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Impaired Podocyte Autophagy Exacerbates Proteinuria in Diabetic Nephropathy

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Overcoming refractory massive proteinuria remains a clinical and research issue in diabetic nephropathy. This study was designed to investigate the pathogenesis of massive proteinuria in diabetic nephropathy, with a special focus on podocyte autophagy, a system of intracellular degradation that maintains cell and organelle homeostasis, using human tissue samples and animal models. Insufficient podocyte autophagy was observed histologically in patients and rats with diabetes and massive proteinuria accompanied by podocyte loss, but not in those with no or minimal proteinuria. Podocyte-specific autophagy-deficient mice developed podocyte loss and massive proteinuria in a high-fat diet (HFD)–induced diabetic model for inducing minimal proteinuria. Interestingly, huge damaged lysosomes were found in the podocytes of diabetic rats with massive proteinuria and HFD-fed, podocyte-specific autophagy-deficient mice. Furthermore, stimulation of cultured podocytes with sera from patients and rats with diabetes and massive proteinuria impaired autophagy, resulting in lysosome dysfunction and apoptosis. These results suggest that autophagy plays a pivotal role in maintaining lysosome homeostasis in podocytes under diabetic conditions, and that its impairment is involved in the pathogenesis of podocyte loss, leading to massive proteinuria in diabetic nephropathy. These results may contribute to the development of a new therapeutic strategy for advanced diabetic nephropathy.

Diabetic nephropathy is a leading cause of end-stage renal disease and is becoming a serious health problem worldwide. The appearance of microalbuminuria, the progression to overt proteinuria, and the resultant renal dysfunction over several years to decades is the typical progressive course of diabetic nephropathy. Recent clinical studies have shown that microalbuminuria and a part of overt proteinuria can be halted and reversed by strict control of glycemia and blood pressure (1–3). However, some patients with diabetes still develop massive proteinuria, resulting in a rapid decline of renal function (4). Thus, a better understanding of the pathogenesis of massive proteinuria in diabetic nephropathy may further improve renal outcomes in patients with diabetes.

During evolution, living organisms developed several systems to overcome starvation in times of scarcity. These systems may be associated with the pathogenesis of diabetes and its vascular complications in times of plenty. Autophagy is an evolutionarily conserved intracellular catabolic process that allows for the degradation of proteins and organelles via the lysosomal pathway (5,6). One major

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role of autophagy is to degrade proteins and reconstitute the intracellular metabolism to cope with starvation and another is to remove damaged organelles such as mitochondria, peroxisomes, and lysosomes (5–8). Autophagy is thus essential to maintain cell homeostasis under various stress conditions. Furthermore, autophagy has been shown to regulate whole-body glucose and lipid metabolism in mammals, with impaired autophagy involved in the pathogenesis of several metabolic diseases (9–12). However, the role of autophagy in the pathogenesis of diabetic nephropathy remains unclear.

Podocytes are pivotal in maintaining glomerular filtration barrier function; thus, alterations in these cells are associated with massive proteinuria (13,14). Podocytes are well-differentiated cells with no capacity to divide. The intracellular degradation system is thus believed to be important in maintaining podocyte homeostasis. Indeed, clinical and experimental evidence has shown that lysosome dysfunction leads to severe podocyte injury and massive proteinuria (15–17). Interestingly, autophagy activity in podocytes is constitutively high, even under nonstress conditions (18,19), suggesting that the autophagy-lysosome system plays a pivotal role in maintaining podocyte homeostasis and that its alteration is involved in the pathogenesis of diabetic nephropathy.

Thus, this study was designed to determine the role of autophagy in maintaining podocyte homeostasis under diabetic conditions and its involvement in the development of massive proteinuria in diabetic nephropathy.

RESEARCH DESIGN AND METHODS

Study Approvals

All procedures in the animal studies were performed in accordance with the guidelines of the Research Center for Animal Life Science of Shiga University of Medical Science. In human studies, all patients provided written informed consent. The protocols for human studies were approved by the scientific-ethical committees of Shiga University of Medical Science and Ikeda City Hospital and adhered to the Declaration of Helsinki guidelines.

Kidney Biopsy Specimens

Human kidney biopsy specimens were obtained from seven patients with type 2 diabetes with massive proteinuria (>3.5 g/day), four patients with type 2 diabetes with minimal proteinuria (<0.5 g/day), six patients with membranous nephropathy with reversible nephrotic-range proteinuria, three patients with minimal change nephrotic syndrome with reversible nephrotic-range proteinuria, and five patients with IgA nephropathy with minimal proteinuria. All patients with diabetes had diabetes for >10 years as well as retinopathy.

Diabetic Rodent Models

Eight-week-old male C57BL/6 mice were obtained from Clea Japan Inc. (Tokyo, Japan) and 8-week-old Long-Evans Tokushima Otsuka (LETO) and Otsuka Long-Evans Tokushima Fatty (OLETF) rats from Shimizu Laboratory Supplies Co., Ltd. (Kyoto, Japan). The mice were fed either a standard diet (STD; 10% of total calories from fat) or a high-fat diet (HFD; 60% of total calories from fat) for 32 weeks. LETO and OLETF rats were fed ad libitum until 50 weeks old, although some OLETF rats were killed at age 32 weeks. The rodents were fasted overnight and killed (20).

In Vivo Autophagy Analysis

GFP-LC3 transgenic mice provided by Noboru Mizushima (Tokyo University, Tokyo, Japan) were used to analyze autophagy activity (19).

HFD-Induced Diabetes in Podocyte-Specific Autophagy-Deficient Mice

Podocyte-specific *Atg5*-deficient mice (Podo-Atg5$^{-/-}$) were generated by crossbreeding Atg5$^{f/f}$ mice (21) with *Nphs2-Cre* transgenic mice (22). Eight-week-old male Atg5$^{f/f}$ mice were used as a control group. All mice were crossed on a C57BL/6 background. To assess the effects of dietary intervention, Podo-Atg5$^{f/f}$ mice were fed an STD (n = 5) or HFD (n = 6) and Podo-Atg5$^{-/-}$ mice were fed an STD (n = 7) or HFD (n = 9) for 32 weeks (23).

Blood and Urine Analysis

Blood glucose concentrations were measured using a Glutest sensor (Sanwa Kagaku, Nagoya, Japan). Plasma insulin was measured by ELISA (Morinaga, Tokyo, Japan). Urinary albumin excretion was measured by immunoblot and ELISA (Exocell, Philadelphia, PA) (20). Glucose and insulin tolerance tests were performed as previously reported (23).

Histological Analyses

Fixed kidney specimens embedded in paraffin were sectioned at 3-μm thickness. Antibodies to p62 (MBL, Tokyo, Japan), fibronectin (Chemicon, Temecula, CA), F4/80 (Serotec, Oxford, U.K.), WT1 (Santa Cruz Biotechnology, Santa Cruz, CA), podocin (Sigma-Aldrich, St. Louis, MO), synaptopodin (Progen, Heidelberg, Germany), lysosome-associated membrane protein-2 (lamp2; Abcam, Cambridge, U.K.), and ubiquitin (Cell Signaling Technology, Beverly, MA) were used. Transmission and scanning electron microscopic analyses were performed with the Hitachi S-570 and H-7500 (Hitachi, Tokyo, Japan). For semiquantitative analysis of p62 accumulation, the glomerular intensity of staining was rated as grades 1 (none), 2 (minor), 3 (moderate), 4 (severe), and 5 (most severe) (20). More than 10 glomeruli in each mouse or human samples were evaluated. To determine podocyte number, serial kidney sections were stained with WT1 antibody, and WT1-positive cells were counted, with the number calculated using the dissector/fractionator combination method (24–26). Histological analyses were performed by three independent nephrologists in a blinded manner.

Western Blot Analysis

Western blot analysis was performed as previously described (20). The membranes were incubated with antibodies against cleaved caspase 3 (Asp175), ubiquitin and Atg7 (Cell Signaling Technology), microtubule-associated protein 1 light chain 3 (LC3; Novus Biologicals, Littleton, CO), β-actin (Sigma-Aldrich), and p62 (MBL).

Cell Culture

The mouse podocyte cell line was cultured as previously described (27,28). Differentiated cells were stimulated with high glucose (500 mg/dL), fatty acids (palmitate and oleate, 150 μmol/L each), tumor necrosis factor-α (TNFα, 10 ng/μL), or serum for 24 h. Sera were collected from the indicated rodent models and patients. Complement was depleted by heat inactivation, and cells were incubated in 10% serum for 24 h (29,30), with/without lysosome inhibitors for 1 h (31). Characteristics of the sera used in this study are shown in Supplementary Tables 1 and 2.

Generation of Immortalized *Atg7*-Deficient Podocyte Cell Line

An immortalized *Atg7*-deficient podocyte cell line was generated with the pMESVTS plasmid containing a SV40 large T antigen (32). Glomeruli of podocyte-specific Atg7-deficient (33,34) and wild-type mice were isolated using Dynabeads M-450 Tosylactivated (Invitrogen, Carlsbad, CA) (35). Podocytes were infected with viral supernatant from PLAT-E cells transfected with pMESVTS plasmid (36) and were maintained in RPMI-1640 with 10% FBS at 33°C. The cells were cultured at 39°C to induce differentiation over 7 days.

Human Serum Samples

Human serum samples were taken from patients with type 2 diabetes in the Shiga Prospective Observational Follow-up Study in 2011 and 2012 (2). The preliminary study included 11 patients, 3 with normoalbuminuria (<30 mg/g Cre), 3 with microalbuminuria (30–300 mg/g Cre), 2 with macroalbuminuria (>300 mg/g Cre), and 3 with massive proteinuria (>3.5 g/g Cre). The validation study included 50 subjects, 10 subjects without diabetes, and 40 patients with type 2 diabetes, including 10 with normoalbuminuria, 11 with microalbuminuria, 10 with macroalbuminuria, and 9 with massive proteinuria (Supplementary Table 2). ELISA was used to measure the concentrations of p62 (Enzo Life Science, Farmingdale, NY).

Statistical Analyses

Results are expressed as the mean ± SEM. ANOVA and a subsequent Tukey test were used to determine the significance of differences in multiple comparisons. Student *t* test was used for comparisons of two groups. A P value <0.05 was considered statistically significant.

RESULTS

Insufficient Autophagy in Podocytes of Patients With Diabetes With Massive Proteinuria

To examine the relationships among levels of proteinuria, podocyte damage, and autophagy, kidney biopsy samples taken from patients with diabetic nephropathy and refractory massive proteinuria (>3.5 g/day) (Fig. 1*A*, patients 1–7) or minimal proteinuria (<0.5 g/day) (Fig. 1*C*, patients 17–20), membranous nephropathy and minima change with reversible massive proteinuria (Fig. 1*B*, patients 8–16), and IgA nephropathy with minimal proteinuria (Fig. 1*C*, patients 21–25) were analyzed. Mesangial expansion in periodic acid Schiff (PAS) staining was observed in all patients with diabetic nephropathy, regardless of proteinuria level (Fig. 1*A*, patients 1–7, and Fig. 1*C*, patients 17–20). To examine podocyte injury, we conducted an immunofluorescent study for podocin protein that is a key protein of the slit diaphragm of podocytes (14). The podocin expression pattern was nearly normal in the patients with minimal proteinuria, whether or not they had diabetic nephropathy (Fig. 1*C*, patients 17–25). In contrast, the podocin expression pattern was granular and irregularly scattered in the patients with massive proteinuria regardless of the underlying disease (Fig. 1*A* and *B*, patients 1–16). In addition, decreases in podocin-positive areas were obviously observed in the patients with refractory massive proteinuria due to diabetes (Fig. 1*A*, patients 1–7).

The protein p62 is a specific target of the autophagy degradation; thus, intracellular accumulation of this protein is indicative of insufficient autophagy (37). Intense accumulation of p62 protein was significantly increased in the glomeruli of the patients with diabetes and massive proteinuria (Fig. 1*A*–*D*).

Insufficient Autophagy and Podocyte Injury in Diabetic Rodents with Massive Proteinuria

We further confirmed the relationship among disease stage of diabetic nephropathy, podocyte injury, and autophagy insufficiency by utilizing two rodent models of diabetic nephropathy. One model, in mice, involved HFD-induced renal injury, resulting in diabetes-associated minimal proteinuria (23,38). The second model involved OLETF rats, which spontaneously develop hyperglycemia and subsequent massive proteinuria with age (39). Western blot analysis of urine samples and PAS staining showed minimal proteinuria with glomerular hypertrophy in HFD-fed C57BL/6 mice, and age-dependent progression of proteinuria with glomerular sclerosis in OLETF rats (Fig. 2*A*–*C*). Podocytes in 50-week-old OLETF rats with massive proteinuria showed a reduction in podocin-positive areas, with severe alterations in foot processes and p62 accumulation (Fig. 2*C* and *E*). However, these alterations were not found in the other rodent models (Fig. 2*C*–*E*).

Exacerbation of HFD-Induced Albuminuria in Podocyte-Specific Autophagy-Deficient Mice

These histological results showed that insufficient podocyte autophagy was associated with severe podocyte injury and massive proteinuria in diabetes. To assess the causal association, we used podocyte-specific autophagy-deficient mice.

The protein coded by the *Atg5* gene is essential for autophagosome formation (21). Thus, podocyte-specific autophagy-deficient mice were generated by crossbreeding $Atg5^{f/f}$ mice (21) with *Nphs2*-Cre transgenic mice (22). LC3, a regulatory protein essential for the induction of autophagy, localizes to autophagosome membranes during activation of autophagy. Thus, autophagy activity in the cells of GFP-LC3 transgenic mice can be monitored as

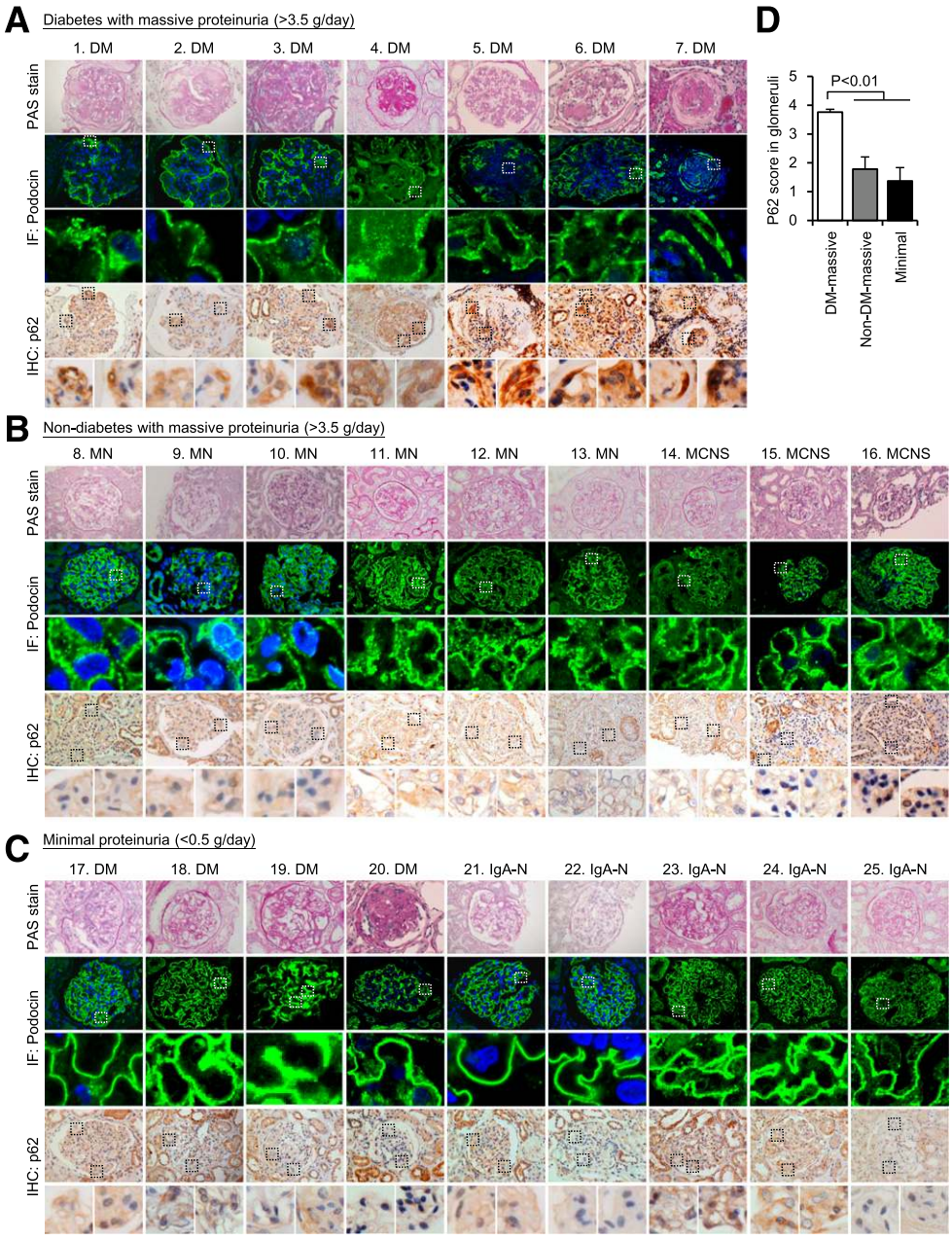

Figure 1—Autophagy insufficiency in podocytes of patients with diabetes and massive proteinuria. *A–C*: Representative pictures of PAS staining, immunofluorescent (IF) assays of podocin protein, and immunohistochemistry (IHC) for p62, a marker of autophagy insufficiency, in kidney biopsy specimens from patients with diabetic nephropathy (DM) and massive proteinuria (patients 1–7) or minimal proteinuria (patients 17–20), membranous nephropathy (MN) with reversible massive proteinuria (patients 8–13), minimal change nephrotic syndrome (MCNS) (patients 14–16), and IgA nephropathy (IgA-N) with minimal proteinuria (patients 21–25). Massive proteinuria was defined as >3.5 g/day and minimal proteinuria as <0.5 g/day. Original magnifications: ×400 for PAS stain and IHC of p62 and ×600 for IF of podocin. *D*: Semiquantitative measurement of p62 intensity in the glomeruli in each group. All results are presented as mean ± SEM and compared by ANOVA and a subsequent Tukey test, with $P < 0.05$ considered statistically significant. The white and black boxes indicate the areas for the magnified pictures.

green dots (19). Impaired formation of GFP-LC3 dots was confirmed in the podocytes of podocyte-specific Atg5-knockout mice (podo-Atg5$^{-/-}$) crossbred with GFP-LC3 transgenic mice (Fig. 3*A*).

To assess the effects of podocyte autophagy deficiency on HFD-induced minimal proteinuria, control Atg5$^{f/f}$ and podo-Atg5$^{-/-}$ mice were fed an STD or HFD for 32 weeks. During 32 weeks, HFD-fed Atg5$^{f/f}$ and podo-Atg5$^{-/-}$ mice showed a similar development of obesity, hyperinsulinemic hyperglycemia, glucose intolerance, and insulin resistance (Fig. 3*B–H*). HFD-fed Atg5$^{f/f}$ mice developed minimal albuminuria, and STD-fed podo-Atg5$^{-/-}$ mice did not show increased urinary albumin excretion (Fig. 3*I* and *J*). However, HFD-fed podo-Atg5$^{-/-}$ mice developed massive albuminuria (Fig. 3*I* and *J*).

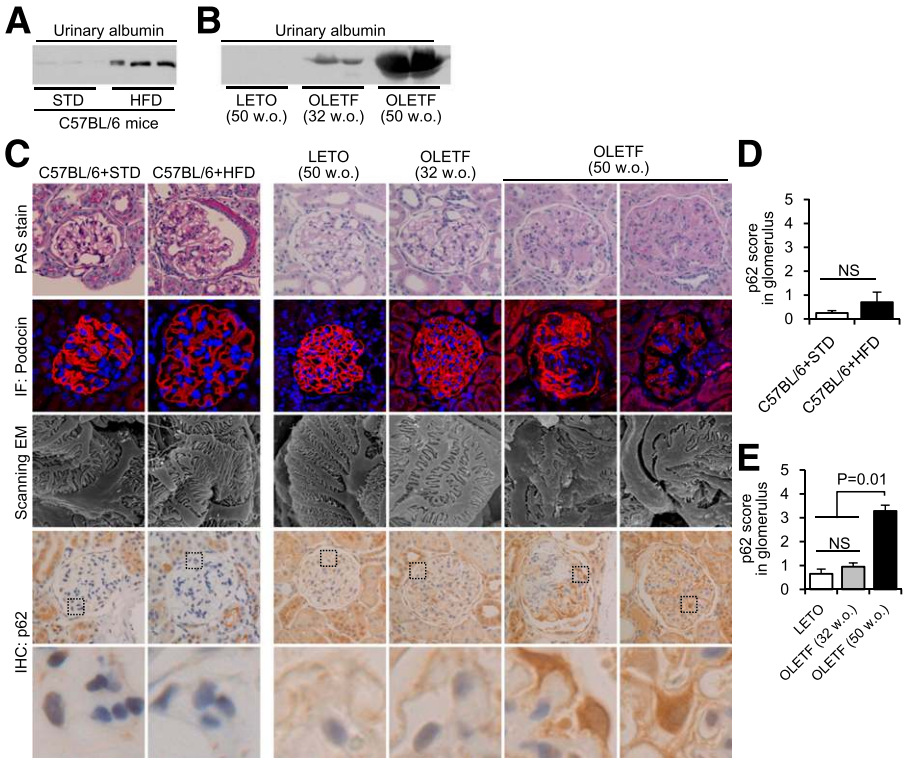

Figure 2—Autophagy insufficiency in podocytes of rodent diabetic models with massive proteinuria. Western blot analysis of urinary albumin in C57BL/6 mice fed an STD or HFD (*A*), and nondiabetic control LETO and diabetic OLETF rats at the indicated ages (weeks old [w.o.]) (*B*). *C*: Representative pictures of PAS, immunofluorescent (IF) assays of podocin, scanning electron microscopy (EM), and immunohistochemistry (IHC) for p62 in kidneys from C57BL/6 mice fed an STD or HFD, and LETO and OLETF rats at the indicated ages. Original magnifications: ×400 for PAS and IHC of p62, ×1,000 (mouse study) and ×600 (rat study) for IF of podocin, and ×8,000 for scanning EM. The black boxes indicate the areas for the magnified pictures. *D* and *E*: Semiquantitative measurement of p62 intensity in glomeruli in each rodent model. All results are presented as mean ± SEM and compared by ANOVA and a subsequent Tukey test, with $P < 0.05$ considered statistically significant. NS, no significance.

Exacerbation of Proteinuria-Related Tubulointerstitial Lesions in HFD-Fed Podocyte-Specific Autophagy-Deficient Mice

Histological analysis of the glomeruli of Atg5$^{f/f}$ mice showed that the HFD increased glomerular size, PAS-positive area, and fibronectin deposition in glomeruli (Fig. 4*A* and *B*). These HFD-induced glomerular alterations tended to be greater in podo-Atg5$^{-/-}$ than in Atg5$^{f/f}$ mice, but the differences were not statistically significant (Fig. 4*A* and *B*). In contrast, renal tubulointerstitial lesions, as determined by hematoxylin-eosin (H-E) staining and immunohistochemical analysis of F4/80-positive macrophages, were exacerbated in HFD-fed podo-Atg5$^{-/-}$ mice (Fig. 4*C* and *D*), suggesting that podocyte-specific autophagy insufficiency under diabetic conditions resulted in massive proteinuria, accompanied by proteinuria-induced tubulointerstitial damage.

There was no evidence of p62 accumulation in the podocytes of HFD-fed Atg5$^{f/f}$ mice, and GFP-LC3 dot formation was not altered in the podocytes of HFD-fed GFP-LC3 mice (Fig. 4*E*–*H*), suggesting that HFD-induced diabetes alone did not affect podocyte autophagy and that autophagy was not related to the onset of HFD-induced minimal proteinuria. In contrast, p62 accumulation was significantly higher in the podocytes of HFD-fed podo-Atg5$^{-/-}$ mice with massive proteinuria (Fig. 4*E* and *F*). These results suggested that insufficient autophagy played a causal role in the progression of proteinuria, from minimal to massive levels, under diabetic conditions.

Podocyte Damage in HFD-Fed Podocyte-Specific Autophagy-Deficient Mice

Transmission electron microscopy showed that both Atg5$^{f/f}$ and podo-Atg5$^{-/-}$ mice fed the HFD for 32 weeks resulted in thickening of the glomerular basement membrane (Fig. 5*A*). STD-fed podo-Atg5$^{-/-}$ mice showed nearly normal podocyte morphology, whereas HFD-fed podo-Atg5$^{-/-}$ mice developed severe foot process effacement (Fig. 5*A*). Furthermore, in scanning electron microscopy, normal foot process structure was drastically disrupted in the podocytes of HFD-fed podo-Atg5$^{-/-}$ mice (Fig. 5*B*).

The podocin expression pattern was normally and linearly aligned in STD- and HFD-fed Atg5$^{f/f}$ mice and in STD-fed podo-Atg5$^{-/-}$ mice (Fig. 5*C*). However, the pattern was visible as dots in HFD-fed podo-Atg5$^{-/-}$ mice (Fig. 5*C*), similar to that observed in patients with diabetes and massive proteinuria. Podocin internalization is an additional marker of alterations in podocyte foot processes (40). Most podocin (red) and synaptopodin

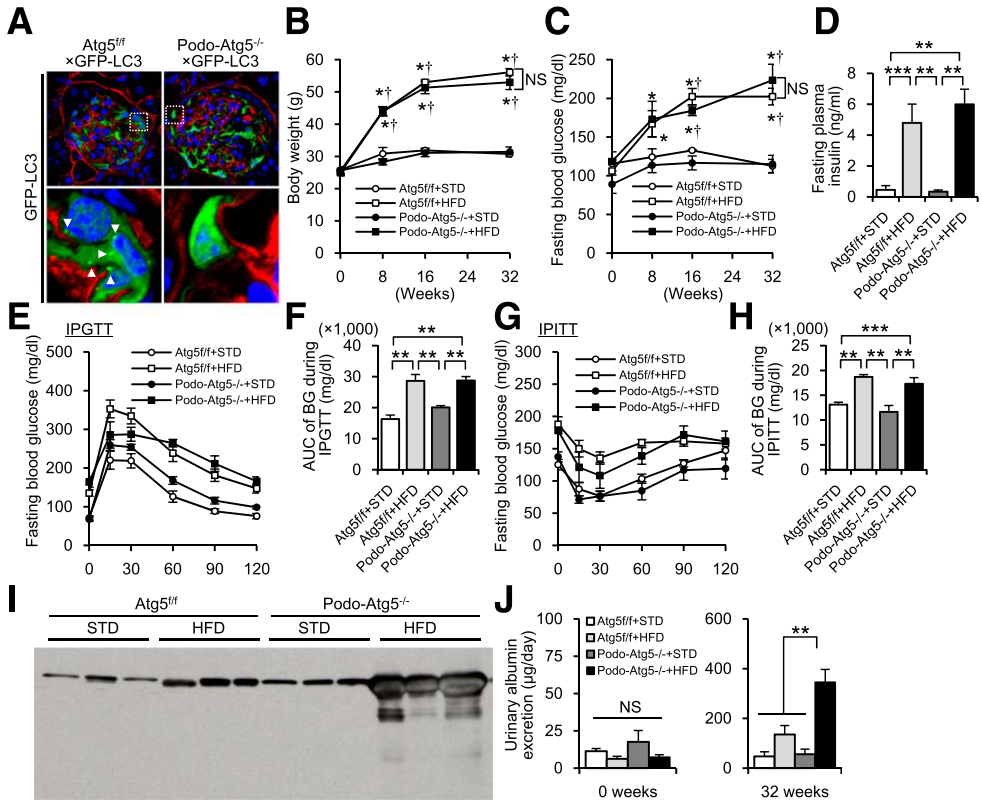

Figure 3—Exacerbation of HFD-induced proteinuria in podocyte-specific autophagy-deficient mice. *A*: GFP-LC3–positive dot analysis in podocytes of GFP-LC3 transgenic mice crossbred with either Atg5$^{f/f}$ or podocyte-specific Atg5 knockout (podo-Atg5$^{-/-}$) mice fed ad libitum. Nidogen staining for visualization of basement membrane. White arrowheads indicate GFP-positive autophagosome dots. Original magnification ×1,000. The white boxes indicate the areas for the magnified pictures. *B* and *C*: Sequential changes in body weight and fasting blood glucose concentrations in the four groups of mice over the 32-week feeding period. *D*: Fasting insulin concentrations in the four groups of mice at the end of the experimental period. *E* and *F*: Glucose change and area under the curve (AUC) of glucose concentrations during intraperitoneal glucose tolerance tests (IPGTTs). *G* and *H*: Glucose change and AUC of glucose concentrations during intraperitoneal insulin tolerance tests (IPITTs). *I*: Western blot analysis of albumin in urine samples from the four groups of mice at the end of the experimental period. *J*: Urinary albumin excretion levels by ELISA at 0 and 32 weeks. All values are shown as mean ± SEM, with levels of significance determined by ANOVA and a subsequent Tukey test. *P < 0.05 vs. Atg5$^{f/f}$ mice fed STD; †P < 0.01 vs. Atg5$^{-/-}$ mice fed STD; **P < 0.01 and ***P < 0.05 vs. the indicated groups. BG, blood glucose; NS, no significance.

(green) signals were visible as normal capillary pattern in the undamaged podocytes of STD- and HFD-fed Atg5$^{f/f}$ mice and STD-fed podo-Atg5$^{-/-}$ mice (Fig. 5*D*). In contrast, increased podocin internalization, as shown by the increased podocin expression in cytosol and the merged yellow signals, was observed in the damaged podocytes of HFD-fed podo-Atg5$^{-/-}$ mice (Fig. 5*D*). In addition, the number of WT1-positive podocytes was significantly reduced in HFD-fed podo-Atg5$^{-/-}$ mice (Fig. 5*E* and *F*). These results suggested that autophagy insufficiency combined with diabetic conditions caused podocyte damage.

Insufficient Autophagy-Related Apoptosis in Cultured Podocytes Stimulated With Serum From Diabetic Rodents

High glucose, fatty acids, and TNFα, an inflammatory cytokine, are major pathogenic factors in diabetic nephropathy. However, each of these, as well as their combination, had no effect on autophagy activity, as determined by p62 accumulation and LC3II conversion in cultured podocytes (Fig. 6*A*–*C*), suggesting that other factors may alter autophagy in podocytes. Several reports showed that some serum factors altered intracellular signaling pathways and autophagy activity in some conditions (29,30), raising a possibility that some serum factors associated with diabetic massive proteinuria might inhibit autophagy in podocytes.

To determine this possibility, mouse podocytes were cultured with serum from STD- or HFD-fed C57BL/6 mice or 50-week-old LETO or OLETF rats, and LC3 dot formation was assessed by immunofluorescence (Fig. 6*D*, protocol 1). LC3 dot formation was lower in cultured podocytes stimulated with serum from 50-week-old OLETF rats (Fig. 6*E*). Insufficient autophagy in cultured podocytes stimulated with serum from 50-week-old OLETF rats (Fig. 6*D*, protocol 2) was also confirmed by an increase in p62 expression levels and a decrease in LC3II bands (Fig. 6*F* and *G*). Furthermore, treatment with OLETF rat serum alone significantly enhanced apoptosis in normal podocytes, as shown by the cleavage of

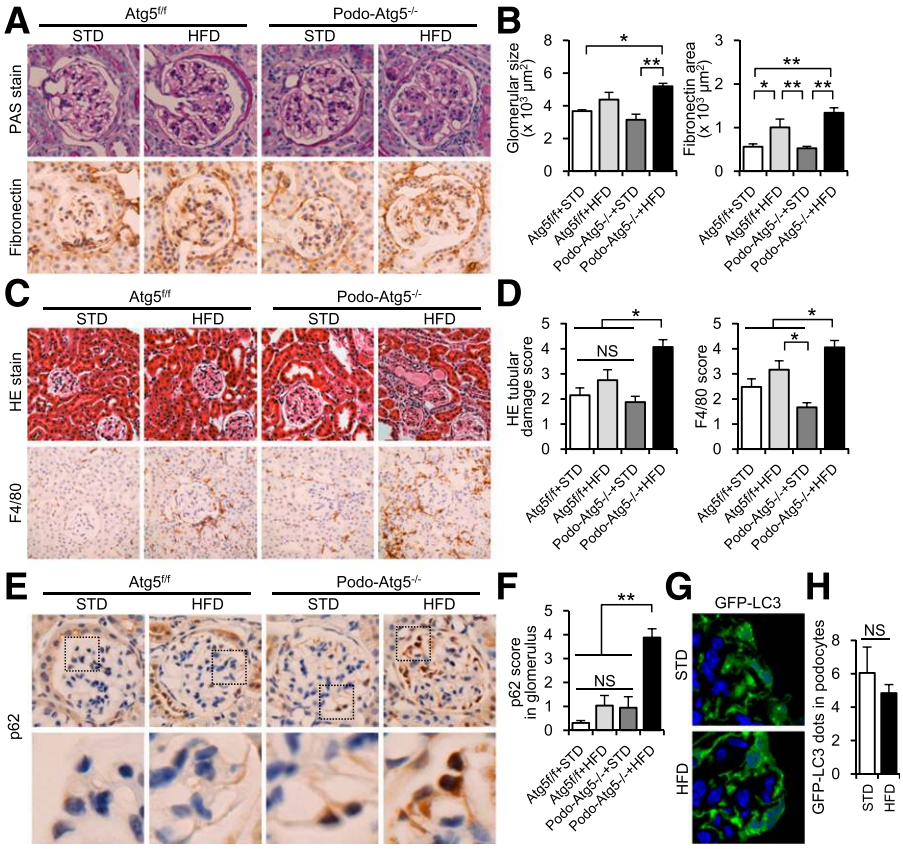

Figure 4—Exacerbation of proteinuria-related tubulointerstitial lesions in HFD-fed, podocyte-specific autophagy-deficient mice. *A*: Representative pictures of PAS staining and immunohistochemical expression of fibronectin in glomeruli of four groups of mice. Original magnification ×400. *B*: Quantitative analysis of glomerular size and fibronectin-positive areas in glomeruli. *C*: Quantitative evaluation of tubulointerstitial lesions by H-E staining and immunohistochemical analysis of F4/80. Original magnification ×200. *D*: Tubular damage scores by H-E staining and F4/80-positive scores. *E*: Representative pictures of immunohistochemical assays for p62. Original magnification ×400. *F*: Quantitation of the numbers of p62-positive cells in the glomeruli of the four groups of mice. The black boxes indicate the areas for the magnified pictures. *G* and *H*: GFP-LC3 dot analysis of GFP-LC3 transgenic mice fed an STD or HFD for 32 weeks. Representative pictures of the glomeruli from GFP-LC3 transgenic mice fed the indicated diet (*G*) and quantitation of numbers of GFP dots in the glomeruli (*H*) (n = 4). Original magnification ×1,000. All values are presented as mean ± SEM, with statistical significance determined by ANOVA and subsequent Tukey tests for multiple comparisons and by Student t tests for pairwise comparisons. $^*P < 0.05$ and $^{**}P < 0.01$ vs. the indicated groups. NS, no significance.

caspase 3 (Fig. 6*F* and *G*). These results suggested that some serum factors of 50-week-old OLETF rats inhibited autophagy and caused apoptosis in podocytes.

We excluded a possibility that serum alterations related to massive proteinuria secondarily caused autophagy insufficiency in podocytes. Stimulation with the serum from podo-Atg5$^{-/-}$ mice fed an HFD had no effect on both p62 accumulation and apoptosis in cultured podocytes (Fig. 6*D*, protocol 3, and Fig. 6*H*), even though they showed massive proteinuria, indicating that massive proteinuria–related serum alteration itself was not a cause of autophagy insufficiency.

To further examine a causal relationship between autophagy insufficiency and apoptosis associated with diabetic serum, we used cultured Atg7-deficient podocytes. The protein coded by the *Atg7* gene is also essential for autophagosome formation (33). Atg7 protein expression was not observed in cultured Atg7-deficient podocytes, and autophagy deficiency was confirmed by a significant increase in p62 protein and a decrease in LC3II bands (Fig. 6*I*). These cells were subsequently incubated with serum from STD- or HFD-fed C57BL/6 mice (Fig. 6*D*, protocol 4). Serum from HFD-fed C57BL/6 mice had no effect on the control Atg7$^{f/f}$ podocytes, whereas it significantly increased the cleavage of caspase 3 in the Atg7-deficient podocytes (Fig. 6*I* and *J*). The study provided further evidence that insufficient autophagy played a causal role in podocyte apoptosis under diabetic conditions.

Accumulation of Damaged Lysosomes in the Podocytes of Diabetic Rodents With Massive Proteinuria

To determine the intracellular component targeted by autophagy in podocytes under diabetic conditions, intracellular structural changes in the podocytes were analyzed. The podocytes of Atg5$^{f/f}$ mice had a normal mitochondrial structure with normal autophagosome formation, regardless of diet (Fig. 7*A*). In the podocytes of STD-fed podo-Atg5$^{-/-}$

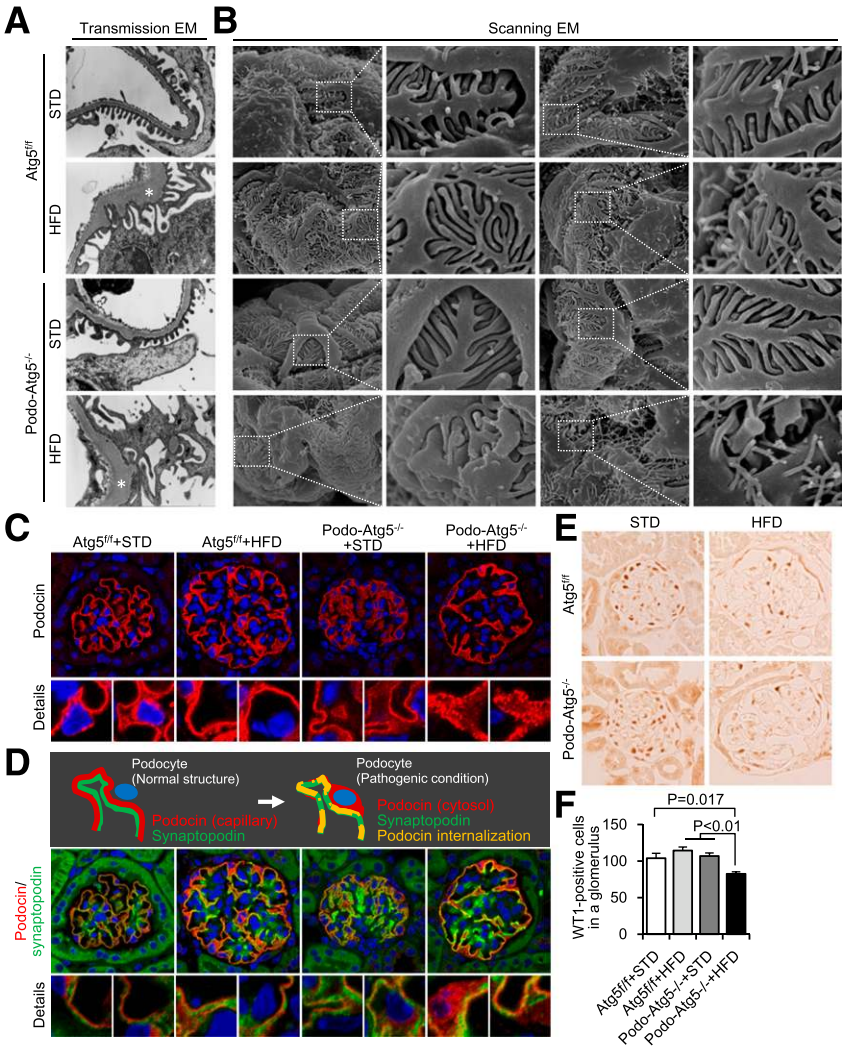

Figure 5—Podocyte dysfunction and loss in HFD-fed, podocyte-specific autophagy-deficient mice. Representative transmission (*A*) and scanning (*B*) electron microscopy (EM) of glomeruli from $Atg5^{f/f}$ and $Atg5^{-/-}$ mice fed an STD or HFD for 32 weeks. The asterisks indicate thickening of the glomerular basement membrane. Original magnifications: ×10,000 for transmission electron microscopy and ×8,000 for scanning electron microscopy. The white boxes indicate the areas for the magnified pictures. *C*: Immunofluorescent determination of podocin expression in glomeruli from the four groups of mice. Original magnification ×1,000. *D*: Double immunofluorescent determination of podocin and synaptopodin in glomeruli from the four groups of mice. Original magnification ×1,000. The red- and green-colored lines represent podocin and synaptopodin, respectively. Under normal conditions, both proteins are visible as a capillary pattern. Once podocyte damage occurs, podocin is internalized, making yellow lines and red-colored dots in cytosol. *E*: Representative pictures of WT1 stain in glomeruli of the four groups of mice. Original magnification ×400. *F*: Quantitation of WT1-positive podocytes in glomeruli. All values are presented as mean ± SEM. Differences were determined by ANOVA and a subsequent Tukey test.

mice, autophagosomes were absent and unidentifiable deposits were observed but mitochondrial structure and nuclear shape were normal (Fig. 7*A*). Interestingly, the cytosol in podocytes of HFD-fed podo-$Atg5^{-/-}$ mice was occupied by a number of huge balloon-like structures, suggesting damaged lysosomes (Fig. 7*A*).

The autophagy system is involved in removing damaged lysosomes and maintaining lysosome homeostasis (8,41), raising a hypothesis that lysosomes damaged by diabetic metabolic loads were targets of autophagy, and that impairment of autophagy by diabetic conditions would result in the accumulation of damaged lysosomes (Fig. 7*B*). Actually, the levels of expression of lamp2, a membrane marker of lysosomes (42), and ubiquitinated proteins to be degraded by lysosomes were increased in the podocytes of HFD-fed podo-$Atg5^{-/-}$ mice (Fig. 7*C*), suggesting that dysfunctional lysosomes accumulated in these podocytes. The accumulation of huge lysosomes with the increase in lamp2-positive areas and the absence of autophagosomes were observed in the podocytes of 50-week-old OLETF rats with massive proteinuria (Fig. 7*D*).

In addition, ubiquitinated proteins were found to accumulate in Atg7-deficient podocytes stimulated with the HFD mouse serum and normal podocytes stimulated with the 50-week-old OLETF rat serum (Fig. 7*E*). Furthermore, a double immunofluorescence assay for LC3 and

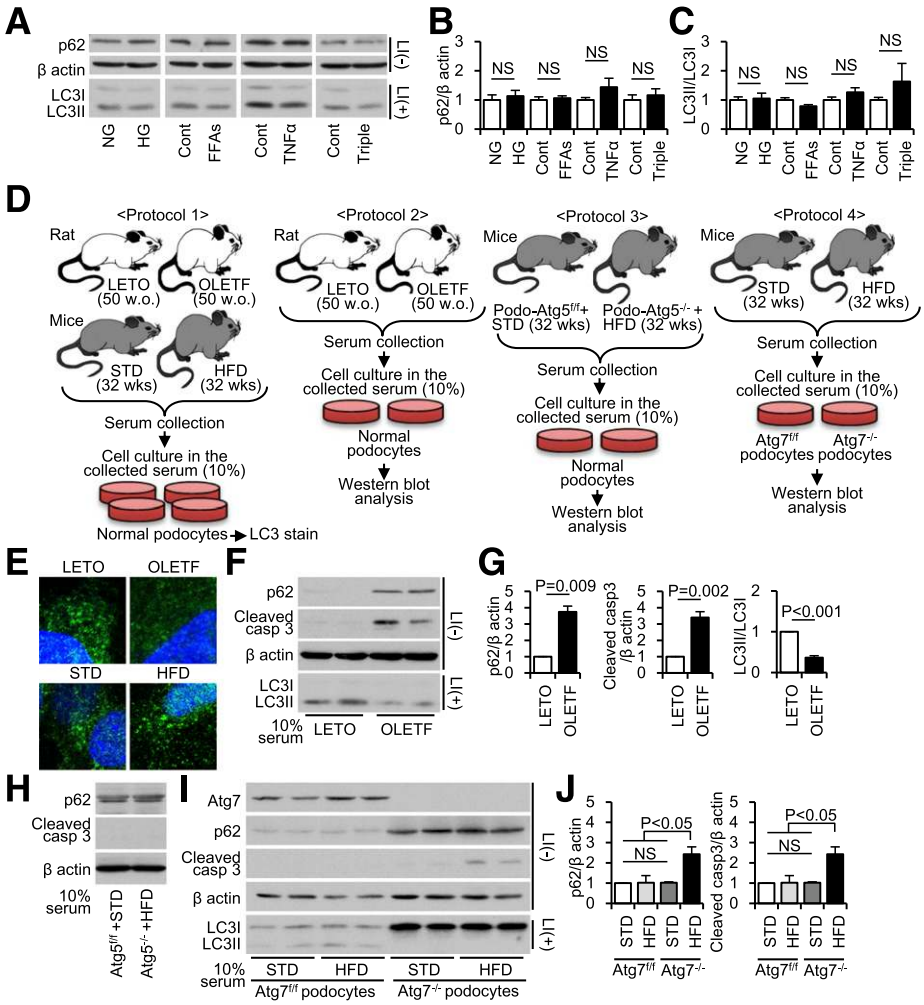

Figure 6—Autophagy insufficiency and apoptosis in cultured podocytes stimulated with serum from diabetic rodent models. *A*: Representative Western blots for p62, β-actin, and LC3 in cultured normal podocytes incubated with high glucose (500 mg/dL), fatty acids (palmitate and oleate, 150 μmol/L each), TNFα, or all three reagents (triple). Quantitative ratios of p62 to β-actin (*B*) and of LC3II to LC3I (*C*) (n = 3). *D*: Study protocols 1–4 for cell culture with sera from the indicated nondiabetic and diabetic rodents. *E*: Representative pictures of LC3 immunofluorescence in cultured normal podocytes incubated with 10% serum from the rodents indicated in protocol 1. *F*: Representative Western blots for p62, cleaved caspase 3, β-actin, and LC3 in cultured normal podocytes incubated with 10% serum of the rats indicated in protocol 2. *G*: Quantitative ratios of p62 and cleaved caspase 3 to β-actin, and ratio of LC3II to LC3I (n = 3). *H*: Representative Western blots for p62 and cleaved caspase 3 in cultured normal podocytes incubated with 10% serum of Atg5$^{f/f}$ mice fed an STD or Atg5-deficient mice fed an HFD indicated in protocol 3. *I*: Representative Western blots of Atg7, p62, cleaved caspase 3, β-actin, and LC3 in cultured Atg7$^{f/f}$ and Atg7-deficient podocytes incubated with 10% sera from the mice indicated in protocol 4. *J*: Quantitative ratios of p62 and cleaved caspase 3 to β-actin (n = 3). All values are presented as mean ± SEM. ANOVA and a subsequent Tukey test were used to determine significance in multiple comparisons, whereas Student t test was used for pairwise comparisons. $P < 0.05$ was considered statistically significant. Cont, control; FFA, free fatty acid; HG, high glucose; NG, normal glucose; NS, no significance; w.o., weeks old.

lamp2 revealed a number of large lamp2-positive signals in these cells, with absent or impaired LC3 dot formation (Fig. 7*F* and *G*). These results confirmed our hypothesis that systemic diabetic changes injure lysosomes, and that diabetic conditions accompanied by massive proteinuria additionally impair autophagy, leading to the accumulation of damaged lysosomes.

Insufficient Autophagy in Cultured Podocytes Stimulated With Serum From Patients With Diabetes With Massive Proteinuria

Finally, to determine the disease stage of human diabetic nephropathy associated with the impaired autophagy-lysosome system, the levels of p62 and ubiquitinated proteins were assessed in cultured podocytes stimulated with serum from 11 patients with diabetes with a varying range of proteinuria. Stimulation of cultured podocytes with serum from patients with diabetes and massive proteinuria resulted in increases in p62 and ubiquitinated protein (Fig. 8*A*).

To validate the relationship between proteinuria progression and autophagy-lysosome dysfunction in 10 subjects without diabetes and 40 patients with diabetes with varying degrees of proteinuria (Supplementary Table 2), the accumulation of p62 protein in cultured podocytes

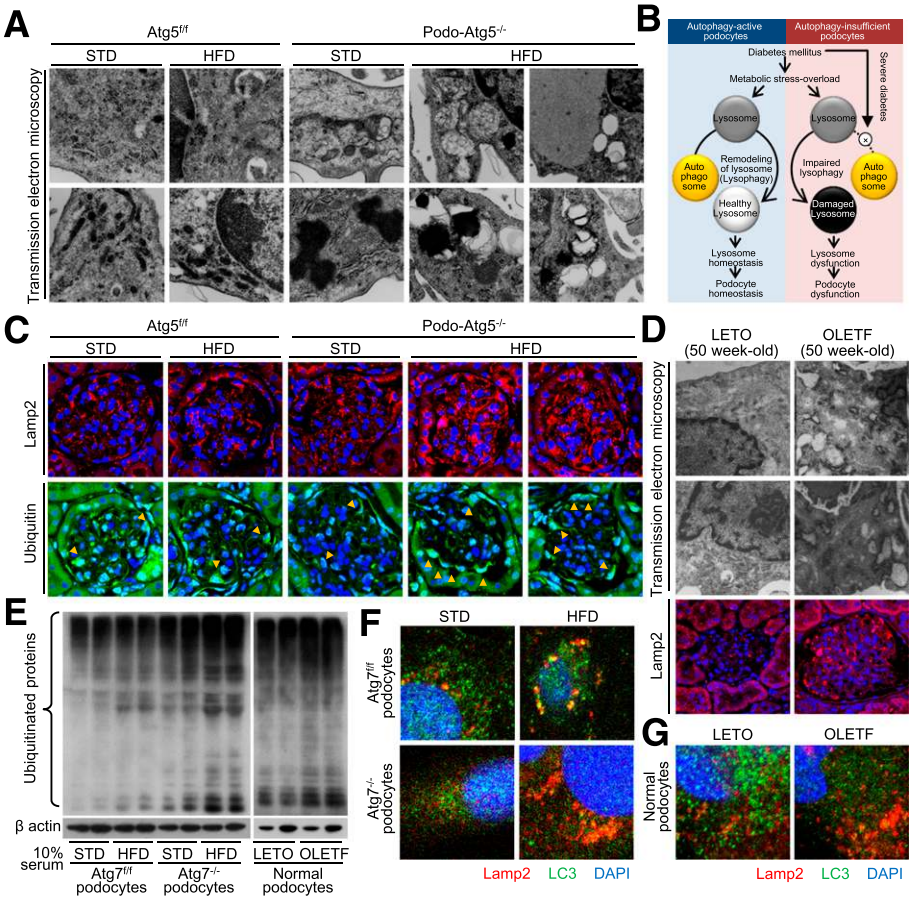

Figure 7—Damaged lysosomes in podocytes of diabetic rodent models with massive proteinuria. *A*: Representative transmission electron microscopy pictures of podocytes from Atg5$^{f/f}$ and Atg5$^{-/-}$ mice fed an STD or HFD for 32 weeks. Original magnification ×20,000. *B*: Hypothetic schema showing that some diabetes conditions increase metabolic loads to lysosomes, requiring damaged lysosomes to be removed by autophagy (lysophagy) and eventual degradation in residual normal lysosomes. *C*: Immunofluorescent expression of lamp2, a lysosome membrane marker, and ubiquitinated proteins in the glomeruli of the four groups of mice. Original magnification ×1,000. Yellow arrowheads, ubiquitin-positive cells. *D*: Representative transmission electron microscopy pictures of podocytes and immunofluorescent expression of lamp2 in 50-week-old LETO and OLETF rats. Original magnifications: ×15,000 for electron microscopy and ×600 for lamp2. *E*: Western blot analysis of ubiquitinated proteins in cultured Atg7$^{f/f}$ and Atg7$^{-/-}$ podocytes incubated with 10% serum from C57BL/6 mice fed an STD or HFD, and from 50-week-old LETO and OLETF rats. *F* and *G*: Double immunofluorescent assay for LC3 and lamp2 in the indicated cells. Original magnification ×1,000.

stimulated with serum was assessed by ELISA (Fig. 8*B*). The levels of p62 protein were significantly higher in cultured podocytes stimulated with the serum from patients with diabetes and massive proteinuria than with serum from other disease stages (Fig. 8*C*). Diabetes alone did not affect p62 accumulation in the cultured podocytes (Fig. 8*C*). These results suggested that insufficient autophagy was particularly associated with progression to massive proteinuria in human diabetic nephropathy.

DISCUSSION

The results presented here have demonstrated that impaired autophagy in podocytes is involved in the pathogenesis of severe podocyte injury, leading to massive proteinuria in diabetic nephropathy. Furthermore, maintenance of lysosome homeostasis by removing damaged lysosomes may be a crucial task of podocyte autophagy under diabetic conditions.

During the past decade, autophagy has been intensively studied in animals with kidney diseases (43). Previous studies using podocyte- and proximal tubular cell–specific autophagy-deficient mice have shown that autophagy insufficiency is related to stress susceptibility (8,44–47), suggesting that autophagy is essential for renoprotection against various pathogenic conditions. Because autophagy is regulated by both nutrients and stress signals, it has been speculated that autophagy is involved in the pathogenesis of diabetic nephropathy (48). The current study utilizing podocyte-specific autophagy-deficient mice provide, for the first time, strong evidence indicating that autophagy is involved in the pathogenesis of diabetic nephropathy.

Autophagy insufficiency was observed specifically in podocytes of patients and rodents with diabetes and massive proteinuria, but not with the other disease stage. Furthermore, HFD-induced diabetes caused minimal

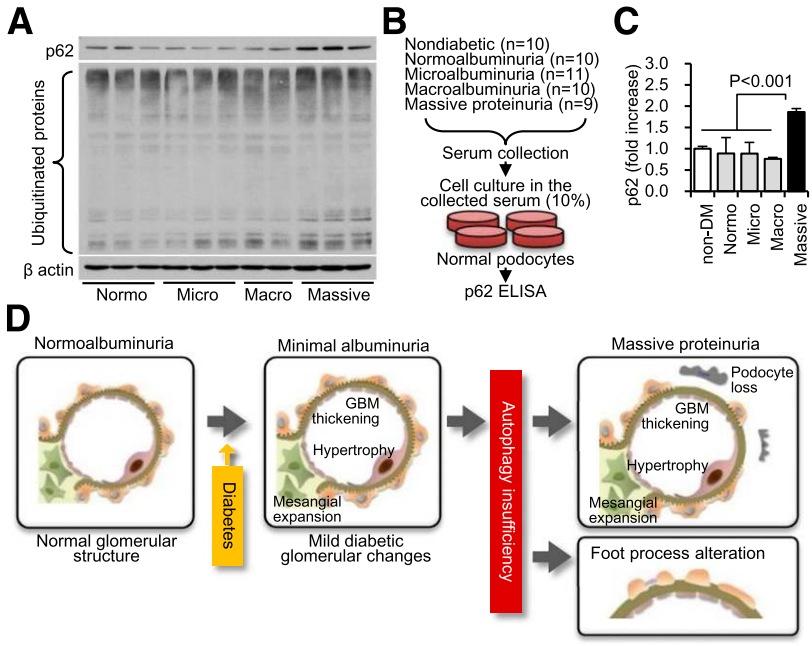

Figure 8—Autophagy-lysosome dysfunction and apoptosis in cultured podocytes stimulated with sera from patients with diabetes and massive proteinuria. *A*: Representative Western blot analyses of p62, ubiquitinated proteins, and β-actin in cultured podocytes incubated with sera from patients with type 2 diabetes and normo- ($n = 3$), micro- ($n = 3$), macro- ($n = 2$), and massive albuminuria ($n = 3$). *B*: Protocol of the cell culture experiment. Cultured normal podocytes were incubated with the sera from subjects without diabetes ($n = 10$), and patients with diabetes and normo- ($n = 10$), micro- ($n = 11$), macro- ($n = 10$), and massive albuminuria ($n = 9$). The collected cell lysates were analyzed by ELISA for expression of p62 protein. *C*: Result of p62 ELISA. All results are presented as mean ± SEM and compared by ANOVA and a subsequent Tukey test, with $P < 0.05$ considered statistically significant. *D*: Hypothetic schema of the current study. Diabetic conditions alone result in mild glomerular lesions with minimal proteinuria. During disease development, however, the combination of insufficient podocyte autophagy and diabetes results in podocyte loss and foot process alteration with lysosome dysfunction, which is associated with the pathogenesis of massive proteinuria. GBM, glomerular basement membrane; non-DM, nondiabetic.

proteinuria in mice with normal podocyte autophagy but massive proteinuria with podocyte loss in mice with podocyte-specific autophagy deficiency. These findings indicate that diabetes alone can cause typical glomerular changes leading to minimal proteinuria, whereas complicated impairment of podocyte autophagy during the course of diabetic nephropathy results in podocyte loss, followed by massive proteinuria (Fig. 8*D*). Thus, insufficient podocyte autophagy may play a pathogenic role particularly in the disease progression to massive proteinuria in diabetic nephropathy. However, this study had several limitations, which may have affected the interpretation of the results. Autophagy deficiency was assessed only by p62 staining and electron microscopic analysis, the number of human kidney biopsy samples was small, and two different rodent species were compared (HFD-fed mice as a model of albuminuria and OLETF rats as a model of massive proteinuria). Additional methods of assessing autophagy deficiency and large clinical cohort and/or animal studies are needed to validate our conclusions.

The mechanism underlying diabetes-related impairment of podocyte autophagy is still unclear. Sera from patients and rodents with massive proteinuria impaired autophagy in cultured podocytes, suggesting that serum factors associated with massive proteinuria in diabetes, but not with diabetes per se, impair podocyte autophagy. The mammalian target of rapamycin complex 1 (mTORC1) is a nutrient-sensing signal that inhibits autophagy (49). Interestingly, mTORC1 activity has been reported to be enhanced in podocytes of humans and animals with advanced diabetic nephropathy (50,51), suggesting that some factors in diabetic serum may activate mTORC1, suppressing podocyte autophagy. Additional studies are required to identify these serum factors and intracellular molecular pathways that inhibit podocyte autophagy, and may contribute to the development of a new therapy for the treatment of refractory diabetic nephropathy. Furthermore, the bioassay involving serum stimulation of cultured cells may be useful in assessing in vivo autophagy activity in human subjects and experimental animals.

Autophagy can degrade many damaged proteins and organelles (7). To date, however, the specific targets of podocyte autophagy have not been identified. Fabry disease, a lysosome disease, causes podocyte damage and proteinuria (15). Moreover, the deletion of a single gene that regulates lysosome function enhanced podocyte damage and proteinuria in mice (16,17). These results indicate that the lysosome is likely important in maintaining podocyte homeostasis. Interestingly, a massive accumulation of lysosomes with abnormal morphology was observed in the podocytes of diabetic rodents with autophagy deficiency. It remains unclear whether the lysosome is also a target of

podocyte autophagy under other pathogenic conditions. Our results, however, suggest that damaged lysosomes are an important degradation target of podocyte autophagy at least under diabetic conditions.

Clinically, there are few effective treatments for patients with diabetes and massive proteinuria; thus, these patients often experience a rapid decline in renal function (4). Because proteinuria is the most likely cause of tubulointerstitial lesions that lead to renal dysfunction, new therapeutic agents are needed to halt the stage progression of proteinuria and/or to protect renal tubular cells against proteinuria-related renotoxicity, thus improving renal outcomes in patients with refractory diabetic nephropathy. We recently reported that autophagy insufficiency in proximal tubular cells was associated with the pathogenesis of obesity- and diabetes-mediated exacerbation of proteinuria-induced tubulointerstitial damage (52). Taken together, our previous and present findings suggest that autophagy activation may be effective for patients with diabetes and massive proteinuria and resultant rapid decline of renal function.

In conclusion, autophagy plays a pivotal role in maintaining lysosome homeostasis in podocytes under diabetic conditions. The impairment of autophagy is involved in the pathogenesis of podocyte loss, leading to massive proteinuria in diabetic nephropathy. These findings suggest a new therapeutic strategy for massive proteinuria in patients with diabetic nephropathy.

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Author Contributions. A.T., M.Y., and S.K. performed the experiments and wrote the manuscript. K.Y., K.A., E.-H.K., and S.U. performed the experiments. J.N., M.C.-K., H.A., and S.-i.A. contributed to the study concept, research design, and interpretation of the results. D.K., M.H., H.M., and T.U. gave conceptual advice. N.K., K.H., and H.O. collected human kidney biopsy samples. All authors contributed to discussion and reviewed and edited the manuscript. S.K. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

References

1. Perkins BA, Ficociello LH, Silva KH, Finkelstein DM, Warram JH, Krolewski AS. Regression of microalbuminuria in type 1 diabetes. N Engl J Med 2003;348:2285–2293

2. Araki S, Haneda M, Sugimoto T, et al. Factors associated with frequent remission of microalbuminuria in patients with type 2 diabetes. Diabetes 2005; 54:2983–2987

3. Yokoyama H, Araki S, Honjo J, et al. Association between remission of macroalbuminuria and preservation of renal function in patients with type 2 diabetes with overt proteinuria. Diabetes Care 2013;36:3227–3233

4. Rossing K, Christensen PK, Hovind P, Tarnow L, Rossing P, Parving HH. Progression of nephropathy in type 2 diabetic patients. Kidney Int 2004;66:1596–1605

5. Mizushima N, Komatsu M. Autophagy: renovation of cells and tissues. Cell 2011;147:728–741

6. Mizushima N, Levine B, Cuervo AM, Klionsky DJ. Autophagy fights disease through cellular self-digestion. Nature 2008;451:1069–1075

7. Kroemer G, Mariño G, Levine B. Autophagy and the integrated stress response. Mol Cell 2010;40:280–293

8. Maejima I, Takahashi A, Omori H, et al. Autophagy sequesters damaged lysosomes to control lysosomal biogenesis and kidney injury. EMBO J 2013;32: 2336–2347

9. Ebato C, Uchida T, Arakawa M, et al. Autophagy is important in islet homeostasis and compensatory increase of beta cell mass in response to high-fat diet. Cell Metab 2008;8:325–332

10. Shigihara N, Fukunaka A, Hara A, et al. Human IAPP-induced pancreatic β cell toxicity and its regulation by autophagy. J Clin Invest 2014;124:3634–3644

11. Rabinowitz JD, White E. Autophagy and metabolism. Science 2010;330: 1344–1348

12. Ezaki J, Matsumoto N, Takeda-Ezaki M, et al. Liver autophagy contributes to the maintenance of blood glucose and amino acid levels. Autophagy 2011;7: 727–736

13. Shih NY, Li J, Karpitskii V, et al. Congenital nephrotic syndrome in mice lacking CD2-associated protein. Science 1999;286:312–315

14. Boute N, Gribouval O, Roselli S, et al. NPHS2, encoding the glomerular protein podocin, is mutated in autosomal recessive steroid-resistant nephrotic syndrome. Nat Genet 2000;24:349–354

15. Kint JA. Fabry's disease: alpha-galactosidase deficiency. Science 1970; 167:1268–1269

16. Oshima Y, Kinouchi K, Ichihara A, et al. Prorenin receptor is essential for normal podocyte structure and function. J Am Soc Nephrol 2011;22:2203–2212

17. Cinà DP, Onay T, Paltoo A, et al. Inhibition of MTOR disrupts autophagic flux in podocytes. J Am Soc Nephrol 2012;23:412–420

18. Asanuma K, Tanida I, Shirato I, et al. MAP-LC3, a promising autophagosomal marker, is processed during the differentiation and recovery of podocytes from PAN nephrosis. FASEB J 2003;17:1165–1167

19. Mizushima N, Yamamoto A, Matsui M, Yoshimori T, Ohsumi Y. In vivo analysis of autophagy in response to nutrient starvation using transgenic mice expressing a fluorescent autophagosome marker. Mol Biol Cell 2004;15:1101–1111

20. Tanaka Y, Kume S, Araki S, et al. Fenofibrate, a PPARα agonist, has renoprotective effects in mice by enhancing renal lipolysis. Kidney Int 2011;79:871–882

21. Kuma A, Hatano M, Matsui M, et al. The role of autophagy during the early neonatal starvation period. Nature 2004;432:1032–1036

22. Moeller MJ, Sanden SK, Soofi A, Wiggins RC, Holzman LB. Podocyte-specific expression of cre recombinase in transgenic mice. Genesis 2003;35: 39–42

23. Deji N, Kume S, Araki S, et al. Structural and functional changes in the kidneys of high-fat diet-induced obese mice. Am J Physiol Renal Physiol 2009; 296:F118–F126

24. Nyengaard JR. Stereologic methods and their application in kidney research. J Am Soc Nephrol 1999;10:1100–1123

25. White KE, Bilous RW, Marshall SM, et al. Podocyte number in normotensive type 1 diabetic patients with albuminuria. Diabetes 2002;51:3083–3089

26. Lemley KV, Bertram JF, Nicholas SB, White K. Estimation of glomerular podocyte number: a selection of valid methods. J Am Soc Nephrol 2013;24:1193–1202
27. Mundel P, Reiser J, Zúñiga Mejía Borja A, et al. Rearrangements of the cytoskeleton and cell contacts induce process formation during differentiation of conditionally immortalized mouse podocyte cell lines. Exp Cell Res 1997;236:248–258
28. Yasuda M, Tanaka Y, Kume S, et al. Fatty acids are novel nutrient factors to regulate mTORC1 lysosomal localization and apoptosis in podocytes. Biochim Biophys Acta 2014;1842:1097–1108
29. Cohen HY, Miller C, Bitterman KJ, et al. Calorie restriction promotes mammalian cell survival by inducing the SIRT1 deacetylase. Science 2004;305:390–392
30. Kume S, Uzu T, Horiike K, et al. Calorie restriction enhances cell adaptation to hypoxia through Sirt1-dependent mitochondrial autophagy in mouse aged kidney. J Clin Invest 2010;120:1043–1055
31. Mizushima N, Yoshimori T, Levine B. Methods in mammalian autophagy research. Cell 2010;140:313–326
32. Lee GH, Ogawa K, Drinkwater NR. Conditional transformation of mouse liver epithelial cells. An in vitro model for analysis of genetic events in hepatocarcinogenesis. Am J Pathol 1995;147:1811–1822
33. Komatsu M, Waguri S, Ueno T, et al. Impairment of starvation-induced and constitutive autophagy in Atg7-deficient mice. J Cell Biol 2005;169:425–434
34. Oliva Trejo JA, Asanuma K, Kim EH, et al. Transient increase in proteinuria, poly-ubiquitylated proteins and ER stress markers in podocyte-specific autophagy-deficient mice following unilateral nephrectomy. Biochem Biophys Res Commun 2014;446:1190–1196
35. Takemoto M, Asker N, Gerhardt H, et al. A new method for large scale isolation of kidney glomeruli from mice. Am J Pathol 2002;161:799–805
36. Morita S, Kojima T, Kitamura T. Plat-E: an efficient and stable system for transient packaging of retroviruses. Gene Ther 2000;7:1063–1066
37. Pankiv S, Clausen TH, Lamark T, et al. p62/SQSTM1 binds directly to Atg8/LC3 to facilitate degradation of ubiquitinated protein aggregates by autophagy. J Biol Chem 2007;282:24131–24145
38. Kume S, Uzu T, Araki S, et al. Role of altered renal lipid metabolism in the development of renal injury induced by a high-fat diet. J Am Soc Nephrol 2007;18:2715–2723
39. Kawano K, Hirashima T, Mori S, Saitoh Y, Kurosumi M, Natori T. Spontaneous long-term hyperglycemic rat with diabetic complications. Otsuka Long-Evans Tokushima Fatty (OLETF) strain. Diabetes 1992;41:1422–1428
40. Fukuda H, Hidaka T, Takagi-Akiba M, et al. Podocin is translocated to cytoplasm in puromycin aminonucleoside nephrosis rats and in poor-prognosis patients with IgA nephropathy. Cell Tissue Res 2015;360:391–400
41. Yu L, McPhee CK, Zheng L, et al. Termination of autophagy and reformation of lysosomes regulated by mTOR. Nature 2010;465:942–946
42. Tanaka Y, Guhde G, Suter A, et al. Accumulation of autophagic vacuoles and cardiomyopathy in LAMP-2-deficient mice. Nature 2000;406:902–906
43. Huber TB, Edelstein CL, Hartleben B, et al. Emerging role of autophagy in kidney function, diseases and aging. Autophagy 2012;8:1009–1031
44. Hartleben B, Gödel M, Meyer-Schwesinger C, et al. Autophagy influences glomerular disease susceptibility and maintains podocyte homeostasis in aging mice. J Clin Invest 2010;120:1084–1096
45. Kimura T, Takabatake Y, Takahashi A, et al. Autophagy protects the proximal tubule from degeneration and acute ischemic injury. J Am Soc Nephrol 2011;22:902–913
46. Liu S, Hartleben B, Kretz O, et al. Autophagy plays a critical role in kidney tubule maintenance, aging and ischemia-reperfusion injury. Autophagy 2012;8:826–837
47. Takahashi A, Kimura T, Takabatake Y, et al. Autophagy guards against cisplatin-induced acute kidney injury. Am J Pathol 2012;180:517–525
48. Kume S, Thomas MC, Koya D. Nutrient sensing, autophagy, and diabetic nephropathy. Diabetes 2012;61:23–29
49. Kim J, Kundu M, Viollet B, Guan KL. AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1. Nat Cell Biol 2011;13:132–141
50. Gödel M, Hartleben B, Herbach N, et al. Role of mTOR in podocyte function and diabetic nephropathy in humans and mice. J Clin Invest 2011;121:2197–2209
51. Inoki K, Mori H, Wang J, et al. mTORC1 activation in podocytes is a critical step in the development of diabetic nephropathy in mice. J Clin Invest 2011;121:2181–2196
52. Yamahara K, Kume S, Koya D, et al. Obesity-mediated autophagy insufficiency exacerbates proteinuria-induced tubulointerstitial lesions. J Am Soc Nephrol 2013;24:1769–1781