Limited capacity of proximal tubular proteolysis in mice with proteinuria

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1Department of Nephrology, Austin Health, Victoria, Australia; 2Department of Medicine, Austin Health, University of Melbourne, Victoria, Australia; 3Department of Renal Medicine, Eastern Health Clinical School, Faculty of Medicine, Nursing and Health Sciences, Monash University, Victoria, Australia; and 4Institute for Breathing and Sleep, Austin Health, Victoria, Australia

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Lee D, Gleich K, Fraser SA, Katerelos M, Mount PF, Power DA. Limited capacity of proximal tubular proteolysis in mice with proteinuria. Am J Physiol Renal Physiol 304: F1009–F1019, 2013. First published January 23, 2013; doi:10.1152/ajprenal.00601.2012.—Albuminuria is associated with the additional loss in the urine of small molecular weight proteins normally degraded by the proximal convoluted tubule (PCT), and competition for binding to the megalin/cubilin reuptake system has been considered the likely cause. We have previously reported that deficiency of the intrinsic lysosomal protein Limp-2 causes tubular proteinuria due to reduced fusion of endosomes with lysosomes in the PCT leading to inadequate proteolysis. To determine whether this mechanism also contributes to the tubular proteinuria induced by albumin overload in normal mice, wild-type (WT) mice received daily BSA injections intraperitoneally for 10 days, using untreated Limp-2−/− mice as positive controls for inadequate proteolysis. BSA overload induced significant urinary loss of megalin and cubilin ligands in WT mice. Tubular uptake of Alexa-conjugated BSA, administered by intravenous injection, was not reduced in the PCT of mice receiving intraperitoneal BSA. Expression of the tubular protein receptor megalin was also unchanged. There was a delay in proteolysis of reabsorbed proteins in WT mice receiving BSA, evidenced by an increased quantity of retinol-binding protein (RBP) in the kidney cortex, increased basal distribution of endocytosed RBP in cells of the PCT, and persistence of exogenous Alexa-conjugated BSA and RBP after injection. Uprogulation of cathepsin L and normal fusion of lysosomes with endosomes were apparently not sufficient to maintain normal clearance of endocytosed proteins. The data suggest that in the presence of competition from albumin overload, reabsorption of filtered proteins is limited by the capacity of lysosomal degradation rather than receptor-mediated endocytosis.

TUBULAR UPTAKE AND PROTEOLYSIS of filtered proteins by the kidney is a receptor-mediated process that becomes abnormal in some clinical situations. One of these is a rare disease called Action Myoclonus-Renal Failure Syndrome, due to mutations of the integral lysosomal protein SCARB2, where patients develop a severe neurological phenotype as well as collapsing focal and segmental glomerulosclerosis in the kidney leading to renal failure (2–4, 35). While most will develop severe proteinuria and kidney failure, a small group of patients have no apparent renal involvement (13). We have recently demonstrated that this group develop tubular proteinuria (11). Studies performed in mice show that mutation of Limp-2, the murine homologue of SCARB2, leads to tubular proteinuria associated with a reduced ability of endosomes containing reabsorbed proteins to fuse with lysosomes in the proximal convoluted tubule (PCT) of the kidney (11). This is a novel potential mechanism for tubular protein loss, as most diseases causing tubular proteinuria affect the megalin/cubilin receptor-mediated uptake in the PCT (7, 12, 18, 21, 23, 30, 38).

To determine whether a similar mechanism might exist in normal mice when challenged with increased loads of filtered proteins, albumin overload proteinuria was induced in wild-type (WT) mice. Repeated injection of BSA into mice and rats has long been known to induce proteinuria, with urinary loss of filtered bovine albumin but also murine albumin and presumably other endogenous proteins (15, 22). While much of this proteinuria is presumed to be of glomerular origin, depending on the glomerular permeability of the mouse strain (22), it has been suggested that exposure to high concentrations of BSA lead to reduced uptake of FITC-BSA due to direct competition for endocytosis (20), as well as reduced expression of megalin on the cell surface (6). Short-term intravenous loading of exogenous proteins in rats has been shown to induce a dose-dependent loss of urinary proteins across a broad spectrum of size and charge, also suggesting a competing process for tubular uptake rather than increased glomerular leakage (5). While urinary loss of tubular proteins in the protein overload model is considered due to competitive inhibition of receptor-mediated endocytosis, it is not clear how proximal tubular cells handle the increased delivery of filtered proteins in vivo. A previous study of protein overload in rats showed upregulation of cathepsin B and L activity (28), suggesting a role for increased lysosomal degradation. Supporting this, preincubation with BSA led to reduced degradation of FITC-BSA in proximal tubular cells (20), suggesting that lysosomal degradation of reabsorbed proteins may be a saturable process.

In the current study, therefore, we attempted to determine whether tubular proteinuria induced in normal mice by short-term albumin overload was associated with competitive inhibition of receptor-mediated endocytosis or lysosomal degradation, using Limp-2−/− mice as positive controls for inadequate proteolysis.

METHODS

Animal studies. The Austin Hospital Animal Ethics Committee approved the experimental protocols in mice. The generation of Limp-2−/− mice has been previously described (19), and WT controls used were littermate siblings. All animals used were males aged between 10 and 14 wk on a mixed 129Sv/Bl6 background. Genotyping was determined by PCR using genomic DNA isolated from a short piece of tail taken at 2–4 wk of age. For BSA overload, WT mice received intraperitoneal injections of fatty acid-free, low-endotoxin BSA (A9543; Sigma-Aldrich) for 8 days over a 10-day period. BSA was administered for 5 consecutive days with increasing doses (2, 4, 6, 8, and 10 mg/g body wt) followed by the maximum daily dose of 10 mg/g body wt recommenced from day 8 onwards. Untreated...
Limp-2−/− mice were used as positive controls for inadequate tubular proteolysis. For examination of the tubular uptake of intravenously injected Alexa-conjugated BSA (Alexa-BSA) and retinol-binding protein (RBP; Alexa-RBP), control groups receiving intraperitoneal saline injections of corresponding volumes were used for comparison. Untreated WT and Limp-2−/− mice were used as controls for the other experiments. BSA-treated WT mice on days 0 and 10, as well as Limp-2−/− mice without BSA treatment, were placed in metabolic cages for 8 h for urinary analysis of proteins, albumin, and creatinine. Mice were then anesthetized, and kidneys were harvested, either fixed in neutral buffered formalin or snap-frozen in liquid nitrogen, and stored at −80°C until analyzed. Urine mouse albumin and creatinine were measured by ELISA (Bethyl Laboratories) and HPLC (10), respectively.

Alexa Fluor 555-conjugated BSA injections. Mice were injected intravenously with 10 μg/g body wt of Alexa Fluor 555-labeled BSA (Alexa-BSA; Molecular Probes) into the tail vein, as described previously (11). Mice were then anesthetized with kidneys harvested at 7 and 30 min and fixed in neutral buffered formalin.

Alexa Fluor 488-conjugated RBP injections. RBP isolated from human urine (GenWay Biotech) was reconstituted in PBS and dialyzed against 100 vol PBS to remove the ammonium carbonate. RBP was then labeled using the Alexa Fluor 488 protein labeling kit (Molecular Probes). One milliliter of protein (at 3 mg/ml) was added to Alexa Fluor 488 and incubated in the dark at room temperature with constant stirring. Excess label was removed by dialysis in 2 × 1,000 vol PBS, and the concentration of RBP was adjusted to 1 mg/ml. Mice were injected intravenously with 5 μg/g body wt of Alexa Fluor 488-conjugated RBP (Alexa-RBP) into the tail vein and then anesthetized. Kidneys were harvested at 30 min and fixed in formalin.

Antibodies. Primary antibodies used included rat anti-LAMP-1 (DSHB), rat anti-cathepsin L (R&D Systems), goat anti-cathepsin B, goat anti-megalin (Santa Cruz), and rabbit anti-β-actin (Cell Signaling). For proximal tubular proteins, rabbit anti-transferrin, rabbit anti-Gc globulin [vitamin D-binding protein (VDBP)], and rabbit anti-RBP (all from DAKO) and goat anti-mouse albumin (Bethyl Laboratories) were used. Significant cross-reactivity with bovine albumin was excluded as the concentration for 1 mg/ml of BSA was measured to be <7.8 ng/ml by ELISA. Secondary antibodies were directed against the primary antibody species and conjugated with horseradish peroxidase (DAKO) and Alexa 488 or Alexa 594 (Molecular Probes) for Western blotting and immunofluorescence microscopy, respectively.

Immunoblotting studies. For urine protein analysis, the urine volume loaded was corrected for creatinine concentration. For assessing the proximal tubular expression of cathepsin B and L, and the quantities of RBP in the kidney cortex, cortex-enriched kidney lysates were used. Samples were separated on 12.5% SDS-PAGE gels. For Western blot analysis, proteins were electrophoretically transferred from the SDS-PAGE gel to PVDF membrane (Millipore), washed, and blocked and immunoreactive proteins were detected with Western Lightning ECL enhanced chemiluminescence substrate (PerkinElmer) according to the manufacturer’s instructions. Densitometry, as a semiquantitative analysis of the expression of immunoreactive proteins, was performed by the software NIH ImageJ 1.34 (http://rsweb.nih.gov/ij), using the “Gel Analysis” functions.

Immunofluorescence microscopy. Four-micrometer paraffin-embedded kidney sections were incubated with primary antibody in 10% BSA [CAS Block (Invitrogen) in the case of anti-BSA antibody] overnight at 4°C, washed three times in PBS, and then incubated in secondary antibodies conjugated with Alexa 594 (Red) or Alexa 488 (Green) in 10% BSA for 30 min. Following three further washes in PBS, sections were mounted with fluorescent mounting medium (DAKO). Images were generated and collected on a confocal laser microscope (Zeiss LSM 510 Pascal).

Quantification of distribution and colocalization of proteins. For tubular protein distribution studies, the fluorescence of RBP in the PCT was quantified using the software NIH ImageJ 1.34, as described previously (11). Briefly, a region of interest was drawn for the apical (excluding the lumen) and basal areas, respectively, divided by a circumferential line midway between the brush border and basolateral surface of the cross section of each PCT, adjacent to the glomerulus. The fluorescence intensity was measured for the apical and basal halves of each tubule, and the mean intensity was calculated. The mean intensity of the background fluorescence was determined by measuring the region of interest in the basal region of each tubule clear of RBP vesicles. The corrected mean fluorescence intensity was calculated by subtracting the background intensity from that of the apical and basal halves of each tubule respectively. A ratio of the corrected basal:apical fluorescence intensity was then calculated for each tubule.

For colocalization studies, the proportion of RBP colocalizing with LAMP-1 or cathepsin B in the PCT was calculated from single optical sections through PCT, all acquired under the same settings, including pinhole (optical section thickness of 0.8 μm) on the confocal microscope. With the use of the ImageJ plugin JACoP (http://rsweb.nih.gov/ij/plugins/jacop.html) as described previously (11), after adjusting the threshold to minimize background noise while retaining the signal of the vesicles, performed in a similar blinded fashion for all tubules by the same operator, Mander’s coefficient was generated as the ratio of the area of pixels of RBP colocalizing with LAMP-1 or cathepsin B to that of the total RBP in each tubule.

Three blinded experiments were performed, and at least 18 proximal tubules in total from at least 3 animals in each group were analyzed. Statistical analysis was performed on the number of proximal tubules in each group.

Real-time PCR. Total RNA was purified from cortex-enriched mouse kidney samples by TRIzol reagent (Invitrogen) in accordance with the manufacturer’s instructions. RNA was then quantified using spectrophotometry and reverse transcribed using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). Real-time PCR was performed on a Stratagene MX3000 real-time PCR system with Solis Biodyne EvaGreen master mix, according to the manufacturer’s instructions. The sequences for the primers used were megalin 5′-AGGCCACAGTTCATGTTG-3′ (sense) and 5′-AGGACACGCCCTATCTCTGTG-3′ (antisense), cubulin 5′-GGAGTTCTTCTCAGGACACA-3′ (sense) and 5′-GATCCGAGGGCTCCTAAAC-3′ (antisense), and the housekeeping gene β-actin 5′-GGGATCCCCGCCCTAGGCACCAGGGTG-3′ (sense) and 5′-GAGTTCCTGCCGGAGGCTTCAAAAC-3′ (antisense). Primer efficiency was measured using standard dilution, and the Pfaffl method (29) was used to calculate relative expression. Data were expressed as fold expression relative to untreated WT controls.

Statistical analysis. GraphPad InStat v.3.06 (GraphPad Software, La Jolla, CA) was used for data analysis. Statistical analysis was performed by unpaired student t-tests with Welch correction for comparisons between two experimental groups for data with parametric distributions (or Mann-Whitney test for data with nonparametric distributions). Kruskal-Wallis test with multiple comparison posttest was used for comparisons across more than two data sets. Error bars represented 1 SD. Data were expressed as means ± SD. A P value of <0.05 was considered significant.

RESULTS

Tubular proteinuria in WT mice following BSA overload. We have previously reported that Limp-2−/− mice had tubular proteinuria and a 13-fold increase in urinary albumin excretion associated with reduced proteolysis in the PCT (11). Following BSA overload at day 10, WT mice developed significantly increased albuminuria (day 0: 17 ± 17; day 10: 271 ± 278 mg/mmol; P < 0.01) to levels not dissimilar to those seen in control Limp-2−/− mice (day 0: 131 ± 98 mg/mmol; P < 0.05;
Fig. 1A). To determine whether there was loss of other proteins usually reabsorbed by the megalin/cubilin receptor complex in the PCT, urine samples were immunoblotted for transferrin, VDBP, and RBP. As expected, no or minimal amounts of these proteins were present in WT mouse urines at day 0. However, each was detected at day 10 of BSA treatment at levels not dissimilar to those seen in untreated Limp-2−/− mice (Fig. 1B). Quantitative analysis by densitometry confirmed the increase in urinary loss of these proteins from WT mice at day 10 compared with day 0 of BSA overload (P < 0.05; Fig. 1C).

The data suggest, therefore, that competition from exogenous BSA exceeded the capacity of proximal tubular uptake or degradation.

**Proximal tubular handling of endogenous mouse albumin and RBP.** Unlike most other urinary proteins, proximal tubular reabsorption of albumin has recently been demonstrated as requiring both megalin and cubulin (1). To determine whether the mouse albuminuria induced by BSA overload was a result of inadequate uptake of filtered proteins by the PCT, immunolabeling for endogenous mouse albumin was performed. Confocal microscopy showed that mouse albumin in the PCT was not reduced but appeared more abundant in the early segments of PCT adjacent to the glomeruli in BSA-treated WT and control Limp-2−/− mice, compared with control WT mice (Fig. 2A). This could be a result of either increased uptake or inadequate degradation. The tubular distribution of endocytosed albumin was, however, largely apically restricted across all three groups, as indicated in the cross sections of the early segments of PCT (Fig. 2A). Most of the albumin immunolabeling was restricted within the peritubular capillaries and along the glomerular capillary walls. No or minimal albumin in the distal segments of the PCT was observed from any of the three groups (Fig. 2A).

Unlike albumin, RBP is a low molecular weight (LMW) protein that is freely filtered and reabsorbed by the PCT exclusively via a megalin-dependent pathway (1, 8). Confocal microscopy showed that similar to mouse albumin, RBP in the PCT was not reduced in BSA-treated WT animals compared with untreated WT or Limp-2−/− mouse controls (Fig. 2B). As previously described (11), the RBP vesicles were distributed more basolaterally in control Limp-2−/− mice in contrast to the apical distribution seen in control WT mice (Fig. 2B), and this was confirmed by quantification of the immunofluorescence signal (P < 0.001; Fig. 2C). RBP vesicles were also distributed significantly more towards the basolateral cell surface in the early segments of PCT of BSA-treated WT mice when compared with their controls (P < 0.05; Fig. 2, B and C), although not to the same extent seen in untreated Limp-2−/− mice (P < 0.05; Fig. 2, B and C). No RBP was detected in the more distal segments of the PCT that were not in close proximity to the glomeruli in any of the three groups (data not shown). Western blots of cortical kidney lysates showed an increased quantity of RBP in kidneys from BSA-treated mice compared with their controls (Fig. 2D), which was statistically significant when quantified by densitometry (P < 0.05; Fig. 2E). The quantity of RBP in kidneys from untreated Limp-2−/− mice also appeared to be increased (Fig. 2D), although this did not reach statistical significance (Fig. 2E). These findings suggest a delay in lysosomal processing of endocytosed RBP within the PCT of mice treated with BSA, resembling the findings in Limp-2−/− mice (11), but do not exclude reduced uptake of RBP by the PCT.
Fig. 2. Endogenous mouse albumin and RBP distribution and quantification in the kidney. A: representative immunofluorescence microscopy showed increased quantity of mouse albumin in early segments of proximal convoluted tubule (PCT) adjacent to the glomeruli (labeled “G”) of BSA-treated WT (+/+ BSA) and control Limp-2−/− (−/− Con) mice compared with control WT mice (+/+ Con; top). The majority of albumin is restricted surrounding the peritubular capillaries and along the glomerular capillary walls. Distribution of mouse albumin remained apical in the cross sections of PCT from all 3 mouse groups (middle). No or minimal mouse albumin was observed in the distal segments of PCT (bottom). Scale bars = 30 μm (top and bottom) and 10 μm (middle). B: representative immunofluorescence microscopy of RBP showed similar tubular uptake in the early segments of PCT adjacent to the glomeruli (labeled “G”) but more basolaterally distributed in BSA-treated WT mice (+/+ BSA) compared with WT controls (+/+ Con), similar to control Limp-2−/− mice (+/+ Con) (top), better appreciated in magnified images of the cross sections of PCT (bottom). Scale bars = 20 μm (top) and 10 μm (bottom). C: quantification of basal to apical distribution of RBP fluorescence signal confirmed the more basal distribution of RBP in BSA-treated WT mice (+/+ BSA, grey bar) compared with their controls (+/+ Con, black bar). Distribution, however, remained more basal in control Limp-2−/− mice (−/− Con, white bar) when compared with BSA-treated WT mice. **P < 0.001; *P < 0.05. Animals examined per group N = 3–4. PCTs examined per group were n = 17, 23, and 24 for WT controls, WT BSA-treated, and Limp-2−/− controls, respectively. D: representative Western blot analysis of cortical kidney lysates immunoblotted for RBP, with β-actin as a loading control, showed an increased quantity of RBP in kidney cortex from BSA-treated WT mice (+/+ BSA) compared with WT controls (+/+ Con). It also appeared that more RBP was present in kidney cortex from Limp-2−/− controls (−/− Con) compared with BSA-treated WT mice. E: densitometry of Western blots for RBP corrected for β-actin confirmed the increased quantity of RBP in BSA-treated WT mice (+/+ BSA, grey bar) compared with their controls (+/+ Con, black bar). Difference between WT and Limp-2−/− controls (−/− Con) was not statistically significant. *P < 0.05; N = 7 for WT controls and WT BSA-treated, and N = 4 for Limp-2−/− controls.
Fig. 3. Representative immunofluorescence microscopy of proximal tubular uptake and degradation of Alexa-BSA and Alexa-RBP. 

A: tubular uptake of Alexa-BSA (red), 7 min after the intravenous injection in BSA-treated WT mice (+/+ BSA) was not reduced compared with saline-treated WT (+/+ Con) or Limp-2<sup>-/-</sup> (+/- Con) controls. Megalin (green) was colabeled to illustrate the apical surface of PCT. Scale bars = 10 μm. B: at 30 min, there was persistence of basolateral Alexa-BSA vesicles in the early segments of PCT adjacent to the glomeruli (labeled “G”) in BSA-treated WT mice (+/+ BSA) compared with WT controls (+/+ Con), where most of the injected Alexa-BSA appeared to have been degraded. The persistence of basolateral Alexa-BSA vesicles in BSA-treated WT mice (+/+ BSA) was, however, not to the same extent seen in Limp-2<sup>-/-</sup> controls (+/- Con) (top), better appreciated in the magnified images of cross sections of PCT (middle). Distal segments showed no detectable Alexa-BSA in any of the mouse groups (bottom). Scale bars = 30 μm (top and bottom) and 10 μm (middle).

C: tubular distribution of Alexa-RBP vesicles (green) 30 min after the intravenous injection was largely restricted to the apical surface in WT controls (+/+ Con). There was an excess of larger and basolaterally distributed Alexa-RBP vesicles spread across the PCT in BSA-treated WT (+/+ BSA) compared with control WT mice, although not to the same extent seen in Limp-2<sup>-/-</sup> controls (+/- Con). Glomeruli were labeled “G” (top). The difference in tubular distribution was more clearly demonstrated in magnified view of cross sections of PCT (middle). These appearances were similar to those of Alexa-BSA at 30 min. No or minimal Alexa-RBP was detected in the distal segments of PCT, although slightly more abundant signals were present in BSA-treated WT mice (bottom). Megalin (red) was colabeled. Scale bars = 30 μm (top and bottom) and 10 μm (middle).
persistence of basolaterally distributed Alexa-BSA vesicles in the early segments of PCT of BSA-treated WT mice compared with WT controls, although not to the extent seen in saline-treated Limp-2<sup>−/−</sup> mice (Fig. 3B). This suggests delayed proteolysis of endocytosed albumin. In the more distal segments still expressing megalin, no Alexa-BSA was identified in any of the three groups (Fig. 3B).

To determine whether inadequate tubular degradation of reabsorbed proteins applied to filtered proteins other than albumin, the mice were injected with intravenous Alexa-RBP and kidneys harvested at 30 min. Confocal microscopy revealed a similar basolateral distribution of undegraded exogenous RBP in the early segments of PCT from BSA-treated WT animals, in contrast to the apical pattern seen in their respective controls, although it was not as prominent as in Limp-2<sup>−/−</sup> mice (Fig. 3C). Similar to the findings of Alexa-BSA, no or minimal Alexa-RBP was observed in the distal segments from all three mouse groups; however, more abundant Alexa-RBP appeared to be present in some areas from BSA-treated WT mice compared with controls (Fig. 3C). The data indicate that delayed proteolysis of endocytosed proteins induced by BSA overload is nonselective, perhaps resulting in urinary loss of proteins other than albumin that are normally reabsorbed by the PCT.

**Megalin expression.** In view of the importance of megalin and cubilin as receptors for proximal tubular reabsorption of urinary proteins, and evidence of reduced megalin expression in cells cultured in high albumin concentrations (6), expression of megalin and cubilin was examined. Quantification of mRNA transcripts for megalin and cubilin showed no difference between any of the three groups of mice (Fig. 4A). Similarly, confocal microscopy using anti-megalin antibody showed no reduction in apical expression in the PCT of BSA-treated WT mice compared with untreated controls (Fig. 4B). No significant intracellular expression of megalin was observed. A change in megalin expression, therefore, was considered unlikely to account for the increase in urinary protein loss seen in WT mice following BSA overload. This was supported by the earlier findings of unchanged tubular uptake of Alexa-BSA at 7 min.

**Expression of lysosomal hydrolases.** Proteins reabsorbed by the PCT are destined for lysosomal degradation (25). Data reported by Olbricht et al. (28) demonstrated increased cathepsin B and L activity in the PCT following LMW protein or dextran overload in rats. Differences in the intrarenal expression of lysosomal hydrolases could, therefore, affect the capacity of the PCT to digest endocytosed proteins. Cortical kidney lysates were immunoblotted for the lysosomal hydrolases cathepsin B and L. Cathepsin L expression was significantly increased in WT mice receiving BSA (Fig. 5A) compared with controls (<i>P</i> &lt; 0.05) (Fig. 5B). Intrarenal cathepsin B expression was, however, unchanged following BSA overload (Fig. 5, A and C).

**Lysosomal structures and trafficking in the proximal tubule.** To evaluate differences in late endosomal/lysosomal structures, immunofluorescence microscopy was used to study the lysosomal membrane protein LAMP-1 and lysosomal hydrolase cathepsin B in the PCT. In control WT mice, both

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**Fig. 4.** Megalin and cubilin expression. <i>A</i>: quantitative RT-PCR revealed no difference in the mRNA expression of megalin or cubilin in the kidney cortex from control WT (+/+ Con, black bars), BSA-treated WT (+/+ BSA, grey bars), and control Limp-2<sup>−/−</sup> mice (−/− Con, white bars). <i>N</i> = 5–6 in each group. <i>B</i>: representative immunofluorescence microscopy showed no difference in the apical expression of megalin in PCT among all 3 groups. Scale bars = 10 μm.

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**Fig. 5.** Expression of lysosomal hydrolases. <i>A</i>: representative Western blots of cathepsin B and L expression in cortical kidney lysates from control WT (+/+ Con), BSA-treated WT (+/+ BSA), and control Limp-2<sup>−/−</sup> mice (−/− Con), with β-actin as a loading control. <i>B</i> and <i>C</i>: densitometry of Western blots of cathepsin L (<i>B</i>) and cathepsin B (<i>C</i>) expression corrected for β-actin. Significant upregulation of cathepsin L was noted in BSA-treated WT mice (+/+ BSA, grey bar) compared with WT (+/+ Con, black bar) and Limp-2<sup>−/−</sup> (−/− Con, white bar) controls, while there was no significant difference in cathepsin B expression among the 3 groups. <i>*P</i> &lt; 0.05; <i>N</i> = 4 in each group.
LAMP-1 and cathepsin B vesicles were largely restricted to the apical surface. Following BSA treatment, the abundance of LAMP-1 and cathepsin B vesicles appeared unchanged. Some of the LAMP-1 and cathepsin B vesicles, however, were more basally distributed across the PCT, although not to the degree seen in control Limp-2−/− mice (Fig. 6, A and D). These appearances were similar to the altered tubular distribution of RBP observed in BSA-treated WT mice (Fig. 2B).

To determine whether the accumulation of endocytosed proteins in mice treated with BSA was due to a failure of fusion of lysosomes with endosomes, so resembling the situation previously identified in Limp-2−/− mice, colocalization studies of endogenous RBP with the late endosomal/lysosomal markers LAMP-1 or cathepsin B were performed. Late endosomes possess LAMP-1 as well as lysosomal hydrolases such as cathepsin B. The majority of hydrolases are thought to be stored in small dense lysosomes (33), and therefore, LAMP-1 and cathepsin B may be more specific markers for late endosomes and lysosomes, respectively (11). There was a similar degree of colocalization of RBP with LAMP-1 in the PCT from all three groups (Fig. 6B), more clearly demonstrated in the magnified images (Fig. 6C), further confirmed by quantification studies (Fig. 6G). As previously shown (11), there was an excess of RBP vesicles that did not colocalize with cathepsin B in the PCT of Limp-2−/− mice compared with control WT mice (Fig. 6E), better appreciated in the magnified images (Fig. 6F). Quantification studies confirmed a lower proportion of RBP that colocalized with cathepsin B in Limp-2−/− mice (P < 0.001; Fig. 6G), suggesting reduced fusion of cathepsin B–containing lysosomes with endosomes containing reabsorbed RBP. In BSA-treated WT mice, however, there was no increase in RBP that failed to colocalize with cathepsin B (Fig. 6, E−G), as occurred in the Limp-2−/− mice. The mechanism of insufficient proteolysis seen in the BSA-treated WT mice was different to that seen in Limp-2−/− mice, as the endocytosed proteins were able to enter the late endosomal/lysosomal compartments but their degradation, once there, was delayed.

**DISCUSSION**

In protein overload models, it has been proposed that tubular proteinuria develops as a result of competition for uptake of filtered proteins (16, 17, 22). In this short-term albumin overload model, WT mice developed a significant increase in mouse albuminuria and urinary loss of ligands of megalin, to levels similar to those seen in Limp-2−/− mice, which have known tubular proteinuria (11). There was, however, no evidence of inadequate tubular uptake of filtered proteins due to the competition from BSA. This was supported by the unchanged quantity of intravenously injected Alexa-BSA at 7 min in the early segments of PCT compared with controls, suggesting the high capacity of the megalin/cubilin receptor complex when challenged with BSA overload. Furthermore, megalin expression in the PCT was unchanged. This is in contrast to in vitro studies showing reduced albumin uptake in cultured proximal tubular cells (20) due to reduced expression of megalin (6). A recent study in dogs with progressive glomerular disease from X-linked Alport Syndrome demonstrated increasing LMW proteinuria associated with increased shedding and urinary loss of megalin and cubilin and reduced endocytosis of their ligands (36). The absence of a change in megalin expression or tubular uptake of Alexa-BSA in the current study might be due to the short duration of the study without histological evidence of tubular damage (data not shown).

BSA-treated WT mice demonstrated features of inadequate degradation of reabsorbed proteins resembling those seen in Limp-2−/− mice. Following BSA overload, WT mice developed an increase in the quantity of RBP within the kidney cortex, increased basal tubular distribution of endogenous RBP, and increased abundance of endogenous mouse albumin in the PCT, and there was persistence of exogenous Alexa-BSA and Alexa-RBP in the PCT at 30 min. The lack of change in the tubular distribution of endogenous albumin, in contrast to RBP, might be due to the much lower permeability of albumin at the glomerulus (27, 34), resulting in less filtered mouse albumin competing with the administered BSA for tubular uptake and degradation. The lower glomerular permeability of mouse albumin, compared with the extensively filtered RBP, is supported by the observation in this study that it was predominantly restricted within the peritubular capillaries and along the glomerular capillary walls. Most of the changes in the abundance and distribution of endocytosed proteins were observed in the early segments of PCT. In the more distal segments, no or minimal immunoreactive proteins were detected in the PCT. This indicates that no significant endocytic uptake occurred in the distal segments of PCT as a result of albumin overload in this study.

Alexa-BSA fluorescence within cells of the PCT at 7 min postinjection presumably reflects a balance between protein uptake and degradation. In the setting of a delay in degradation of endocytosed proteins, it is possible that a reduction in the rate of uptake of Alexa-BSA also occurred in the PCT of BSA-treated WT mice, resulting in no net change in fluorescence. On the other hand, it is also possible that albumin overload induced an increase in Alexa-BSA uptake by the PCT at 7 min that was not observed due to more rapid protein degradation, and the appearance of more abundant labeled proteins at 30 min might simply reflect an increase in tubular processing of an increased quantity of endocytosed proteins. It has recently been proposed, however, that endocytic tracer arrives in the early endosomes after 1–5 min, late endosomes after 10–15 min, and lysosomes after 30 min (32). In addition, it has previously been demonstrated in mice with defective proteolysis but intact endocytosis that the quantity of fluorescence-labeled proteins in the proximal tubular cells was not increased at 7–10 min but increased at 30 min when compared with normal proximal tubular cells (11, 37), similar to findings in the Limp-2−/− mouse controls used in this study. This suggests that endocytosis is the predominant process at 7 min after injection of a tracer, while at 30 min the balance tips towards degradation as the more important process. Nevertheless, a complete separation of protein uptake and degradation is not possible.

Consistent with previous studies (28), cathepsin L expression was increased in the kidney cortex of BSA-treated WT mice. Despite this, proteolysis in the PCT was apparently still inadequate to process the increased load of endocytosed proteins. Unlike cathepsin L, cathepsin B expression was unchanged, perhaps due to a disturbance in the recycling mechanism of tubular uptake and lysosomal targeting of filtered proteins.
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**Figure 1:** TUBULAR PROTEINURIA AND PROTEOLYSIS

**A** LAMP-1

**B** LAMP-1 & RBP

**C** LAMP-1 & RBP

**D** Cathepsin B

**E** Cathepsin B & RBP

**F** Cathepsin B & RBP

**G** Mander’s coefficient

**Legend:**

- **+/+ Con:** Wild-type control
- **+/+ BSA:** Wild-type with BSA
- **/- Con:** Knockout control

**Graph:**

- **RBP with LAMP-1**
- **RBP with Cat B**

**Statistical Significance:**

- ***:** Significant difference at p < 0.05
procathespin B, which has previously been demonstrated in ClC-5-deficient mice (26).

An increase in creatinine clearance was observed compared with previously published data for normal mice using the same methodology (418 ± 160 vs. 255 ± 68 l/min) (14). Hyperfiltration could lead to increased filtration and, therefore, increased urinary excretion of proteins. However, this is unlikely to account for the increased urinary excretion of RBP and other endogenous proteins in this study because the volume of urine loaded to the SDS-PAGE gels was corrected for urinary creatinine concentrations, and mouse albuminuria measured by ELISA was also corrected for creatinine concentration and reported as urinary mouse albumin-to-creatinine ratios. It is possible that hyperfiltration could lead to increased delivery and subsequent uptake of labeled RBP and albumin by the PCT, resulting in the persistence and basal distribution of partially degraded but immunoreactive proteins. However, the findings in this study still support the key observation that the degradation of endocytosed proteins was not sufficient to match the rate of tubular uptake (whether unchanged or increased), associated with increased urinary loss. Even if the partially degraded proteins were immunoreactive, it would still suggest that tubular proteolysis was inadequate to match the uptake of proteins when compared with control WT mice.

Western blotting identified an increase in the quantity of RBP within the kidney cortex of BSA-treated WT mice, supporting inadequate degradation. The vast majority of RBP is synthesized in the liver and extensively filtered at the glomerulus. No RBP is detectable in the PCT of megalin-deficient mice (8), suggesting that it is unlikely for PCT to endogenously synthesize a significant quantity of RBP. In the current study, endogenous RBP was detected in only a limited number of the early segments of PCT in all three mouse groups. However, the possibility that albumin overload induced synthesis of RBP in the PCT leading to the increased quantity identified in the kidney cortex cannot be completely excluded.

Limp-2−/− mice have defective proteolysis due to failure of fusion of lysosomes with endosomes containing reabsorbed proteins in the PCT, leading to tubular proteinuria (11). Interestingly, these mice have normal expression of megalin and cubulin and show no defect in the tubular uptake of filtered proteins. We proposed that reabsorbed but undegraded proteins were recycled to the brush border along with their receptors megalin and cubulin (7) and subsequently released back into the tubular lumen. However, there is no direct evidence for this proposal although such a mechanism is known to exist for megalin (7). WT mice subjected to protein overload also exhibited features of inadequate proteolysis in the PCT. Endocytosed RBP colocalized with LAMP-1, expressed in lysosomes as well as the late endosomes before fusion with lysosomes, to the same degree in control WT and Limp-2−/− mice. In addition to this, the appearance of basolateral LAMP-1 and cathepsin B in some areas of the PCT suggests that the basolateral vesicles containing RBP might have been late endosomes and lysosomes. The difference when compared with Limp-2−/− mice, however, was that endosomes containing reabsorbed proteins were still able to fuse with lysosomes, as evidenced by the unchanged degree of colocalization of endocytosed RBP with lysosomes labeled with cathepsin B. Whether the mechanism proposed for tubular proteinuria in Limp-2−/− mice would be applicable to mice with BSA overload, therefore, is arguable.

Proteinuria is a predictor for progression of human kidney disease (31) and correlates with the degree of tubulointerstitial injury, itself predictive of the likelihood of progression (9). Investigating how the proximal tubules handle an increase in filtration of proteins may, therefore, help understand the mechanism of tubular injury in proteinuric kidney disease. A previous in vivo study showed reduced expression of tubular injury markers from megalin-deficient proximal tubular cells following induction of nonselective proteinuria (24). In the current study of protein overload, the reabsorption of filtered proteins was likely limited by the capacity of protein degradation rather than tubular uptake. Although the study was not designed to address this issue specifically, the findings suggest that inadequate degradation of reabsorbed proteins may contribute to tubular injury.

In summary, we have demonstrated that normal mice develop tubular proteinuria following BSA overload. This was associated with delayed degradation of endocytosed proteins, but no abnormality in albumin uptake. It would suggest that, in the setting of increased glomerular leakage of proteins, proteinuria may develop as a result of the limited capacity of lysosomes in the proximal tubules to process an increased load of endocytosed proteins.

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GRANTS

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Fig. 6. Tubular distribution of LAMP-1 and cathepsin B and colocalization studies of RBP with LAMP-1 and cathepsin B. A: representative immunofluorescence microscopy of LAMP-1 vesicles showed a small area of more basolateral distribution (inside the boxes) in the PCT of BSA-treated WT mice (+/+ + BSA) compared with apical restriction seen in WT controls (+/+ Con), although not to the same extent in Limp-2−/− controls (−/− Con). B: colocalization studies of RBP (in red) with LAMP-1 (in green) showed good colocalization in all 3 groups. C: magnified images of areas inside the boxes in B. D: representative immunofluorescence microscopy of cathepsin B vesicles similarly showed more basolateral distribution (area inside the box) across some areas of the PCT in BSA-treated WT mice (+/+ + BSA) compared with the restriction to the apical surface in WT controls (+/+ Con), although not to the same extent seen in Limp-2−/− controls (−/− Con). E: colocalization studies of RBP (in red) with cathepsin B (in green) demonstrated good colocalization of RBP with cathepsin B in both control (+/+ Con) and BSA-treated WT (+/+ BSA) mice, in contrast to the excess of RBP not colocalizing with cathepsin B seen in the PCT of Limp-2−/− controls (−/− Con). F: magnified images of areas inside the boxes in E. Scale bars = 10 μm (A, B, D, and E), 20 μm (C and F). G: quantification studies of colocalization showed that there was a lower proportion of RBP colocalizing with cathepsin B, measured by the Mander’s coefficient. In control mice, RBP within the PCT was present in low abundance in the setting of increased glomerular leakage of proteins, and subsequent uptake of labeled RBP and albumin by the PCT, resulting in the persistence and basal distribution of partially degraded but immunoreactive proteins. However, the findings in this study still support the key observation that the degradation of endocytosed proteins was not sufficient to match the rate of tubular uptake (whether unchanged or increased), associated with increased urinary loss. Even if the partially degraded proteins were immunoreactive, it would still suggest that tubular proteolysis was inadequate to match the uptake of proteins when compared with control WT mice.
DISCLOSURES

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AUTHOR CONTRIBUTIONS


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