Imaging mass spectrometry reveals direct albumin fragmentation within the diabetic kidney

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Albumin degradation in the renal tubules is impaired in diabetic nephropathy such that levels of the resulting albumin fragments increase with the degree of renal injury. However, the mechanism of albumin degradation is unknown. In particular, fragmentation of the endogenous native albumin has not been demonstrated in the kidney and the enzymes that may contribute to fragmentation have not been identified. To explore this we utilized matrix-assisted laser desorption/ionization imaging mass spectrometry for molecular profiling of specific renal regions without disturbing distinct tissue morphology. Changes in protein expression were measured in kidney sections of eNOS$^{-/-}$ *db/db* mice, a model of diabetic nephropathy, by high spatial resolution imaging allowing molecular localizations at the level of single glomeruli and tubules. Significant increases were found in the relative abundances of several albumin fragments in the kidney of the mice with diabetic nephropathy compared with control nondiabetic mice. The relative abundance of fragments detected correlated positively with the degree of nephropathy. Furthermore, specific albumin fragments accumulating in the lumen of diabetic renal tubules were identified and predicted the enzymatic action of cathepsin D based on cleavage specificity and *in vitro* digestions. Importantly, this was demonstrated directly in the renal tissue with the endogenous nonlabeled murine albumin. Thus, our results provide molecular insights into the mechanism of albumin degradation in diabetic nephropathy.

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While diabetic nephropathy (DN) takes years to develop, it is largely irreversible and remains a major health complication for diabetic patients. The pathogenic mechanisms underlying this disease remain unclear. Uncovering molecular events that define disease progression can improve early detection and provide potential targets for drug therapy. Proteomic technologies have been used in this vein to discover proteins and pathways that contribute to the pathophysiology of the kidney. Previous proteomic studies have looked for biomarkers in the urine and blood.[1–4] A number of studies have analyzed renal tissues directly using either total kidney lysates or laser capture micro-dissection technique.[5–8] However, the ability to localize molecular changes in the morphologically intact renal tissue, at the site of disease, provides a more specific insight into disease mechanisms.[9–12]

It is well established that processing of albumin is mishandled in the diabetic kidney. Detection of albumin in the urine indicates kidney damage and is in fact the main diagnostic tool used to assess early disease and disease progression.[13,14] In healthy kidneys, only a small amount of albumin passes through the glomerular filter. The majority of it is then reabsorbed by the proximal tubules and degraded, while the remainder is excreted in the urine.[15] While intact urinary albumin is the main measure in the clinic, more studies are showing that albumin fragments increase as well, and these are not always detected by traditional assays.[16–18] The origin of albumin fragments is of interest, but the mechanism of albumin fragmentation is unknown. Some studies suggest fragments are made in the urine by urine proteases,[19,20] while others found fragments present in the kidney as well.[21–24] However, the fragmentation of the endogenous native albumin has not been demonstrated in the kidney, and the enzymes that may contribute to fragmentation have not been identified.

In the present study, we used matrix-assisted laser desorption/ionization (MALDI) imaging mass spectrometry (IMS) for molecular profiling of specific tissue regions

without disturbing the distinct tissue morphology.[25] Briefly, IMS acquires mass spectra at discrete coordinates directly from an intact tissue coated in an organic matrix. The data set can be queried to monitor the location of molecules spatially where the relative intensity of the molecular ion detected is represented in color as a heat map. A more detailed description of the MALDI IMS workflow is depicted in Supplementary Figure S1.[26] High–spatial resolution IMS, sufficient to analyze individual renal glomeruli and tubules, was performed to determine albumin fragments in the kidney of the eNOS$^{-/-}$ *db/db* mouse, a DN model.[27,28] This is the first report showing localization of endogenous unlabeled albumin fragments in the kidney with the identification of the fragments and the protease involved, cathepsin D, using specificity of the cleavage sites, immunohistochemistry (IHC) staining, and *in vitro* digestions.

RESULTS

Albumin excretion in eNOS$^{-/-}$ db/db mice

As expected, eNOS$^{-/-}$ *db/db* mice developed hyperglycemia and albuminuria by 12 weeks of age, which significantly progressed by 22 weeks of age (Figure 1).

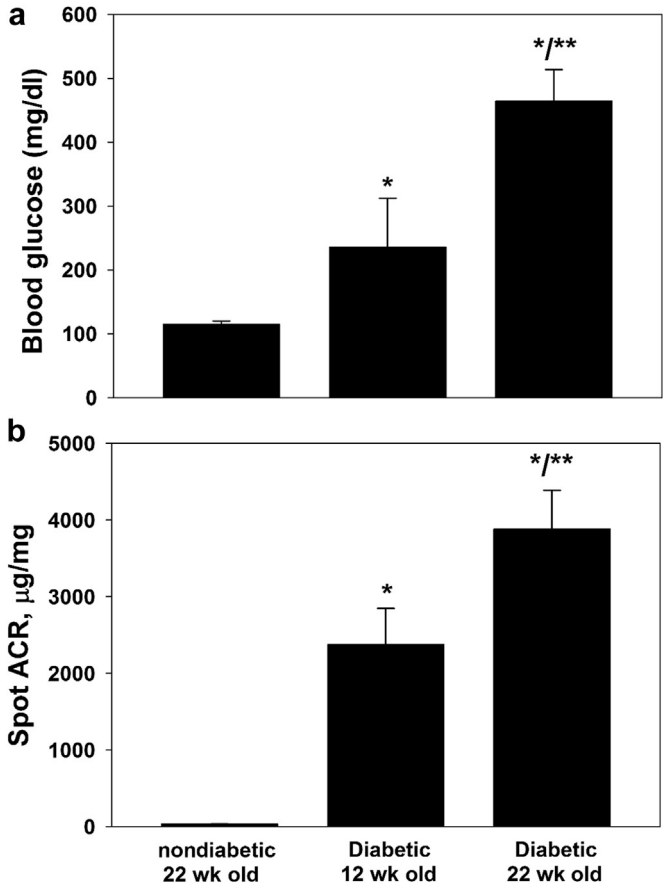

Figure 1 | Hyperglycemia and albuminuria in diabetic mice. (**a**) Blood glucose levels were determined in nondiabetic wild-type mice and eNOS$^{-/-}$ *db/db* mice as described in the Materials and Methods section. (**b**) Urinary albumin-to-creatinine ratio (ACR) was determined in nondiabetic wild-type mice and in eNOS$^{-/-}$ *db/db* mice as described in the Materials and Methods section. Each bar graph represents the mean ± SEM ($n = 8$). *$P < 0.05$ versus nondiabetic; **$P < 0.05$ versus 12-week-old diabetic.

Detection and identification of albumin fragments in the kidney

We have used IMS to determine the presence and localization of albumin fragments in the kidney of diabetic mice and nondiabetic controls. Several albumin fragments were detected directly from the kidney section of a diabetic kidney (Figure 2a). Based on accurate mass measurements and tandem mass spectrometry (MS/MS) experiments (Figure 2b), 18 peaks were determined to originate from albumin (Table 1). Interestingly, all of the detected albumin fragments originated from 3 surface regions of the albumin molecule (Supplementary Figures S2 and S3).

Imaging albumin fragments with IMS

Kidneys from control, 12-week DN, and 22-week DN mice were imaged at either 10 µm or 25 µm spatial resolution, and albumin fragments were tracked. We found that degradation of albumin was increased in the DN kidney and was not detected in control tissue. Additionally, the relative abundance of fragments increased with DN progression (Figure 3). Several selected fragments had specific localized distributions within the diabetic kidney (Figure 3).

Immunofluorescence staining and tubular localization of albumin fragments

After determining the presence of albumin fragments in the DN kidney, we wanted to determine localization of these fragments to specific renal structures. First we found that albumin fragments were not detected in glomeruli by comparing IMS images with periodic acid–Schiff staining of the same section (Figure 4a) and confirmed tubular localization of the fragments using 10-µm spatial resolution MALDI IMS (Figure 4b). Next, we compared IMS images with immunofluorescence tissue staining for tubular specific proteins (Figure 4c and d). Albumin fragments identified by IMS colocalized with dolichos biflorus agglutinin, a distal tubule and collecting duct marker (Figure 4c), and lotus tetragonolobus lectin, a proximal tubule marker (Figure 4d).

Major N-terminal albumin fragment in urine

We sought to determine whether the fragments that we identified in the kidney were also present in urine. We found that the major albumin fragment in the kidney at *m/z* 2791 is also the major albumin fragment in the urine (Figure 5). This was detected in the urine of 12- and 22-week-old DN mice but not in the control urine (Figure 5). Interestingly, a similar N-terminal albumin fragment has been reported in urine of humans with renal disease.[29,30] Other albumin fragments were not detected in urine, possibly because they were either further degraded or reabsorbed by the renal tubules and not excreted.

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