Hypertensive individuals are at greater risk for developing chronic kidney disease (CKD). Reducing proteinuria has been suggested as a possible therapeutic approach to treat CKD. However, the mechanisms underlying the development of proteinuria in hypertensive conditions are incompletely understood. Cardiac and vascular dysfunction is associated with changes in the O-GlcNAcylation pathway in hypertensive models. We hypothesized that O-GlcNAcylation is also involved in renal damage, especially development of proteinuria, associated with hypertension. Using the spontaneously hypertensive rat (SHR) model, we observed higher renal cortex O-GlcNAcylation, glutamine–fructose aminotransferase (GFAT), and O-GlcNAc transferase (OGT) protein expression, which positively correlated with proteinuria. Interestingly, this was observed in hypertensive, but not pre-hypertensive, rats. Pharmacological inhibition of GFAT decreased renal cortex O-GlcNAcylation, proteinuria, and albuminuria in SHR. Using a proximal tubule cell line, we observed that increased O-GlcNAcylation reduced megalin surface expression and albumin endocytosis in vitro, and the effects were correlated in vivo. Moreover, megalin is O-GlcNAcylated both in vitro and in vivo. In conclusion, our results demonstrate a new mechanism involved in hypertension-associated proteinuria.

Hypertension is a highly prevalent chronic disease, directly associated with the development of chronic kidney disease (CKD)1–3. Proteinuria associated with hypertension is a good predictor of higher cardiovascular risk and renal injury (3). The origin of proteinuria in hypertension-induced CKD remains an open question in the literature. Classically, proteinuria is associated with modifications in glomerular structure, leading to overload of protein in proximal tubule (PT) cells and, consequently, urinary excretion (4, 5). However, the role of PT cells in this process has been pointed out (6, 7).

Proteins are reabsorbed in the PT cells by receptor-mediated endocytosis, in which megalin plays a pivotal role (8). Megalin/LRP2 is a scavenger receptor of the low-density lipoprotein receptor family located at the PT luminal membrane (9). This receptor has a short C-terminal cytoplasmic tail, which contains several domains involved in protein–protein interactions and motifs phosphorylated by serine/threonine kinases, indicating that protein reabsorption in PT cells is a highly dynamic and regulated process (9).

Following this idea, it is possible to imagine that intracellular O-GlcNAcylation could also play an important role in protein reabsorption in PT cells. Patients with diabetic nephropathy have increased glomerular and tubular O-GlcNAcylation (10). In diabetic nephropathy models, decreased PT protein reabsorption is correlated with proteinuria and albuminuria (7).

Intracellular O-GlcNAcylation is a post-translational modification involved in a variety of physiologic and pathophysiologic processes (11, 12). O-GlcNAc transferase (OGT) requires UDP-GlcNAc produced by the hexosamine biosynthetic pathway (HBP) to promote O-GlcNAcylation. The removal of O-GlcNAc is promoted by O-GlcNAcase (OGA). The rate-limiting step in HBP is the conversion of fructose 6-phosphate to glucosamine 6-phosphate induced by glutamine–fructose aminotransferase (GFAT) activity (11). Lunde et al. (13) showed a correlation among increased O-GlcNAcylation, GFAT expression, and cardiac hypertrophy in spontaneously hypertensive rats (SHRs). However, the contribution of O-GlcNAcylation to spontaneously hypertensive rat; WGA, wheat germ agglutinin; BP, blood pressure; SBP, systolic blood pressure; UP/Cr, urinary protein/creatinine ratio; DON, 6-diazo-5-oxo-L-norleucine; HA, hemagglutinin; PNGase F, peptide:N-glycosidase F; ANOVA, analysis of variance; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
the development of proteinuria in essential hypertension is still to be determined. In the present study, we aimed to evaluate the possible role of O-GlcNAcylation in the PT protein reabsorption during hypertension. SHRs were used as a model of essential hypertension, the most clinically relevant form of hypertension (14). Interestingly, we demonstrate a correlation between renal cortex O-GlcNAcylation and proteinuria in adult SHRs. The mechanism underlying this process involves an increase in renal cortex O-GlcNAcylation machinery, leading to an increase in O-GlcNAcylation, internalization of megalin, and, consequently, decrease in protein reabsorption in PT cells.

Results

O-GlcNAcylation is increased in the renal cortex of adult SHRs

Initially, we studied possible changes in O-GlcNAcylation of proteins in the renal cortex of adult SHRs. We used 14-week-old SHRs with established hypertension and proteinuria (15). Age-matched Wistar rats were used as controls. An increased area of wheat germ agglutinin (WGA)-positive staining was observed in the renal cortical sections of SHRs (Fig. 1, A and B). Pre-incubation of WGA with 0.5 M GlcNAc blocked the positive signal, confirming the specificity of GlcNAc staining. Using RL-2 antibody, which recognizes intracellular O-GlcNAcylation, we also observed increased positive staining in adult SHRs (Fig. 1, C and D). Importantly, 0.5 M GlcNAc blocked the positive signal.

Although most O-GlcNAcylated bands increased, a band of 51 kDa decreased in SHR animals (Fig. 2A). However, the global O-GlcNAcylation increased in hypertensive animals (Fig. 2D). Accordingly, O-GlcNAcylation cycling proteins, OGT and OGA, showed increased expression in the renal cortex of SHRs (Fig. 2, B and D). Interestingly, OGT showed a higher increase than OGA (2.5-fold increase versus 2.0-fold increase, respectively). When we assessed GFAT protein expression, the rate-limiting step in UDP-GlcNAc production in the HBP, a remarkable 10-fold increase was observed in adult SHRs (Fig. 2, C and D). This effect was correlated to a specific increase in GFAT-1 expression. Under these conditions, GFAT-2 expression was not detected, as described previously (16). These results demonstrate that adult SHRs present higher renal cortex O-GlcNAcylation, associated with increased GFAT-1 and OGT renal expression.
O-GlcNAcylation inhibits PT protein reabsorption in SHRs

In the next step, we assessed whether SHRs have a dysregulated renal cortical O-GlcNAcylation pathway before the development of hypertension. We used young (4-week-old), pre-hypertensive SHRs, with normal levels of blood pressure (BP) and mild urinary protein/creatinine ratio (UP/Cr) of 4-week-old SHRs, with normal levels of blood pressure (BP) and mild urinary protein/creatinine ratio (UP/Cr) ([48],[49]). Importantly, incubation with 0.5 M GlcNAc blocked RL-2 signal on Western blotting ([50],[51]), for 3 days. DON treatment significantly reduced renal O-GlcNAcylation in adult SHRs, demonstrating inhibition of GFAT in the renal tissue ([52],[53]). Proteinuria, measured by UP/Cr, was reduced after DON treatment ([54],[55]). Urinary SDS-PAGE analyses and urine Western blotting confirmed that middle- and low-molecular weight proteinuria, as well as albuminuria, was reduced after DON treatment ([56],[57],[58],[59]). The UP/Cr and SBP values were not affected by DON treatment in Wistar rats ([60],[61],[62],[63]).

Interestingly, DON-treated SHRs showed decreased SBP compared with vehicle-treated SHR ([64],[65]). These results could suggest that the effect of DON treatment on proteinuria could be a result of lower BP. To test this hypothesis, 14-week-old SHRs were treated with hydralazine, which is known to decrease BP, for 3 days ([66],[67]). At this condition, UP/Cr ([68],[69]) and renal cortex O-GlcNAcylation ([70],[71],[72]) were not changed. 0.5 M GlcNAc blocked RL-2 signal on Western blotting ([73],[74]). Together, these results show that the increase in O-GlcNAcylation in the renal cortex mediates, at least in part, the proteinuria observed in adult SHRs.

Protein reabsorption in PT cells is inhibited by increased O-GlcNAcylation

In Fig. 7A, we observed that SHRs have a significant decrease in PT protein reabsorption, evaluated by in vivo BSA-FITC uptake. In addition, we also measured albumin endocytosis in LLC-PK1 cells, a porcine PT cell line, treated with glucosamine (5 mmol/liter) or PugNAc (5 μmol/liter), an OGA inhibitor, for 24 h. Both compounds increased O-GlcNAcylation ([75],[76]) and inhibited BSA-FITC endocytosis in LLC-PK1 cells ([77],[78],[79]). Importantly, incubation with 0.5 M GlcNAc blocked RL-2 signal on Western blotting ([80],[81]). Incubation of these cells with 5 mmol/liter mannitol (as an osmotic control) for 24 h
O-GlcNAcylation inhibits PT protein reabsorption in SHRs

Figure 5. Acute treatment of 14-week-old SHRs with GFAT inhibitor DON ameliorates proteinuria and hypertension. Representative Western blot for O-GlcNAc residue (RL-2), indicative of O-GlcNAcylation, in renal cortex homogenate of 14-week-old SHRs treated with vehicle or 0.5 mg/kg DON for 3 days (n = 4) is shown (A). Urine SDS-PAGE analysis was performed in 14-week-old Wistar rats and vehicle- or DON-treated SHRs. Proteins are stained with Coomassie Blue (C). Urinary albumin content was assessed by Western blotting (D). Urinary albumin content was increased compared with the cells not incubated with glucosamine. Under those conditions, megalin was immunoprecipitated, and Western blotting was carried out. Interestingly, basal megalin was observed in Wistar rats. This modification was markedly increased in SHRs (Fig. 8A), and a reduced BSA-FITC uptake, observed by confocal microscopy (Fig. 8B).

Increased O-GlcNAcylation reduces megalin surface expression in PT cells

Protein is reabsorbed in PT cells by receptor-mediated endocytosis, and megalin is a key receptor involved in this process (8, 9). We tested whether the level of megalin could be altered in adult SHRs. Total megalin expression was increased in adult SHRs (Fig. 9A), but its cellular distribution was modified (Fig. 9A, white arrows). Using surface plots from confocal microscopy, it was detected that megalin is internalized in PTs of SHRs (Fig. 9B), which correlates with the decreased protein reabsorption observed previously. Interestingly, DON treatment, which decreases renal cortex O-GlcNAcylation, induced redistribution of megalin to the surface of the luminal membrane, as observed in control animals. Furthermore, treatment of LLC-PK1 cells with glucosamine or PugNAc for 24 h promoted megalin internalization, as observed through confocal microscopy (Fig. 10A). These results corroborate the inhibition of albumin endocytosis in LLC-PK1 cells induced by O-GlcNAcylation (Figs. 7B and C) and 8B). Therefore, our results strongly suggest that O-GlcNAcylation in PT cells decreases megalin expression at the surface of the luminal membrane, leading to a reduction in protein reabsorption that could be involved, at least in part, with proteinuria observed in adult SHR.

Finally, we decided to measure whether megalin could be directly O-GlcNAcylated in PTs of SHRs. To address this question, megalin was immunoprecipitated, and Western blotting to O-GlcNAcylation was carried out. Interestingly, basal megalin O-GlcNAcylation was observed in Wistar rats. This modification was markedly increased in SHRs (Fig. 10B). Similar experiments were performed on LLC-PK1 cells incubated with glucosamine. Under those conditions, megalin O-GlcNAcylation was increased compared with the cells not incubated with glucosamine (Fig. 10C). In agreement, purified megalin treated with PNGase F, an endoglycosidase that removes N-glycans, maintained a positive signal for RL-2 antibody, demonstrating specific intracellular O-GlcNAc signal (Fig. 10D). These results indicate that increased megalin O-GlcNAcylation could be involved in the distribution of megalin at the luminal plasma membrane and, consequently, in decreasing protein reabsorption in PTs.
**Figure 7. Increased O-GlcNAcylation inhibits proximal tubule protein endocytosis.** Intravenous administration of 5 μg/g BSA-FITC was carried out in 14-week-old SHRs or Wistar rats. After 15 min, rats were perfused with saline, and renal cortex BSA-FITC-specific fluorescence was quantified (A). LLC-PK1 cells were treated with 5 mmol/liter D-glucosamine (GlcN) or 5 μmol/liter PugNAc for 24 h. Representative Western blot for O-GlcNAc residue (RL-2) in cell lysates is shown. Incubation of RL-2 with 0.5M GlcNAc was used as a specificity control (B). In vitro BSA-FITC uptake was performed in cells treated as described. Total BSA-FITC-specific fluorescence was quantified (n = 7) (C) or visualized through inverted microscopy (n = 3) (D). Nuclei were stained with DAPI. Magnification, ×400. Scale bar, 50 μm. Values are presented as mean ± S.E. (error bars) *, p < 0.05 versus control or Wistar rats. Data were analyzed through one-way ANOVA with Bonferroni post hoc test.

**Figure 8. OGT overexpression reduces PT albumin endocytosis.** LLC-PK1 cells were transiently transfected with HA (control) or OGT-HA plasmids. A representative Western blot for O-GlcNAc residue (RL-2) and HA in cell lysates is shown (A). In vitro BSA-FITC uptake was performed, and representative images of confocal microscopy analysis are shown. HA was stained with Alexa Fluor 546. Cell morphology was accessed by differential interference contrast (DIC) (B). Magnification, ×630. Scale bar, 20 μm.
Proteinuria is associated with a greater risk of CKD progression and mortality in patients with hypertension (3, 18, 19). In this context, understanding the mechanisms involved in the development of proteinuria in hypertension-induced CKD is a relevant issue. In the present work, we demonstrated that proteinuria observed in adult SHRs could be, at least in part, a consequence of changes in PT protein reabsorption. This phenomenon is associated with an increase in \( \text{O-GlcNAcylation} \) in the renal cortex of adult SHRs, leading to internalization of megalin from the PT luminal membrane and, consequently, a decrease in protein reabsorption in these cells. These results reveal a novel regulatory mechanism of protein reabsorption in PT cells and may lead to the development of new treatment approaches for CKD associated with essential hypertension (20).

Essential hypertension has been associated with glomerular damage and podocyte injury, leading to an increase in protein filtration (3). Consequently, there is a protein overload in PT cells, leading to saturation of protein reabsorption and proteinuria. However, it has been proposed that PT cells could have a role in the albuminuria observed in kidney disease (6, 7). This idea is reinforced by the observation that megalin-deficient PT cells lead to albuminuria and proteinuria (21). Our findings did not rule out the role of glomerular dysfunction in proteinuria but bring new insights to our understanding of the role of PT cells with hypertension-associated albuminuria.

Defective PT function has been observed in patients and animal models of essential hypertension (15, 22), creating a dangerous loop between hypertension and renal disease. Our results show that adult SHRs, which have established hypertension, have increased renal cortical \( \text{O-GlcNAcylation} \) machinery. The involvement of extracellular glycosylation, however, cannot be ruled out. The main product of the HBP, UDP-GlcNAc, is a precursor of UDP-GalNAc and CMP-Neu5Ac production (23), thus regulating the pattern of extracellular glycosylation. Recently, it was shown that the megalin glycoprotein modifies binding properties to different ligands, including albumin (24).

The increase in renal cortex \( \text{O-GlcNAcylation} \) machinery did not occur in young SHRs, which do not have established hypertension. This observation suggests that the increase in renal \( \text{O-GlcNAcylation} \) could be correlated with hypertension establishment. Interestingly, our results showed an increase in \( \text{O-GlcNAcylated bands} \) at \( \sim 60 \text{kDa} \), and a decrease in \( \sim 51 \text{kDa} \) bands, indicating that proteins are dynamically \( \text{O-GlcNAcylated} \) in hypertensive conditions. Using deoxycorticosterone acetate salt–induced hypertension, Lima et al. (25) proposed that the increase in vascular GFAT expression and \( \text{O-GlcNAcylation} \) was temporally correlated with increased BP and vascular dys-

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**Discussion**

Proteinuria is associated with a greater risk of CKD progression and mortality in patients with hypertension (3, 18, 19). In this context, understanding the mechanisms involved in the development of proteinuria in hypertension-induced CKD is a relevant issue. In the present work, we demonstrated that proteinuria observed in adult SHRs could be, at least in part, a consequence of changes in PT protein reabsorption. This phenomenon is associated with an increase in \( \text{O-GlcNAcylation} \) in the renal cortex of adult SHRs, leading to internalization of megalin from the PT luminal membrane and, consequently, a decrease in protein reabsorption in these cells. These results reveal a novel regulatory mechanism of protein reabsorption in PT cells and may lead to the development of new treatment approaches for CKD associated with essential hypertension (20).

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**Figure 9. Increased O-GlcNAcylation promotes megalin internalization in vivo.** A, Megalin (Alexa Fluor 546) protein expression in 14-week-old Wistar rats and SHRs was observed through immunofluorescence. Nuclei were stained with DAPI. Representative sections from three independent animals per group are presented. Magnification, \( \times 400 \). Scale bar, 50 \( \mu \text{m} \). Arrows, altered localization of megalin-positive signal. B, confocal microscopy analysis of megalin expression in renal sections of adult Wistar rats, SHRs, or SHRs treated with DON. Representative images of two independent experiments are shown. Cellular distribution of megalin-positive signal is shown in a three-dimensional surface plot. Magnification, \( \times 630 \). Zoom, \( \times 6 \). Scale bar, 10 \( \mu \text{m} \).
function. In agreement, we observed increased renal O-GlcNAcylation and GFAT expression only in hypertensive adult SHR. Similarly, an increase in GFAT and OGT protein expression was observed, followed by higher O-GlcNAcylation, in the cardiac tissue of adult SHRs (12).

An important correlation has been shown between protein filtration at glomerular membrane and increase in BP (3). In this way, it is relevant to address whether the decrease in proteinuria observed in DON-treated SHR is due to changes in O-GlcNAcylation or in the SBP. Our previous results demonstrated that the decrease in SBP by hydralazine treatment did not change proteinuria in adult SHRs (15). Here, we showed that the treatment SHR with DON or hydralazine for 3 days decreased the BP. However, only DON treatment decreased proteinuria. These results indicate that the effect of DON treatment on proteinuria is due to changes in O-GlcNAcylation rather than in BP. In agreement, a positive correlation between renal cortical O-GlcNAcylation and proteinuria was observed in offspring F1 hybrid animals.

O-GlcNAcylation has been proposed to act as a conserved stress-response mechanism in several tissues, in which the final effect depends on several conditions (26, 27). Glucosamine-induced O-GlcNAcylation promoted renal protection in a model of acute kidney injury (28) and prevented inflammation-induced vascular dysfunction (29), indicating a protective role of acutely increased O-GlcNAcylation. However, chronically increased O-GlcNAcylation was positively correlated with different pathologic conditions, such as diabetic cardiomyopathy (30), idiopathic pulmonary hypertension (31), insulin resistance (32), and high glucose–induced renal damage (33). Therefore, we can speculate that increased BP drives stress-response–related O-GlcNAcylation in tissues affected by blood pressure homeostasis, such as renal tissue. The establishment of hypertension could lead to chronically increased tissue O-GlcNAcylation, which promotes end-organ damage, such as massive proteinuria. Accordingly, inhibition of GFAT activity reduced renal cortical O-GlcNAcylation, UP/Cr, and albuminuria in SHRs.

Megalin is an LDL-like family receptor responsible for receptor-mediated endocytosis and reabsorption of several plasma proteins, including albumin, in the PT (9). Megalin was recently described as a fast-recycling, long-lived receptor, which completes its cycle through the different endosomal compartments in ~10 min (34). Different mechanisms were shown to regulate megalin trafficking, such as adaptor proteins ARH (35) and Dab2 (36), and GSK3β-dependent phosphorylation in the PPPSP domain (37), explaining the fast cycling kinetics of this receptor. Here, we demonstrated, both in vitro and in vivo, that

Figure 10. Megalin is O-GlcNAcylated in vivo and in vitro. In vitro megalin expression was assessed by confocal microscopy. Megalin (Alexa Fluor 488) was detected in LLC-PK1 cells grown in transwell inserts (A). A representative photomicrograph of two independent experiments is shown. Magnification, ×630. Scale bar, 20 µm. Immunoprecipitation of megalin was performed as described under “Experimental procedures.” Western blotting for O-GlcNAc residue (RL-2) and megalin was performed in immunoprecipitated (IP) samples from adult Wistar rats and SHRs (B) and LLC-PK1 control cells or cells treated with glucosamine (GlcN) (5 mmol/liter) (C). A nonspecific antibody was used as a control for immunoprecipitation specificity (IgG). The predicted band size is marked with an arrowhead. Megalin immunoprecipitated from SHR renal cortex was subjected or not to PNGase F treatment for 2 h at 37 °C, followed by Western blotting for O-GlcNAc residue (RL-2) and megalin. Mouse IgG size shift was used as a positive control for PNGase F treatment (D). Densitometric quantification was obtained as a ratio of O-GlcNAc/megalin expression, and the relative expression is represented as mean ± S.E. in -fold change.
increased O-GlcNAcylation promotes megalin internalization, which decreases PT protein reabsorption. It was previously reported that receptor O-GlcNAcylation reduces surface expression of E-cadherin (38), suggesting that O-GlcNAcylation modulates membrane trafficking. It is plausible to postulate that direct megalin O-GlcNAcylation could also regulate its trafficking, because megalin O-GlcNAcylation was correlated with its internalization.

O-GlcNAcylation may have a possible role in glomerular podocytes. The presence of megalin was recently observed in rat podocytes (39). It was proposed that megalin-mediated albumin endocytosis in podocytes clears nonfiltered albumin from the glomerular membrane, consequently avoiding clogging during filtration. Some studies have obtained evidence indicating the presence of receptor-mediated albumin endocytosis in human podocytes (40, 41). However, the expression of megalin in human podocytes is still a controversial issue.

Ono et al. (42) proposed that glomerular O-GlcNAcylation is critical for maturation of foot processes and podocyte function. Thus, megalin could undergo additional O-GlcNAcylation in essential hypertension similar to that observed in PT cells. Further studies are necessary to clarify this issue.

At this moment, it is important to address the correlation between expression of megalin with PT albumin endocytosis and its possible impact on proteinuria in SHR. Previous results have shown that megalin is able to compensate for increased protein filtration, increasing PT protein reabsorption (43). Here, despite increased megalin expression, lower megalin surface expression was demonstrated at the PT luminal membrane in SHRs. In agreement, Sun et al. (44) recently observed an increased megalin expression and internalization in renal biopsies of patients with proteinuria. Finally, our results show that megalin internalization in PT of SHR was associated with increased renal O-GlcNAcylation, leading to reduced PT protein reabsorption and proteinuria in SHR.

**Experimental procedures**

**Animals**

All procedures were performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the institutional ethics committee (approval number 045–17). Male Wistar rats and SHRs aged 4 and 14 weeks were used in the experiments. When indicated, 14-week-old Wistar rats and SHRs were treated with a daily intraperitoneal injection of either PBS as vehicle or 0.5 mg/kg/day of DON (Sigma-Aldrich) for 3 days. Another experimental group was treated with a daily oral dose of water as vehicle or 15 mg/kg/day of hydralazine for 3 days. For the hybrid animal experiments, a male Wistar rat and a female SHR were crossed, and F1 hybrid animals from the same parents were analyzed when they reached 14 weeks.

**Cell culture and transfection**

LLC-PK1 (ATCC, Manassas, VA) cells, a porcine PT cell line, were maintained in low-glucose Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin (37 °C and 5% CO₂). For confocal microscopy, cells were grown on transwell inserts (Corning, Inc.) for 6 days to reach complete polarization. When indicated, LLC-PK1 cells were transfected with plasmid DNA using Lipofectamine 2000 (Invitrogen) for 48 h according to the manufacturer's instructions. The human OGT cDNA was obtained from the ATCC and subcloned into pEF-HA (45) to create HA–OGT. Cells were then serum-starved and incubated as indicated.

**Functional parameters**

SBP was measured by noninvasive, tail-cuff plethysmography (Harvard Apparatus, Holliston, MA). Urinary protein (Labtest, Minas Gerais, Brazil) and urinary creatinine (GoldAnala, Minas Gerais, Brazil) were measured by colorimetric reaction.

**Renal cortex isolation and preparation**

Renal cortex isolation was performed as described previously (46). The tissue was homogenized in ice-cold preparation solution containing 250 mm sucrose, 10 mm HEPES, 5 mm phenylmethanesulfonyl fluoride, and protease inhibitor mixture (Sigma-Aldrich). Samples were centrifuged twice at 10,000 × g for 10 min. The supernatant obtained was stored at −80 °C until used. Protein concentration was accessed by the Lowry method as described previously (47).

**Western blotting, urine SDS-PAGE, and immunoprecipitation**

Immunoblotting was performed as described previously (48). Immunodetection was performed for GFAT-1 (catalog no. 5322, clone D12F4, Cell Signaling, Danvers, MA), GFAT-2 (catalog no. SC134710, Santa Cruz Biotechnology, Inc.), OGAT (catalog no. 345), OGT (catalog no. AL-28), O-GlcNAcylation (RL-2, catalog no. SC59624, Santa Cruz Biotechnology), or GAPDH (catalog no. 2118, clone 14C10, Cell Signaling). Urinary albumin (catalog no. ab106582, Abcam, Cambridge, UK) or total urinary protein content was detected through SDS-PAGE. For immunoprecipitation, freshly prepared renal homogenates or LLC-PK1 samples (1 mg) were resuspended in 1 ml of lysis buffer containing 20 mm HEPES, 2 mm EDTA, 5 mm Na₃VO₄, protease inhibitor mixture (Sigma-Aldrich), and OGA inhibitor (PugNAc, 1 μM). Samples were incubated with 1 μg/ml anti-rabbit megalin antibody (catalog no. EPRS875, Abcam) for 2 h at 4 °C with slow rotation. Control samples were incubated with nonspecific antibody (catalog no. 7074, anti-rabbit IgG, Cell Signaling). When indicated, immunoprecipitated megalin was incubated or not with 5 units of PNGase F (Sigma-Aldrich) for 2 h at 37 °C. Mouse IgG (Cell Signaling) was used as a control for PNGase F activity. Proteins were resolved in 4% (megalin) or 12% (mouse IgG) SDS-PAGE and then immunodetected as described above. Pre-incubation of RL-2 with 0.5 m GlcNAc (Sigma-Aldrich) for 1 h was performed before incubation of the membrane as a specificity control for GlcNAc, when indicated.

**BSA-FITC uptake in vivo and in vitro**

For in vivo measurement of PT protein uptake, anesthetized Wistar rats and SHRs aged 14 weeks were infused intravenously with BSA-FITC (Sigma-Aldrich) at a dose of 5 μg/g of body weight. After 15 min, rats were perfused with saline to extract serum contaminants, and renal cortex homogenate was prepared as described above. Fluorescence was quantified with a
O-GlcNAcylation inhibits PT protein reabsorption in SHRs

SpectraMax M2 microplate reader (Molecular Devices, Sunnyvale, CA). For in vitro analysis, LLC-PK1 cells (ATCC) grown on glass coverslips were incubated with 5 mM D-glucosamine (Sigma-Aldrich), 5 μM PugNAc, or 5 mM mannosyl in serum-free Dulbecco’s modified Eagle’s medium ( Gibco) for 24 h. Cells were incubated with BSA-FITC (15 μg/ml) diluted in Ringer solution for 30 min at 37 °C. Fluorescence was either quantified in a SpectraMax M2 microplate reader (Molecular Devices) or visualized by fluorescence microscopy (80i Eclipse, Nikon, Tokyo, Japan).

Histochemistry and immunofluorescence

Sample fixation and preparation were performed as described previously (15). WGA lectin (Vector Laboratories) was incubated at a final concentration of 2.5 μg/ml for 1 h. RL-2 (catalog no. SC59624, Santa Cruz Biotechnology) both for 1 h at room temperature. Results were revealed with 3,3’-diaminobenzidine (Pierce). Sections were counterstained with Harry’s hematoxylin. Pre-incubation of 2.5 μg/ml WGA with 0.5 μM GlcNAc (Sigma-Aldrich) for 1 h was performed before incubation of the sections as a specificity control for GlcNAc. Immunofluorescence analyses were performed in paraffin-embedded sections and LLC-PK1 cells grown in transwells with rabbit anti-megalin antibody (catalog no. ab76969, Abcam). Renal sections were analyzed by confocal microscopy (Leica TCS SP8, Leica, Wetzlar, Germany), and LLC-PK1 cells were analyzed in Spinning Disk Cell Observer (Carl Zeiss, Germany). For GlcNAc. Immunofluorescence analyses were performed in paraffin-embedded sections and LLC-PK1 cells grown in transwells with rabbit anti-megalin antibody (catalog no. ab76969, Abcam). Renal sections were analyzed by confocal microscopy (Leica TCS SP8, Leica, Wetzlar, Germany), and LLC-PK1 cells were analyzed in Spinning Disk Cell Observer (Carl Zeiss, Germany); the final images were analyzed with Fiji or ZEN software, respectively. When indicated, the magnification was increased ×6.

Statistical analysis

All values are presented as means ± S.E. Unpaired Student’s t test was used to compare two groups, whereas one-way ANOVA with Bonferroni post hoc test was used to compare more than two groups. Correlation was obtained through Pearson’s coefficient. p < 0.05 was considered statistically significant. GraphPad Prism version 5 software was used for the statistical analysis.

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