tests (Figure 1, A–D). Our previous report showed an absence of renal phenotypes in mice fed a HFD for 4 weeks,²³ although these became obese and glucose intolerant. Thus, to examine the effects of obesity on proteinuria-induced tubulointerstitial lesions, equal levels of proteinuria were induced in nonobese and obese mice using an intraperitoneal FFA-bound albumin-overload model (Figure 1E), an established model for proteinuria-induced tubulointerstitial lesions.^{24,25} In nonobese mice, increased proteinuria induced renal histologic damage, characterized by tubular cell flattening and lumen dilation, tubular cell vacuolization, and cast formation (Figure 1, F and G), and increased expression of neutrophil gelatinase-associated lipocalin, a marker of renal damage²⁶ (Figure 1H). FFA-albumin overload also induced apoptosis, as shown by the cleavage of caspase 3 (Figure 1, I and J). All markers of FFA-albumin-overload-induced PTEC damage were significantly exacerbated in the kidneys of HFD-induced obese mice (Figure 1, F–J).

HFD-Induced Obesity Suppressed Proteinuria-Induced Autophagy in PTECs

We next examined the effects of fasting, proteinuria, and obesity on autophagy in PTECs using a green fluorescent protein (GFP)-LC3 transgenic mouse model generated to monitor autophagy activity.²⁷ In tissue sections from this transgenic line, autophagosomes are observed as GFP-LC3 dots.¹³ Mice fed *ad libitum* showed no GFP-LC3 dots in all tissues including kidney (Figure 2A). Mice fasted for 48 hours induced autophagy in PTECs, as well as in other organs such as the skeletal muscle, liver, and heart, whereas FFA-albumin overload induced autophagy only in PTECs (Figure 2A). Furthermore, FFA-depleted albumin, which was reported to be unable to induce tubulointerstitial lesion,²⁵ did not induce GFP dot formation in PTECs (Supplemental Figure 1).

To examine the relationship between albumin reabsorption from urinary lumen and autophagy induction in the PTECs, Texas Red (TR)-conjugated albumin was intravenously injected into GFP-LC3 transgenic mice treated with an intraperitoneal injection of FFA-albumin, with PTECs that reabsorbed albumin showing TR-albumin positivity at the brush border (Figure 2B). Proteinuria-induced GFP-LC3 dots were induced in TR-positive PTECs (Figure 2, C and D), but not in TR-negative PTECs (Figure 2, E and F), suggesting that proteinuria-induced autophagy is especially prominent in PTECs reabsorbing urinary albumin.

We next examined the effect of obesity on proteinuria-induced autophagy in PTECs. Although HFD-induced obesity did not alter basal levels of LC3 dot formation in the kidney, it suppressed FFA-albumin-overload-induced GFP-LC3 dot formation in PTECs (Figure 2G). A similar finding was obtained by Western blot analysis for LC3, in which an increased LC3II/LC3I ratio indicates activation of autophagy.²⁶ Although HFD-induced obesity did not increase LC3II formation (Figure 2, H and I), FFA-albumin-overload-induced LC3II

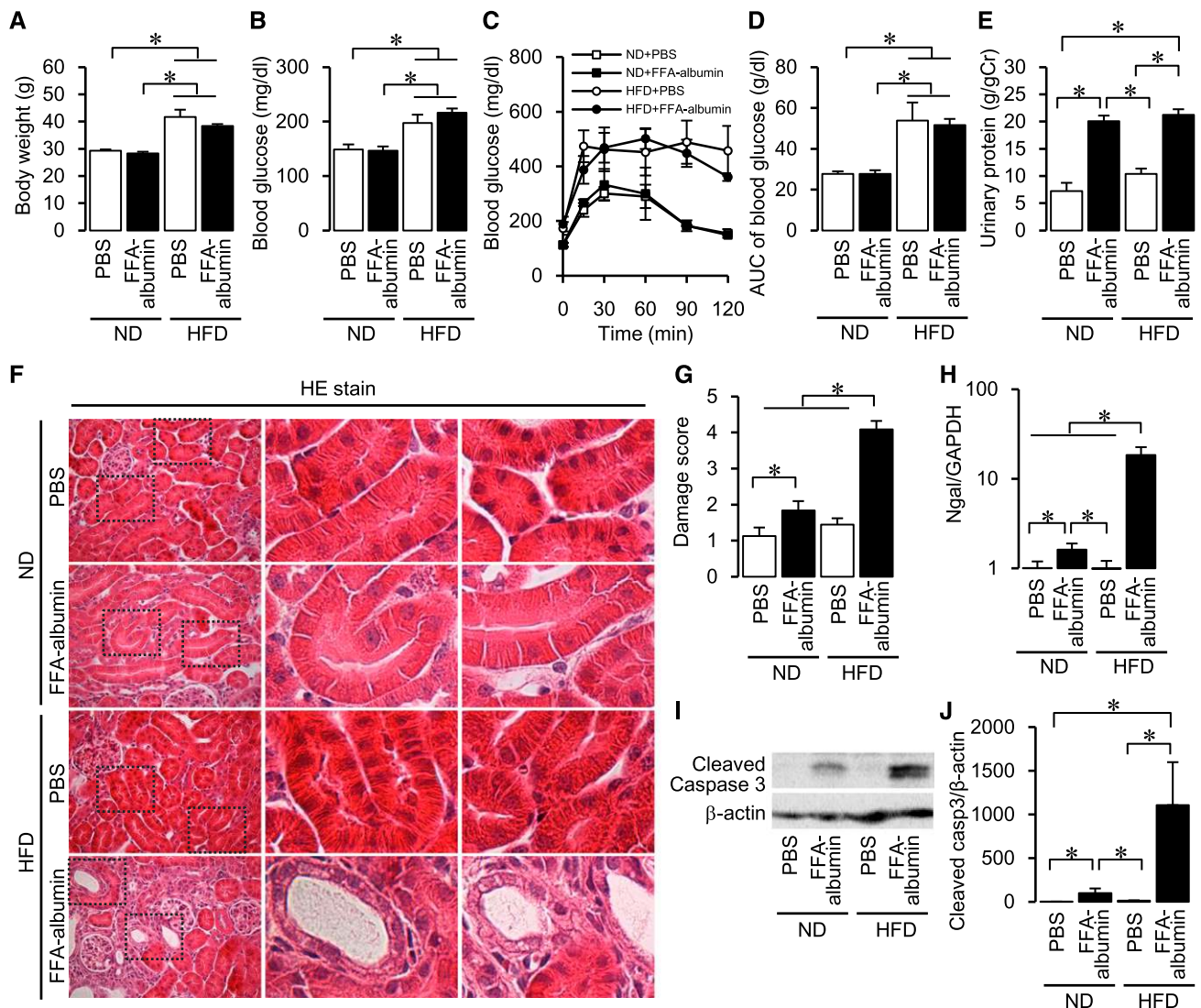


Figure 1. HFD-induced obesity exacerbates proteinuria-induced proximal tubular cell damage in mice. (A–D) Metabolic characteristics of mice fed a ND or HFD for 4 weeks before injection of PBS or FFA-nondepleted albumin (FFA-albumin): body weight (A), fasting blood glucose concentration (B), change in blood glucose concentration (C), and area under the curve (AUC) during intraperitoneal glucose tolerance tests (D). (E) Urinary protein excretion levels. (F) Histologic analysis, determined by hematoxylin and eosin (HE) staining of kidney sections. (G) Renal damage scores determined by HE staining. (H) Quantitative analysis of expression of neutrophil gelatinase-associated lipocalin (Ngal) mRNA. (I) Representative Western blots for cleaved caspase 3 and β actin as a loading control. (J) Quantitative analysis of the ratio of cleaved caspase 3 to β actin. Data are mean \pm SEM. * $P < 0.05$. Original magnification, $\times 400$ in F (upper lane, original; lower two lanes, details).

formation was significantly suppressed in obese mice (Figure 2, J and K). Furthermore, HFD-induced suppression of GFP dot and LC3II formation was restored in PTECs of mice returned to a lean condition (Figure 2, G, L, and M), suggesting that obesity-mediated inhibition of autophagy was reversible.

Autophagy Deficiency Exacerbated Proteinuria-Induced PTEC Damage

To examine the pathologic significance of autophagy deficiency on proteinuria-induced PTEC damage, we investigated

whether FFA-albumin overload exacerbated PTEC damage in the kidneys of PTEC-specific *Atg5*-deficient (*Atg5*^{-/-}) mice. *Atg5* mRNA and protein were absent from the kidneys, but not other organs, of these PTEC-specific *Atg5*^{-/-} mice (Figure 3, A and B). Although an LC3II band was found in homogenates from the renal cortexes of control (*Atg5*^{+/+}) mice, this band was absent from samples of PTEC-specific *Atg5*^{-/-} mice (Figure 3B). Functional deletion of autophagy in PTECs was confirmed by a lack of autophagosomal formation in kidney sections of PTEC-specific *Atg5*^{-/-} mice

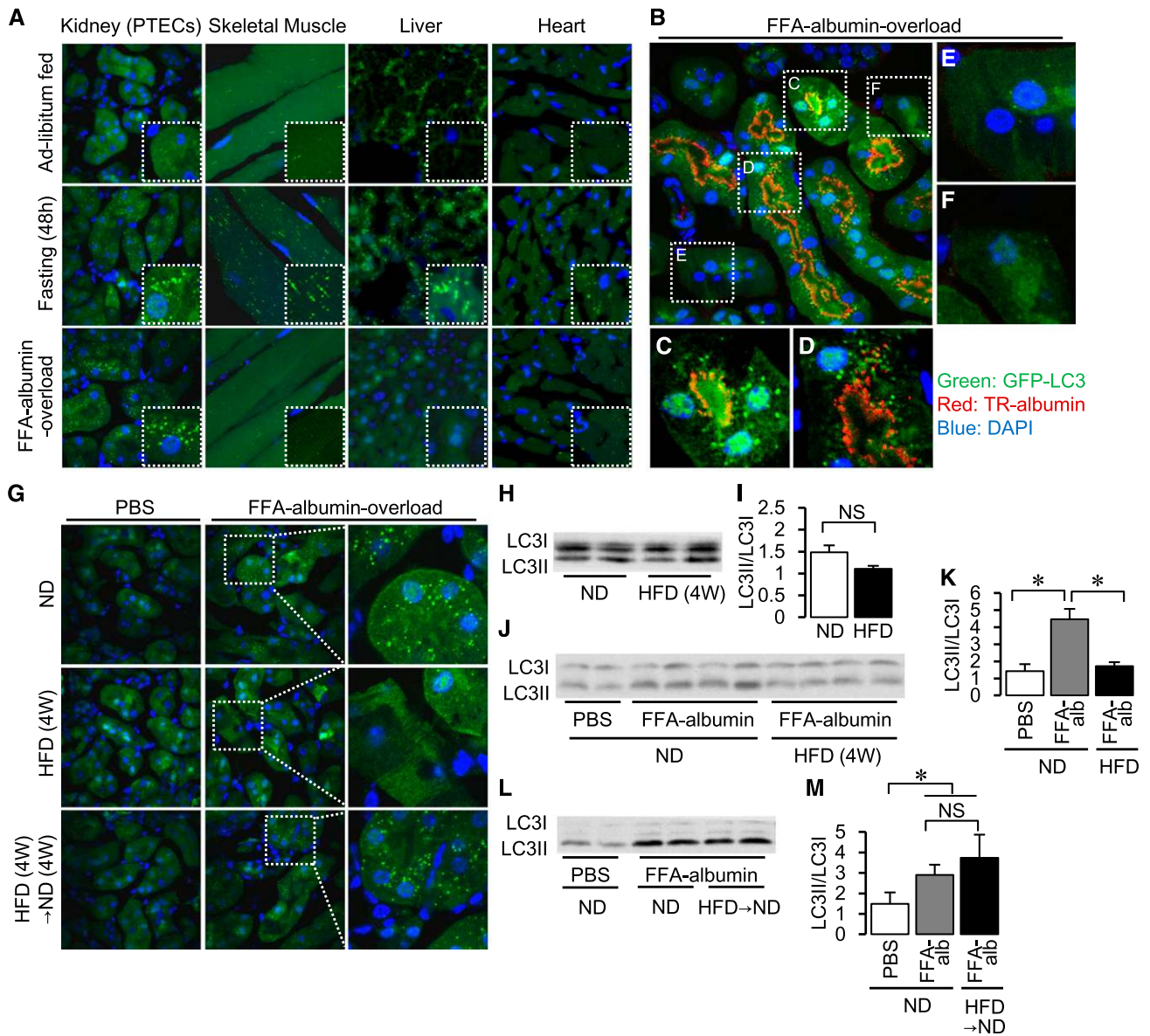


Figure 2. HFD-induced obesity suppressed proteinuria-induced autophagy in proximal tubular cells. (A) Autophagy activation is not observed in tissue from GFP-LC3 transgenic mice fed *ad libitum* (upper lanes). Forty-eight hours of fasting elicits autophagy, defined as GFP-LC3 dots in PTECs, skeletal muscle, liver, and heart (middle lanes). FFA-albumin overload induces GFP-LC3 dots only in PTECs, but not in other tissues (lower lanes). (B) PTECs reabsorbing urinary albumin are visualized as TR-conjugated albumin-positive cells, with FFA-albumin-overload-induced autophagy observed as GFP dots. (C and D) FFA-albumin-overload-induced GFP-LC3 dots are observed in TR-positive PTECs. (E and F) FFA-albumin-overload-induced GFP-LC3 dots are not observed in TR-negative PTECs. (G) FFA-albumin-overload-induced GFP-LC3 dots are not observed in PTECs of GFP-LC3 transgenic mice fed a HFD for 4 weeks (middle lanes), but HFD-induced suppression of autophagy is recovered in PTECs after mice are switched to a ND for an additional 4 weeks (lower lanes). (H–M) Representative figures and quantitative results of Western blots for LC3II and LC3I proteins in kidney samples from the indicated groups of mice. An increase in the LC3II/LC3I ratio indicates active autophagy. Data are mean ± SEM. **P*<0.05.

crossbred with GFP-LC3 transgenic mice (Figure 3C). PTECs of *Atg5*^{+/+} mice, but not of PTEC-specific *Atg5*^{-/-} mice, showed proteinuria-induced GFP-LC3 dots, with the latter showing diffuse expansion of GFP signals (Figure 3C).

FFA-albumin overload of PTEC-specific *Atg5*^{-/-} mice exacerbated histologic damage (Figure 3, D and E) and

significantly increased neutrophil gelatinase-associated lipocalin expression levels and apoptosis, as determined by the cleavage of poly ADP ribose polymerase and caspase 3, (Figure 3, F–I), compared with control (*Atg5*^{+/+}) mice. These results suggest that obesity-mediated autophagy insufficiency plays a pathogenic role in PTECs exposed to proteinuria.

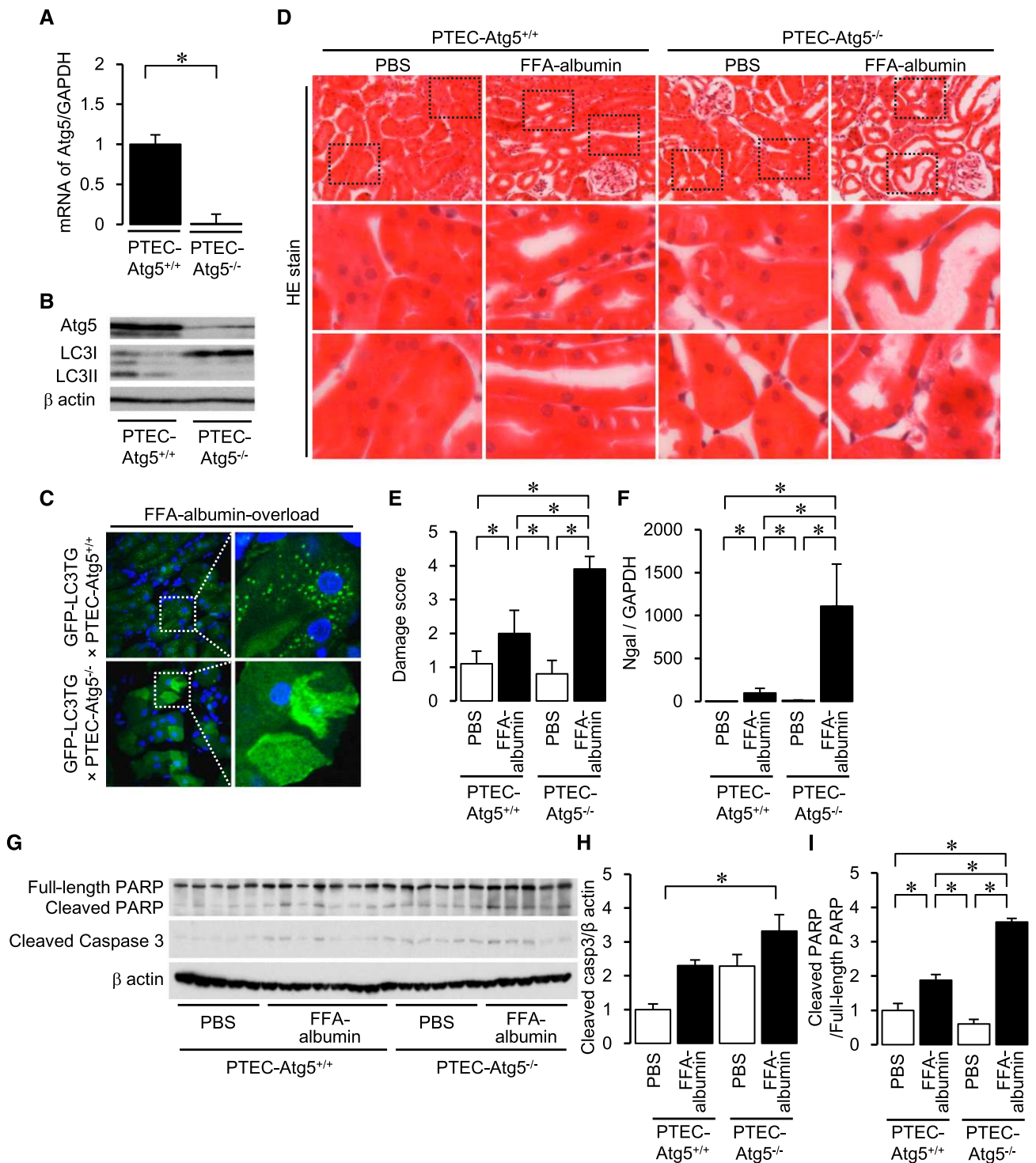


Figure 3. PTEC-specific *Atg5*-deficient mice develop more severe proteinuria-induced tubulointerstitial lesions. (A) Quantitative real-time PCR showing deletion of the *Atg5* gene in renal cortex samples. (B) Representative Western blots for *Atg5* and LC3I/LC3II proteins. An absence of LC3II protein and increased LC3I protein indicate complete loss-of-function of autophagy; β actin is used as an internal control. (C) Representative pictures showing albumin-overload-induced GFP-LC3 dots in kidney sections from GFP-LC3 transgenic mice (upper lanes) and GFP-LC3 transgenic mice crossbred with PTEC-specific *Atg5*-deficient mice (lower lanes). (D) Histologic analysis by hematoxylin and eosin (HE) staining of kidney sections from four groups of mice. (E) Renal damage scores determined by HE staining. (F) Quantitative analysis of neutrophil gelatinase-associated lipocalin (Ngal) mRNA expression. (G) Representative Western blots for poly ADP ribose polymerase (PARP) and cleaved caspase 3, with β actin as loading control. (H) Quantitative analysis of the ratio of cleaved caspase 3 to β actin. (I) Quantitative analysis of the ratio of cleaved PARP to full-length PARP. Increased cleavage of caspase 3 and PARP indicate active apoptosis. Data are mean \pm SEM. * P <0.05. Original magnification, \times 400 in C and D.

ER Stress Was Not Associated with Proteinuria-Induced Autophagy in PTECs

To assess the mechanism by which proteinuria induced, and obesity suppressed, autophagy in PTECs, we assayed the levels of expression of mRNAs encoded by genes associated with autophagy-lysosomal degradation, finding no significant differences in renal expression levels of all genes among the four groups of mice (Figure 4A). Because autophagy activity is regulated by intracellular stress- and nutrient-sensing signals,^{13,14} we examined the molecular signaling pathways that regulate proteinuria-induced autophagy in cultured mouse PTECs. Because saturated FFAs such as palmitate have cytotoxic effects on PTECs,^{24,28} we used palmitate-bound albumin (pal-albumin) to mimic and magnify the cytotoxic effects of FFA-albumin on these cells. We found that pal-albumin significantly increased LC3II formation in cultured PTECs only when pretreated with lysosomal inhibitors

(Figure 4B), and that this increase was completely inhibited by pretreatment with 3-MA, an autophagy inhibitor (Figure 4B). These results suggest that FFA-albumin has direct cytotoxic activity on cultured PTECs, along with increasing autophagic influx.

ER stress has been reported to induce stress-responsive autophagy.¹⁴ We found that pal-albumin induced ER stress in cultured PTECs, as determined by increases in phosphorylation of the PKR-like ER kinase (Figure 4C). We therefore investigated whether suppression of ER stress by supplementation with a molecular chaperone inhibited pal-albumin-induced autophagy in cultured PTECs. We found that the pal-albumin-induced increase in the LC3II/LC3I ratio remained unchanged even in cells pretreated with tauroursodeoxycholic acid (TUDCA), a molecular chaperone that was able to reduce ER stress (Figure 4, C and D). Furthermore, TUDCA failed to inhibit FFA-albumin-induced autophagosome

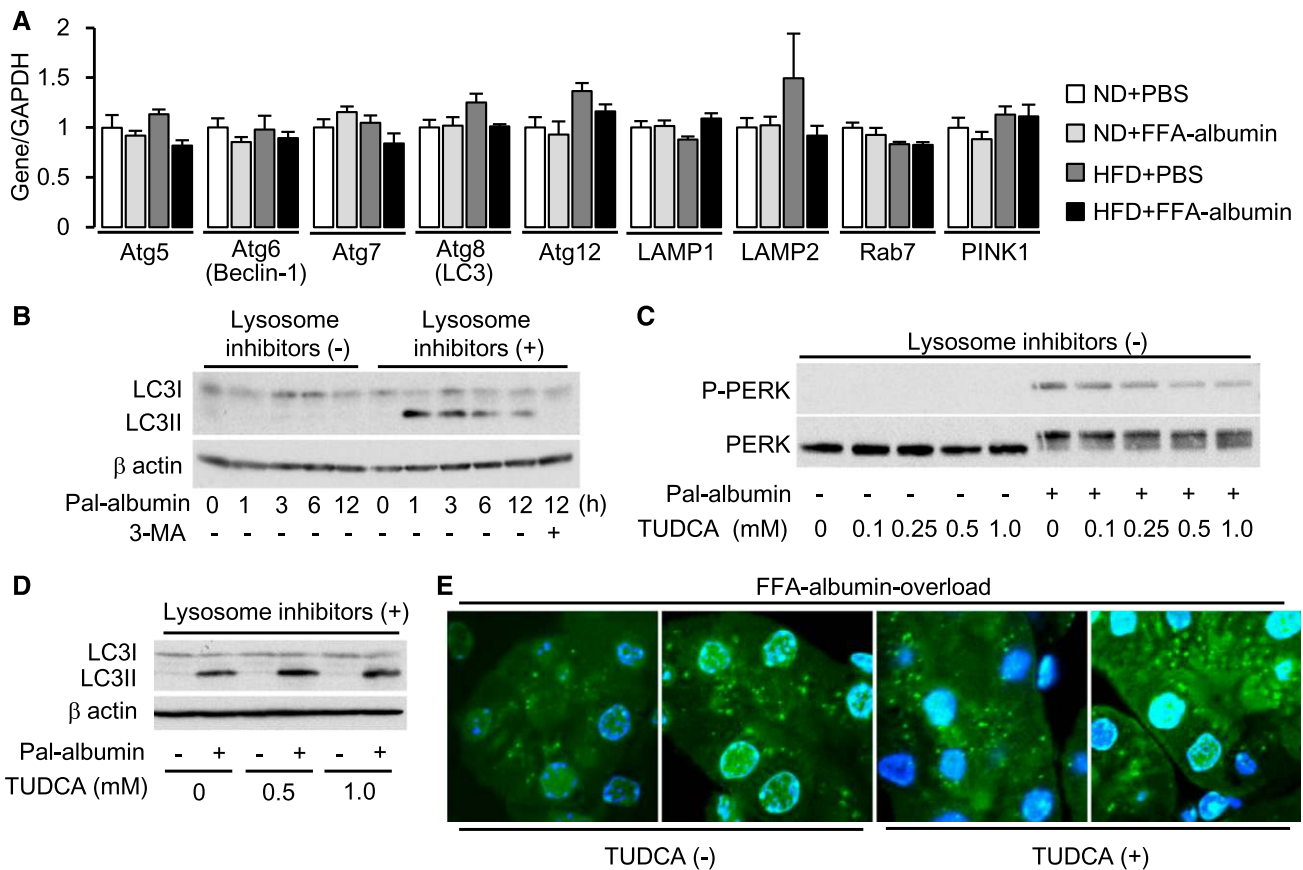


Figure 4. ER stress is not involved in the mechanism underlying proteinuria-induced autophagy in PTECs. (A) Quantitative analysis of levels of mRNA expression encoded by genes associated with autophagy-lysosomal degradation in the kidney samples from four groups of mice. (B) Representative Western blots of palmitate-albumin-induced LC3II formation, a marker of active autophagy, in cultured PTECs. LC3II is detected only in cells pretreated with the lysosome inhibitors E64d and pepstatin A. Pretreatment with 3-MA, an inhibitor of autophagy, abolishes palmitate-albumin-induced LC3II formation. (C) Representative Western blots of phosphorylated PKR-like ER kinase (P-PERK) and PERK showing the effects of TUDCA on palmitate-albumin-induced ER stress. (D) Representative Western blot showing that pretreatment with TUDCA at any concentrations do not alter palmitate-albumin-induced LC3II formation in cultured PTECs. (E) Representative blots showing that TUDCA fails to inhibit albumin-overload-induced GFP-LC3 dots in PTECs of GFP-LC3 transgenic mice fed a ND.

formation in kidneys of GFP-LC3 transgenic mice (Figure 4E). These results indicated that ER stress was not associated with proteinuria-induced autophagy activation in PTECs.

Hyperactivation of mTORC1 was Associated with Obesity-Mediated Inhibition of Autophagy in PTECs

Autophagy has been reported to be regulated by three nutrient-sensing signals,^{13,14} to be suppressed by hyperactivation of the mammalian target of rapamycin (mTORC1), and to be induced by activation of the AMP-activated protein kinase (AMPK) and silent information regulator 2 (SIRT1). In addition, mTORC1 is negatively regulated by tuberous sclerosis 1 (TSC1).²⁹ To clarify the involvement of each signal in the mechanism of FFA-albumin-induced autophagy, we transfected small interfering RNAs (siRNAs) against TSC1, AMPK α , and SIRT1 into cultured PTECs, finding that each of these siRNAs decreased the expression of its encoded protein (Figure 5A). In cultured PTECs, siRNAs against TSC1 and AMPK α , but not against SIRT1, significantly reduced the pal-albumin-induced increase in the LC3II/LC3I ratio and autophagosome formation, as determined by endogenous LC3 staining (Figure 5, A–C). Pal-albumin did not significantly affect the phosphorylation of P70S6K and S6 proteins, both downstream molecules of mTORC1, but significantly increased the phosphorylation of AMPK α in cultured PTECs (Figure 5, D–F). Taken together, these findings suggest that AMPK α activation was essential for the induction of proteinuria-induced autophagy, which was modified by mTORC1 in response to environmental factors.

Because these cell culture results, focusing on nutrient-sensing signals, suggested that both AMPK and mTORC1 are involved in FFA-albumin-induced autophagy in PTECs, we investigated the mechanisms by which proteinuria and obesity affect AMPK and mTORC1 activities in mouse kidneys. The activation of AMPK, as determined by the phosphorylation of AMPK α in kidneys, was induced equally in non-obese and obese mice by FFA-albumin overload, suggesting that stress-responsive AMPK activation was not altered by obesity (Figure 5G). In contrast, inappropriate hyperactivation of the mTORC1 signal, as determined by staining for phosphorylated S6 (pS6^{ser235/236}), was observed only in PTECs of HFD-induced obese mice (Figure 5H). Suppression of FFA-albumin-induced autophagy was observed especially in PTECs showing an active mTORC1 signal (pS6^{ser235/236}-positive). However, FFA-albumin-overload-induced GFP dot formation was observed in pS6^{ser235/236}-negative PTECs, even in the kidneys of HFD-induced obese mice (Figure 5H). Furthermore, pretreatment with rapamycin, a specific inhibitor of mTORC1, diminished mTORC1 activation and restored autophagy activity in PTECs of obese mice (Figure 5H). These results indicated that hyperactivation of the mTORC1 signal was associated with obesity-mediated suppression of autophagy in PTECs of obese mice.

Accumulation of p62 Protein and Activity of mTORC1 Were Enhanced in Kidneys of Obese Patients with Overt Proteinuria

Because p62 protein has been reported to be degraded by autophagy, intracellular accumulation of p62 represents impaired autophagy.^{30,31} We therefore measured p62 accumulation and S6^{ser235/236} phosphorylation in renal biopsy specimens from nonobese and obese patients with overt proteinuria (>1 g/d), including two nonobese patients with IgA nephropathy, one obese patient with IgA nephropathy, and an obese patient with type II diabetes (Figure 6). The PTECs from the two nonobese patients with overt proteinuria (patients 1 and 2) showed little positive staining for p62 and phosphorylated S6^{ser235/236}, whereas both were found in PTECs from the two obese patients with overt proteinuria (patients 3 and 4).

DISCUSSION

This study confirmed that more severe proteinuria-induced PTEC damage occurred in obese than in nonobese conditions. Furthermore, the renoprotective activity of proteinuria-induced autophagy in PTECs was impaired in obese mice and humans, and inappropriate hyperactivation of the mTORC1 signal was associated with obesity-mediated insufficiency of autophagy, suggesting that intrarenal nutrient-sensing signals are essential to maintain homeostasis in PTECs under stress conditions (Figure 7). These results provide valuable new insight and contribute to a better understanding of the pathogenesis of obesity-related PTEC vulnerability and the poor renal outcomes observed in obese patients with CKDs such as diabetic nephropathy.

Proteinuria-induced autophagy was especially induced in PTECs reabsorbing urinary albumin. In addition, FFA-nondepleted albumin, but not FFA-depleted albumin, induced autophagy in mouse kidneys, and palmitate-conjugated albumin induced autophagy in cultured PTECs. These results suggest that FFA-bound albumin reabsorbed in PTECs directly induces autophagy. Autophagy has cell- and tissue-protective roles in various disease conditions, including AKI models mediated by ischemic-reperfusion^{19,22} and nephrotoxic agents.^{21,32} We found that proteinuria-induced PTEC damage was more severe in PTEC-specific autophagy-deficient mice than in control mice, suggesting that autophagy also plays a renoprotective role in proteinuric kidney disease.

Although studies have shown that autophagy has renoprotective activity against various nephrotoxic stresses, few studies have assessed the conditions that impair autophagy activity in the kidney. We found that obesity significantly suppressed autophagy in PTECs of both mice and humans. During the course of evolution, organisms have developed mechanisms by which autophagy can be induced by energy depletion and various intracellular stresses to overcome stress conditions during prolonged starvation. It was therefore understandable that a hypernutrient state would suppress fasting-induced

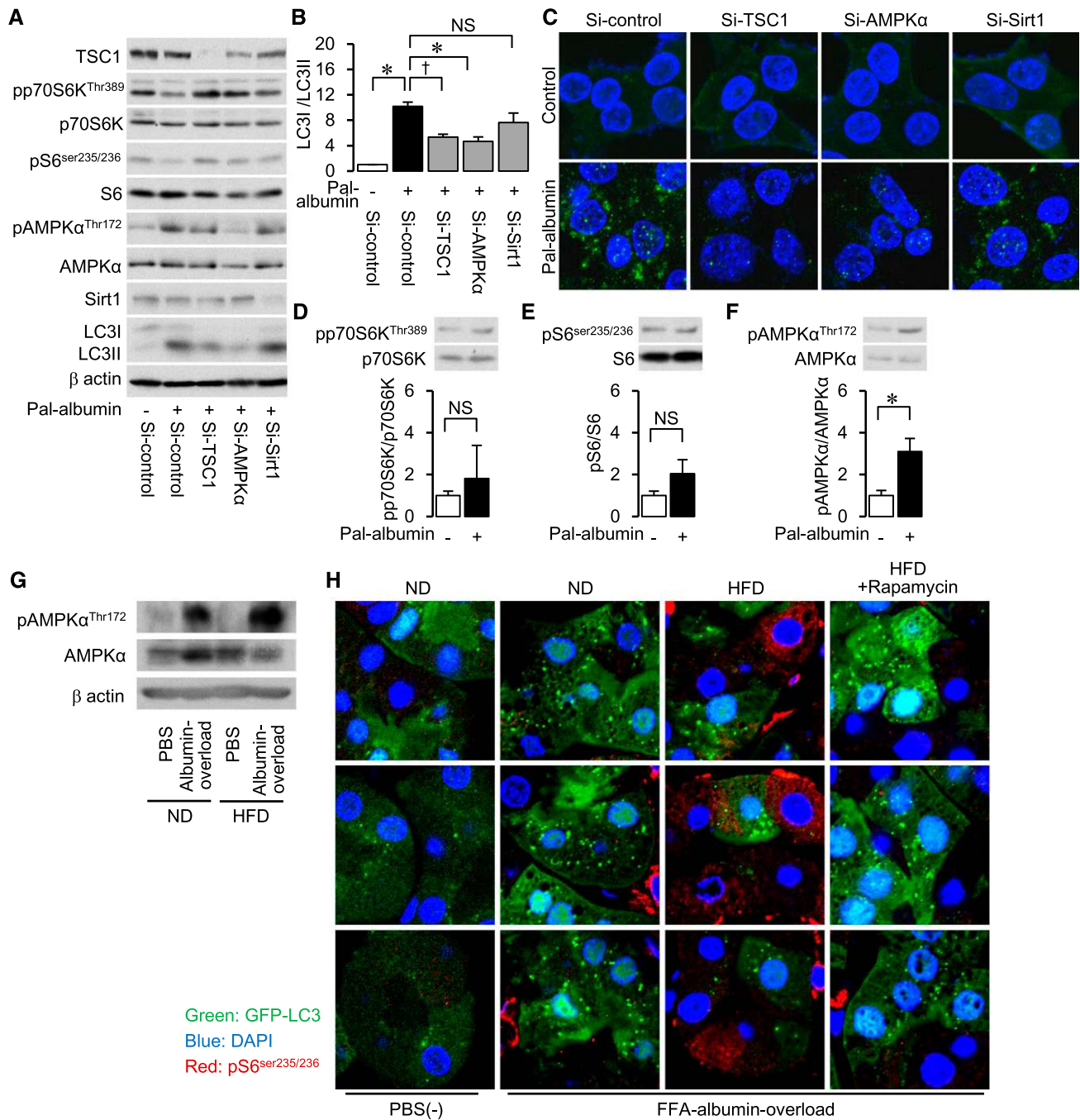


Figure 5. Nutrient-sensing signals are involved in the regulatory mechanism underlying proteinuria-induced autophagy in PTECs. (A) Representative Western blots showing the levels of TSC1, phosphorylated p70S6K, p70S6K, phosphorylated S6, S6, phosphorylated AMPK α , AMPK α , Sirt1, LC3, and β actin proteins in cultured PTECs transfected with siRNA against the TSC1, AMPK α , and Sirt1 genes. (B) LC3II/LC3I ratios in palmitate-albumin-stimulated PTECs transfected with siRNA against the TSC1, AMPK α , and Sirt1 genes. (C) Representative pictures showing LC3 staining of cultured PTECs transfected with siRNA against the TSC1, AMPK α , and Sirt1 genes. Green signals indicate staining for endogenous LC3 proteins, and blue signals indicate nuclei by 4',6-diamidino-2-phenylindole (DAPI) staining. (D–F) Representative Western blots showing the effects of palmitate-albumin on the phosphorylation of p70S6K (D), S6 (E), and AMPK α (F). (G) Representative Western blots for phosphorylated AMPK α and total AMPK α in kidney samples from mice fed a ND and injected with PBS (–), mice fed a ND with albumin overload, mice fed a HFD and injected with PBS(–), and mice fed a HFD with albumin overload. (H) Three representative pictures showing GFP-LC3 dots in PTECs of mice fed a ND and injected with PBS(–), mice fed a ND with albumin overload, mice fed a HFD with albumin overload, and albumin-overloaded HFD mice treated with rapamycin. Red signals represent phosphorylated S6 proteins, indicating hyperactivation of mTORC1; blue signals indicate nuclei stained with DAPI. Rapamycin is used to inhibit mTORC1 activity in GFP-LC3 transgenic mice fed a HFD for 4 weeks (right lanes). Data are mean \pm SEM. * $P < 0.01$; † $P < 0.01$.

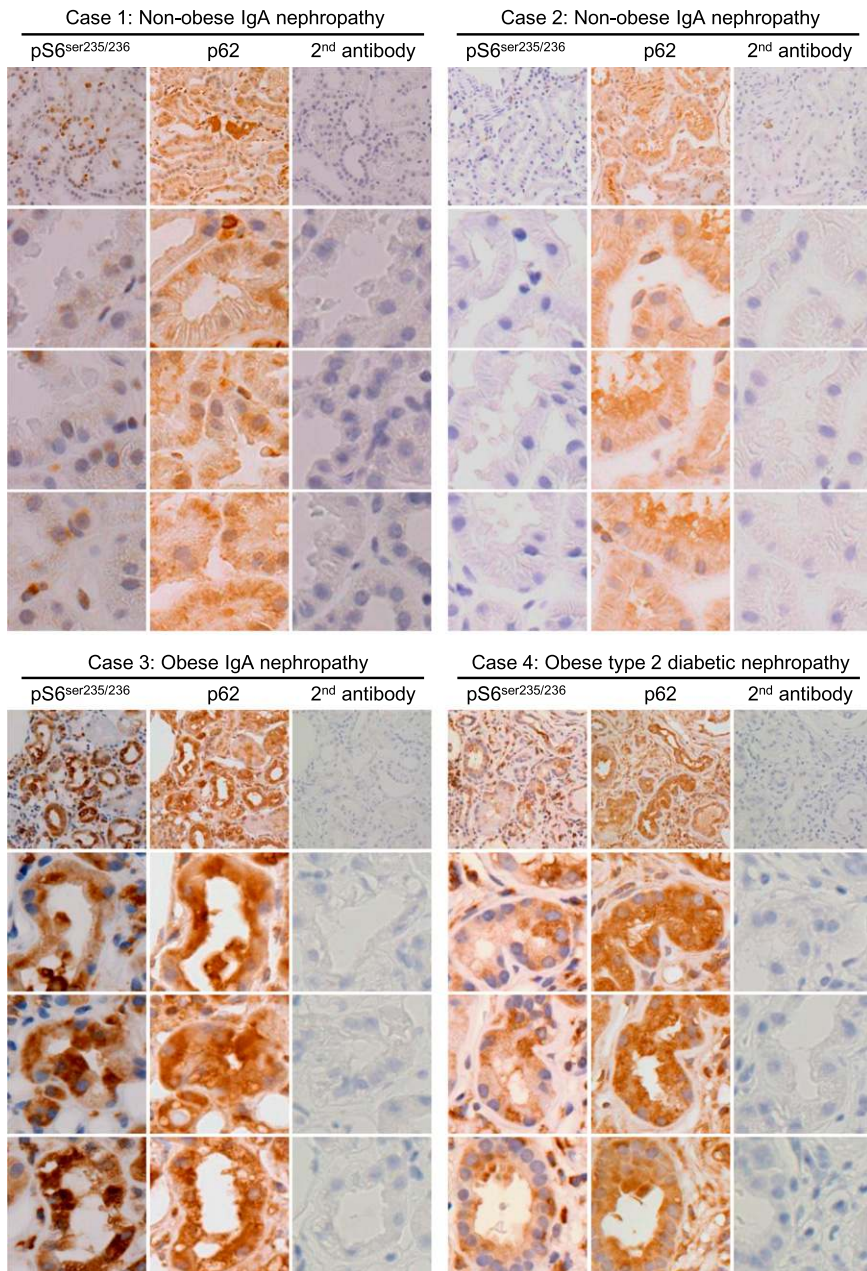


Figure 6. Immunohistochemical assessment of p62 accumulation and S6^{ser235/236} phosphorylation in kidney specimens obtained from nonobese (body mass index [BMI] <21 kg/m²) and obese (BMI >25 kg/m²) patients with overt proteinuria (>1.0 g/d). Samples are obtained from four patients: two nonobese patients with IgA nephropathy (patients 1 and 2), an obese patient with IgA nephropathy (patient 3), and an obese patient with type II diabetes with overt proteinuria (patient 4). Specific staining for p62 and phosphorylated S6^{ser235/236} in kidney sections is confirmed by the absence of nonspecific staining with secondary antibody (right lane in each panel). Original magnification, ×400 (upper lane; original, lower three lanes; details).

autophagy in the tissues of obese mice. Under these conditions, mTORC1 was also hyperactivated, suppressing stress-responsive autophagy in PTECs and suggesting that tight linkages exist between intracellular nutrient-sensing signals and stress-resistant

mechanisms. Furthermore, a recent report showed that chronic exposure to a HFD inhibited chaperone-mediated autophagy, another type of autophagy-associated intracellular degradation system.³³ The alteration of the stress-responsive intracellular degradation system by hypernutrition suggests that people with excess energy from food are at risk. This phenomenon may provide an additional and mechanistic explanation for obesity being an independent risk factor for AKI as well as glomerular diseases, because autophagy was found necessary to maintain cellular homeostasis in PTECs exposed to AKI.^{19,21,22,32}

ER stress and some nutrient-sensing signals are recognized as the main regulators of autophagy.¹⁴ We found that ER stress was not associated with proteinuria-induced autophagy, whereas AMPK may be essential, and that inappropriate hyperactivation of mTORC1 was associated with obesity-mediated suppression of autophagy in PTECs. Our results agree with previous research, which suggested that AMPK and mTORC1 have opposite effects on the induction of autophagy.^{13,14,34}

Hyperactivation of the mTORC1 signal has been reported to play a pathogenic role in podocyte damage and subsequent glomerular lesions in diabetic animal models^{35,36} and to be associated with the etiology of various metabolic diseases.³⁷ In addition, this study was the first to show an association between hyperactivation mTORC1 and the obesity-mediated vulnerability of PTECs. Collectively, amelioration of the mTORC1 signal may be an appropriate therapeutic target in modern diseases mediated by hypernutrition. However, because inhibition of the mTORC1 signal also causes podocyte dysfunction and subsequent albuminuria by disrupting autophagic flux,³⁸ agents activating autophagy, other than mTORC1 inhibitors, may provide a more effective and safer therapeutic strategy in diseases related to obesity and autophagy deficiency.

The mechanism underlying the activation of mTORC1 in PTECs of obese mice remains unclear. Because mTORC1 activity is positively regulated by growth factors including insulin,³⁹ hyperinsulinemia mediated by insulin resistance in HFD-induced obese mice may explain the obesity-mediated mTORC1 activation in these PTECs. Although the exact roles of insulin signaling,

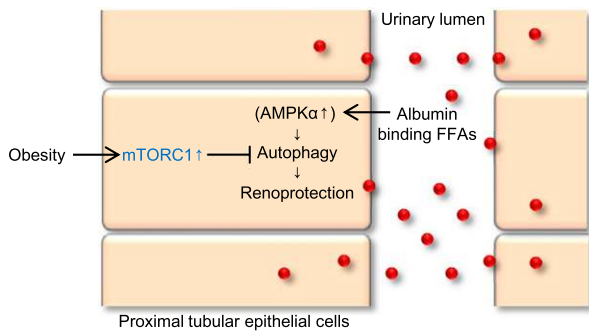


Figure 7. Proposed mechanisms of proteinuria-induced autophagy in proximal tubular cells. Proteinuria renoprotectively elicits autophagy, as well as activates AMPK α in proximal tubular cells. Obesity suppresses proteinuria-induced autophagy by hyperactivating mTORC1 in proximal tubular cells, leading to the obesity-mediated exacerbation of proteinuria-induced tubulointerstitial damage.

mTORC1, and other nutrient-sensing signals in podocytes have recently been revealed,^{35,36,38,40–43} their roles in PTECs remain obscure. Detailed determination of the roles of these nutrient-sensing pathways in PTECs should provide a better understanding of the pathogenesis of obesity-related kidney damage.

The principal therapeutic target in patients with glomerular disease is a reduction in urinary albumin excretion. This may be difficult to achieve in obese patients, making it necessary to establish new therapeutic concepts that improve renal outcomes in obese patients with persistent proteinuria. Our results clearly show that obesity is strongly associated with a worsening in proteinuria-induced PTEC damage, suggesting that protection of PTECs against proteinuria should be a therapeutic target in patients with treatment-resistant proteinuria. Moreover, we found that the obesity-related suppression of renoprotective autophagy was reversible, suggesting that restoration of autophagy may be a possible therapeutic target.²⁰ Because obesity-related exacerbation of proteinuria-induced renal damage may involve molecular mechanisms other than impaired autophagy, efforts to identify these mechanisms may contribute to improved renal outcomes and increased healthy life expectancy in obese patients with proteinuria.

In conclusion, obesity significantly impaired the renoprotective action of autophagy in PTECs, leading to more severe proteinuria-induced tubulointerstitial lesions. Our results suggest that new therapeutic agents or diet therapy restoring autophagy activity may protect PTECs against proteinuria, improving renal outcomes in obese patients with proteinuria.

CONCISE METHODS

Animal Models

All procedures were performed in accordance with the guidelines of the Research Center for Animal Life Science of Shiga University of Medical Science.

Protein-Overload Model in HFD-Induced Obese Mice

The FFA-nondepleted BSA-induced renal injury model in HFD-fed mice was established as follows. Seven-week-old male C57BL/6 mice were divided into four groups: ND + PBS, ND + FFA-nondepleted albumin, HFD + PBS, and HFD + FFA-nondepleted albumin ($n=6, 5, 6, \text{ and } 7$, respectively). Mice were fed either a ND (5% of total calories from fat) or a HFD (60% of total calories from fat), purchased from Research Diets (New Brunswick, NJ). After 4 weeks, the mice were given consecutive daily intraperitoneal bolus injections of either 0.3 g/30 g body weight FFA-nondepleted albumin (Sigma Chemical, St Louis, MO) diluted in sterile PBS or equal volumes of sterile PBS for 11 days.²⁴ After the injection on day 11, the mice were anesthetized and perfused as described. Blood and kidney samples were collected and stored at -80°C until assayed.²⁴

Monitoring Autophagosomes in Tissue Sections of GFP-LC3 Transgenic Mice

To assess the effects of fasting and proteinuria on autophagy, GFP-LC3 transgenic mice were starved or intraperitoneally injected with FFA-nondepleted albumin. After 48 hours of fasting or 12 hours after FFA-albumin overload, the mice were euthanized, frozen tissue sections were prepared, and autophagosomes were observed by confocal laser microscopy (LSM 510; Zeiss, Thornwood, NY).^{11,44} To double-stain cells with TR-conjugated albumin and GFP-LC3, FFA-albumin-overloaded GFP-LC3 transgenic mice were intravenously injected with TR-conjugated albumin (Molecular Probes, Eugene, OR) 1 hour before euthanasia.⁴⁵

To assess the effects of obesity on proteinuria-induced autophagy in PTECs, GFP-LC3 transgenic mice were fed a HFD for 4 weeks and intraperitoneally injected with FFA-nondepleted albumin 12 hours before analysis. Other mice, treated similarly, were switched to a ND for 4 weeks, to assess the reversibility of HFD-induced suppression of autophagy in PTECs. To determine the effects of TUDCA supplementation on obesity-mediated suppression of autophagy in PTECs, GFP-LC3 transgenic mice fed a HFD were given twice-daily intraperitoneal bolus injections of 250 mg/kg (500 mg/kg per day) TUDCA in sterile PBS and intraperitoneally injected with FFA-nondepleted albumin 12 hours before analysis.^{46,47} To analyze the effects of rapamycin on HFD-mediated suppression of autophagy in PTECs, GFP-LC3 transgenic mice fed a HFD for 4 weeks were given once-daily intraperitoneal injections of 0.2 mg/kg per day rapamycin for 3 consecutive days before an intraperitoneal injection of FFA-nondepleted albumin.⁴⁸

Protein-Overload Model in PTEC-Specific Autophagy-Deficient Mice

PTEC-specific *Atg5*-deficient mice (PTEC-*Atg5*^{-/-}) were generated by crossbreeding *Atg5*-floxed mice with *kidney androgen-regulated protein (KAP)-Cre* mice, in which Cre recombinase is expressed only in PTECs.^{49,50} Seven-week-old male *Atg5*-floxed mice not cross-bred with Cre mice (PTEC-*Atg5*^{+/+}) were used as a control. PTEC-*Atg5*^{+/+} and PTEC-*Atg5*^{-/-} mice were divided into four groups: PTEC-*Atg5*^{+/+} + PBS, PTEC-*Atg5*^{+/+} + FFA-albumin overload, PTEC-*Atg5*^{-/-} + PBS, and PTEC-*Atg5*^{-/-} + FFA-albumin overload ($n=5, 7, 6, \text{ and } 5$, respectively).

Blood and Urinary Analyses

Blood glucose concentrations were measured using a Glutest sensor (Sanwa Kagaku, Nagoya, Japan). Urinary albumin was measured by the Bradford method.

Intraperitoneal Glucose Tolerance Tests

Mice were fasted for 8 hours followed by intraperitoneal glucose injection (1 g/kg body weight). Blood glucose was measured using tail blood collected at 0, 15, 30, 60, 90, and 120 minutes after the injection.

Histologic Analyses

Fixed kidney specimens embedded in paraffin were sectioned at 3- μ m thickness and stained with hematoxylin and eosin to evaluate PTEC damage, which was characterized by tubular cell flattening and tubular lumen dilation, tubular cell vacuolization, and cast formation. Two blinded nephrologists experienced in renal histopathology independently quantified and rated the morphologic changes. PTEC damage was graded as follows: 1, none; 2, minor; 3, moderate; 4, severe; and 5, most severe.²⁴

Cell Culture

Immortalized murine proximal tubular cells (mProx24 cells), established as described,^{24,28} were cultured in DMEM containing 10% FBS, 100 U/ml penicillin, and 100 μ g/ml streptomycin at 37°C and 5% CO₂ thermostat. Because we previously reported that of several types of FFAs, only palmitate showed cytotoxic effects in cultured PTECs,²⁸ we assessed the effects of palmitate-bound BSA on autophagy in cultured PTECs. Subconfluent cells were starved by incubation in 0.2% FCS DMEM for 12 hours, followed by transfection of siRNA or stimulation with 150 μ M of palmitate bound to 8.0% BSA for the indicated periods (9 hours). Where indicated, cells were pretreated with 3-MA (10 mM) or TUDCA (1 mM) 3 hours before palmitate stimulation. To evaluate LC3II formation by Western blotting, cells were pretreated 1 hour before harvest with pepstatin A and E64d to inhibit the degradation of autophagosomes by lysosomes, followed by Western blotting to detect LC3II.^{11,15} Autophagy and ER stress were inhibited by 3-MA and TUDCA, respectively. We purchased 3-MA, TUDCA, pepstatin A, and E64d from Sigma Chemical.

Transfection of siRNA

Cultured PTECs were seeded in 6-well plates and incubated for 24 hours. Cells were transfected with 100 nM siRNAs against TSC1, AMPK, and SIRT1 (SMARTpool reagent; Dharmacon, Chicago, IL) or control siRNA (nontargeting siRNA; Dharmacon) using DharmaFECT 4 transfection reagent (Dharmacon) and incubated for 24 hours in medium containing 0.2% FCS-containing DMEM. After transfection, the cells were starved for 24 hours, treated with 150 μ M palmitate-conjugated albumin, and analyzed as indicated.¹¹ The siRNA sequences are described in Supplemental Table 1.

RNA Extraction and Quantitative Real-Time PCR

Total RNA was isolated from renal cortices or cultured PTECs, and cDNA was synthesized as described. iQSYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA) was used for real-time PCR (ABI Prism TM 7500 Sequence Detection System; PerkinElmer Applied

Biosystems, Foster City, CA), and the levels of expression of each mRNA were quantified using the standard curve method, as described, and normalized relative to the level of expression of glyceraldehyde 3-phosphate dehydrogenase mRNA in the same sample.²⁴ Primer sequences are described in Supplemental Table 2.

Protein Extraction and Western Blot Analyses

Renal cortices and cultured PTECs were homogenized in an ice-cold lysis buffer containing 150 mmol/L NaCl, 50 mmol/L Tris-HCl (pH 8.0), 0.1% SDS, 1% Nonidet P-40, and protease inhibitor cocktail (Boehringer Mannheim, Lewes, UK). The samples were resolved by 10% or 15% SDS-PAGE and transferred to polyvinylidene fluoride membranes (Immobilon, Bedford, MA). The membranes were incubated with the antibodies indicated below, washed, and incubated with horseradish peroxidase-coupled secondary antibodies (Amersham, Buckinghamshire, UK). Blots were visualized using an enhanced chemiluminescence detection system (PerkinElmer Life Science, Boston, MA).²⁴ Antibodies used in this study included those against cleaved caspase 3 (Asp175), poly ADP ribose polymerase, AMPK, phosphorylated AMPK^{Thr172}, P70S6K, phosphorylated P70S6K^{Thr389}, S6, phosphorylated S6^{ser235/236}, and TSC1 (all from Cell Signaling Technology, Beverly, MA); antibodies against LC3 and Atg5 (Novus Biologicals, Littleton, CO); anti- β actin (Sigma-Aldrich); anti-SIRT1 (Upstate Biotechnology, Lake Placid, NY); and anti-p62 (MBL International Corporation, Woburn, MA).

LC3 Staining in Cultured PTECs

PTECs were seeded on gelatin-coated glass covers (stored in 70% ethanol) in 12-well plates. After treatment, the cells were washed with cold PBS, fixed in 4% paraformaldehyde, treated with 0.1% Triton X in PBS(–) for 15 minutes and with 0.1% Tween-20 in 1% BSA-PBS(–) for 30 minutes, and incubated with primary antibody diluted into 1% BSA-PBS(–) for 1 hour. The cells were washed and incubated with secondary antibody diluted into 1% BSA-PBS(–) for 1 hour in the dark. After incubation with 4',6-diamidino-2-phenylindole solution for 5 minutes, the cells were washed with cold PBS(–), mounted onto slide glass with Vectashield Mounting Medium and visualized under confocal microscopy.⁵¹

Immunohistochemical Assessment of p62 Protein Accumulation and Phosphorylation of S6^{ser235/236} in Kidney Specimens Obtained from Patients with Overt Proteinuria

Human kidney biopsy specimens were collected from two nonobese patients (body mass index <21 kg/m²) with IgA nephropathy, one obese patient (body mass index >25 kg/m²) with IgA nephropathy and one obese patient with type II diabetes with overt proteinuria (1.0 g/d). All patients provided written informed consent, and the study protocol was approved by the Scientific-Ethical Committee of Shiga University of Medical Science and adhered to the Declaration of Helsinki guidelines regarding ethical principles for medical research involving human subjects. The kidney sections were subsequently stained with antibodies to p62 (MBL) and phosphorylated S6^{ser235/236} (Cell Signaling Technology).

Statistical Analyses

Results are expressed as the mean \pm SEM. ANOVA and a subsequent Scheffé's test were used to determine the significance of differences in multiple comparisons. A *P* value <0.05 was considered statistically significant.

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DISCLOSURES

None.

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