

Proteomic Identification and Immunolocalization of Increased Renal Calbindin-D28k Expression in OVE26 Diabetic Mice

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■ Abstract

Diabetic nephropathy is a common diabetic complication that is associated with alterations in the expression of several renal proteins and abnormal calcium homeostasis. We performed proteomic analysis to screen for global changes of renal protein expression in diabetic kidney. Proteins extracted from the whole kidney of 120-day-old OVE26 (a transgenic model of Type 1 diabetes) and FVB (non-diabetic background strain) mice were separated by two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) and visualized by SYPRO Ruby staining (n = 5 in each group). Quantitative intensity analysis revealed 41 differentially expressed proteins, of which 30 were identified by matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF MS) followed by peptide mass fingerprinting. One of the altered proteins with the greatest magnitude of

change was the calcium-binding protein, calbindin-D28k, whose expression was increased 6.7-fold in diabetic kidney. We confirmed the increase in calbindin-D28k expression in diabetic kidney by Western blot analysis. Immunohistochemical study demonstrated that calbindin-D28k expression was markedly increased in tubular epithelial cells of distal convoluted tubules (DCT), collecting ducts (CD), and proximal convoluted tubules (PCT) in diabetic kidney. Calbindin-D28k plays a critical role in maintaining calcium homeostasis. The elevation in renal calbindin-D28k expression in our model may indicate a compensatory mechanism to overcome hypercalciuria in diabetes.

Keywords: proteomics · proteome · proteomic analysis · kidney · diabetic nephropathy · diabetes · calbindin-D28k · calcium · vitamin D · renal tubules

Introduction

Diabetic nephropathy is a major diabetic complication and accounts for more than 40% of cases with end-stage renal disease [1]. The number of diabetic patients with renal failure is expected to increase in the coming years. Common histopathological changes in diabetic nephropathy can affect all intrarenal structures, including glomeruli, tubulointerstitium,

and blood vessels [2, 3]. All these changes accompany alterations in many different proteins, leading to decreased renal function and ultimately renal failure. Diabetes is also associated with altered Ca²⁺ homeostasis, characterized by hypercalciuria, decreased plasma 1,25-dihydroxy-vitamin D₃ (1,25(OH)₂D₃), and osteopenia [4-9]. This metabolic complication is more common in type 1 (insulin-dependent), especially in long-standing cases who require high-dose insulin [10].

To date, the pathogenic mechanisms of this diabetic complication remain unclear and alterations in kidney proteins may play a pivotal role in this metabolic derangement as the kidney is one of the major organs that maintain Ca^{2+} homeostasis. Global analysis of alterations in renal protein expression during diabetes may lead to better understanding of the pathogenesis and pathophysiology of these diabetic renal complications.

We performed gel-based proteomic analysis to examine the renal proteome of diabetic OVE26 transgenic mice, which display many characteristics of Type 1 diabetes, compared to non-diabetic FVB background strain ($n = 5$). In the OVE26 diabetic model, diabetes occurs due to overexpression of calmodulin in pancreatic β -cells that causes metabolic derangements, including hyperglycemia, hypoinsulinemia, and hypertriglyceridemia [11, 12]. The OVE26 diabetic mice have polyuria, albuminuria, and renal histopathological changes that provide the best rodent model for simulating human diabetic nephropathy [13]. Initial proteomic analysis revealed 41 differentially expressed proteins in diabetic kidney [14]. In the present communication, we describe identification of calbindin-D28k as a highly regulated protein in diabetic nephropathy and demonstrate that the strikingly increased calbindin-D28k expression occurs in renal tubular epithelial cells of DCT, CD, and PCT. These findings may indicate a compensatory mechanism to overcome hypercalciuria in diabetes.

Methods

The initial production, characterization, and maintenance of the diabetic OVE26 line were performed as described previously [11, 12]. Control or non-diabetic mice were from a non-transgenic FVB background strain. Ten animals (5 in each group) at 120 days of age were studied. Renal proteins derived from a whole kidney from each animal were separated and visualized on individual 2-D gel. Quantitative and statistical analyses were performed to determine differential protein expression. All animal studies were approved by the University of Louisville Institutional Animal Care and Use Committee and were in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Extraction of Renal Proteins

Mice were sacrificed by injection with Ketamine HCl/Xylazine HCl solution (Sigma Chemical Co., St. Louis, MO). Protein extraction from the whole kidney was performed as described previously [15, 16]. Briefly, the kidney was rapidly frozen in liquid nitrogen,

ground to powder, resuspended in a buffer containing 50 mM Tris, 0.3% SDS, and 200 mM DTT, and incubated at 100 °C for 5 min. DNA and RNA were removed by a buffer containing 500 mM Tris, 50 mM MgCl_2 , 1 mg/ml DNase I, and 0.25 mg/ml RNase A. Excess salts were removed by TCA precipitation and the protein pellet was finally resuspended in a buffer containing 40 mM Tris, 7.92 M urea, 0.06% SDS, 1.76% ampholytes, 120 mM DTT, and 3.2% Triton X-100. Protein concentration was measured by spectrophotometry using the HP 8453 UV-visible system (Hewlett-Packard Company, Palo Alto, CA) and Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA).

2-D PAGE Protein Separation

A tube-gel running system (Genomic Solutions Inc., Ann Arbor, MI) was employed for isoelectric focusing (IEF), using 100 mM sodium hydroxide as the cathode buffer and 10 mM phosphoric acid as the anode buffer. Pre-cast carrier ampholyte tube gels, pH 3-10, 1 mm x 18 cm, were prefocused with maximal 1,500 V and 110 μA per tube. Samples containing 100 μg total protein from individual animals were loaded into individual tubes and were focused for 17 h and 30 min to reach 18,000 volt-hours. After completion of the IEF, tube gels were extruded and incubated in premixed Tris/acetate equilibration buffer with 0.01% bromophenol blue and 50 mM DTT for 2 min before loading onto pre-cast 10% homogeneous 22 x 22 cm slab gels (Genomic Solutions Inc., Ann Arbor, MI). The upper running buffer contained 0.2 M Tris base, 0.2 M Tricine, and 0.4% SDS, and the lower running buffer was 0.625 M Tris/acetate. The second dimensional separation was performed with a maximum of 500 V and 20,000 mW per gel.

SYPRO Ruby Staining and Visualization

Separated proteins in slab gels were fixed with 10% methanol and 7% acetic acid for 30 min. The fixed solution was removed and 500 ml of SYPRO Ruby gel stain (Bio-Rad Laboratories, Hercules, CA) was added to each gel and incubated on a gentle continuous rocker in a dark room at room temperature for 18 h. A 12-bit camera with a UV light box system (Genomic Solutions Inc., Ann Arbor, MI) was used to visualize gel images with 1-sec controlled exposure time.

Quantitative Analysis of Protein Expression

Investigator HT analyzer (Genomic Solutions Inc., Ann Arbor, MI) software was used for the matching and analysis of protein spots. The principles of meas-

uring intensity values by 2-D analysis software were similar to those of densitometric measurement. The average mode of background subtraction was used to normalize intensity value, which represents the relative amount of protein. After completion of the spot matching, the normalized intensity values of individual protein spots from each animal were then compared between groups using statistical analysis.

Statistical Analysis

Because the sample size was relatively small, the Exact mode of the Mann-Whitney Test by SPSS software v. 10.0 was used for statistical analysis. Spot intensities were compared between control and diabetic groups, and $p < 0.05$ were considered statistically significant. This significance level obtained by the Exact Test is based on the reassignment of a test statistic, which is more accurate than using the ordinary or asymptotic significance value, especially in the case of a small sample size [17]. The data were reported as mean \pm SEM.

In-Gel Tryptic Digestion, MALDI-TOF MS, and Peptide Mass Fingerprinting

In-gel tryptic digestion and MALDI-TOF MS were performed using the techniques described previously [18]. Peptide mass fingerprinting was performed using the "ProFound" search engine (http://129.85.19.192/profound_bin/WebProFound.exe). The NCBI protein database was restricted to mammalian entries, and peptides were assumed to be monoisotopic, oxidized at methionine residues, and carbamidomethylated at cysteine residues. Up to 1 missed trypsin cleavage and 150-ppm mass tolerance (error window) were allowed for matching of peptide mass values. The Z score was calculated by a comparison of search results against estimated random match population and was a distance to the population mean in unit of standard deviation. A score greater than 1.65 was considered statistically significant ($p < 0.05$).

Western Blot Analysis

Renal proteins were mixed 1:1 with 2X Laemmli sample buffer and heated at 100 °C for 5 min, and 50 μ g proteins were separated by 10% SDS-PAGE. Proteins were transferred onto a nitrocellulose membrane and non-specific bindings were blocked with 5 % milk/TTBS. The membrane was treated with mouse monoclonal anti-calbindin-D28k (Sigma Chemical Co., St. Louis, MO), 1:1,000 in 5% milk/TTBS at 4 °C overnight. Immunoreactive calbindin-D28k band was

detected by autoradiography using horseradish peroxidase-conjugated antibody and chemiluminescent substrate. Quantitative intensity analysis was performed using a PDSI Densitometer (Amersham Bioscience, Piscataway, NJ).

Immunohistochemistry

Immunohistochemistry was performed on 5- μ m-thick kidney sections from 6 individual animals (3 in each group). The sections were de-paraffinized, rehydrated, and digested with 0.1% Trypsin/PBS at room temperature for 10 min. Non-specific bindings were blocked with 5% donkey serum in 0.2% Triton X-100/PBS at room temperature for 2 h. The sections were then incubated with rabbit polyclonal anti-calbindin-D28k (Sigma Chemical Co., St. Louis, MO), 1:500 in PBS at 4°C overnight. Slides were washed 3 times with PBS before incubating with CyTM3-conjugated AffiniPure donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories Inc., West Grove, PA), 1:500 in PBS at room temperature for 2 h. After washing with PBS, immunoreactive protein was observed under fluorescence microscopy.

Results

Diabetes in OVE26 mice occurred within one week after birth and random plasma glucose levels were over 600 mg/dl from 10 weeks of age. Plasma insulin levels of adult OVE26 mice were reduced to approximately 30% of normal values [12]. The animals survived without insulin therapy or any other treatment for at least a year. They exhibited pronounced polyuria and significant albuminuria by 2 months of age [13]. Albuminuria coincided with an elevation in blood pressure as measured by tail cuff [13]. Measurement of fluorescein isothiocyanate inulin clearance demonstrated that GFR increased significantly from 2 to 3 months of age but then decreased significantly after 5 months of age [13]. Renal histopathological changes observed in 120-day-old OVE26 diabetic mice were a thickening of glomerular basement membrane and glomerular hypertrophy [11]. There was no intrarenal leukocyte infiltration or tubulointerstitial injury observed in OVE26 diabetic mice at this age.

Renal proteins derived from a whole kidney from each animal were separated by 2-D PAGE and visualized by SYPRO Ruby staining on individual 2-D gels ($n = 5$ in each group). Protein spot pattern visualized on 2-D gels was reproducible and essentially identical among the individuals, as examined by 2-D analysis software. Approximately 300 protein spots were visual-

ized on each gel (Figure 1A). By quantitative and statistical analyses, a total of 41 visualized protein spots were differentially expressed between the two groups [14]. These differentially expressed spots were then excised and subjected to in-gel tryptic digestion and identification by MALDI-TOF MS followed by peptide mass fingerprinting. From 41 differentially expressed spots, 30 were identified [14]. Positions of all proteins identified on 2-D gels were in the expected range of their theoretical isoelectric points and molecular sizes.

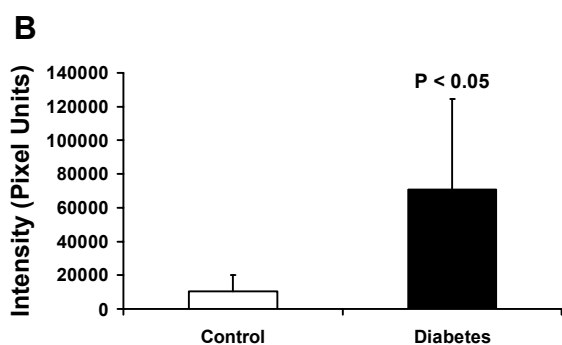
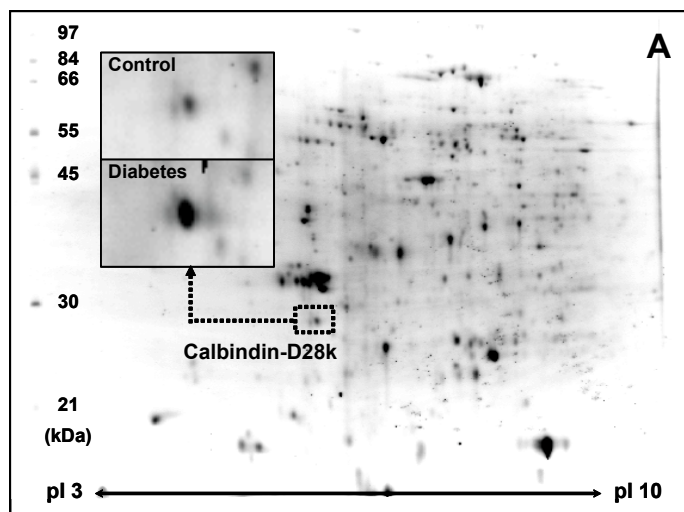


Figure 1. Proteomic identification of increased calbindin-D28k expression in diabetic kidney. **A:** Renal proteins derived from a whole kidney from each animal were separated by 2-D PAGE and visualized by SYPRO Ruby staining on individual 2-D gels ($n = 5$ in each group). Quantitative intensity analysis was performed to determine differential protein expression and differentially expressed proteins were identified by MALDI-TOF MS followed by peptide mass fingerprinting. One differentially expressed protein was calbindin-D28k, of which the expression level increased 6.7-fold in diabetic kidney (**B**).

Among 30 identified proteins, we focused our attention on the change in renal expression of calbindin-D28k in the present study. Calbindin-D28k (NCBI identification number: gi|115396 and accession num-

ber: P07171) was identified as the altered protein with the greatest magnitude of change (6.7-fold increase: $70,654 \pm 53,779$ versus $10,538 \pm 9,629$ intensity units; $p < 0.05$; $n = 5$; Figures 1A and 1B). We confirmed our MALDI-MS-based proteomic data by Western blot analysis. Figure 2 clearly confirms that calbindin-D28k expression was increased in diabetic kidney and the magnitude of change was approximately 2.6-fold ($1,594 \pm 200$ versus 660 ± 102 intensity units, $p < 0.01$; $n = 4$), as determined by densitometric analysis. Because proteomic and immunoblot data did not provide any information regarding localization of changes in protein expression, we performed an immunohistochemical study to localize the increase in calbindin-D28k expression in diabetic kidney. Figure 3 clearly demonstrates that renal calbindin-D28k expression was markedly increased in tubular epithelial cells of DCT, CD, and PCT in diabetic kidney.

Discussion

We performed gel-based proteomic analysis to screen for global changes in renal protein expression in diabetic kidney. One of the altered proteins with the greatest magnitude of change was calbindin-D28k, whose expression level increased 6.7-fold in diabetic kidney, as determined by 2-D proteomic analysis. Although MALDI-TOF MS followed by peptide mass fingerprinting is not a definitive protein identification method, we have frequently demonstrated that proteins identified by this method, with careful analysis using a vigorous scoring system to determine a significant hit (match), are consistent with those identified by other methods, especially 1-D and 2-D Western blot analyses [15, 16, 19-21]. In the present study, the MALDI-MS-based proteomic data were again confirmed by Western blot analysis. However, the magnitude of change in renal calbindin-D28k expression determined by Western blot analysis was only 2.6-fold. The difference in degree of changes between these two methods was most likely due to differential dynamic ranges between antibody-based detection and visualization using SYPRO ruthenium-based staining. Additionally, differences in imaging systems and quantitative software used should account for these different results.

Calbindin-D28k is generally known as a vitamin-D-dependent Ca^{2+} -binding protein that plays an important role in renal tubular Ca^{2+} reabsorption [22]. In the normal kidney, calbindin-D28k distributes along DCT,

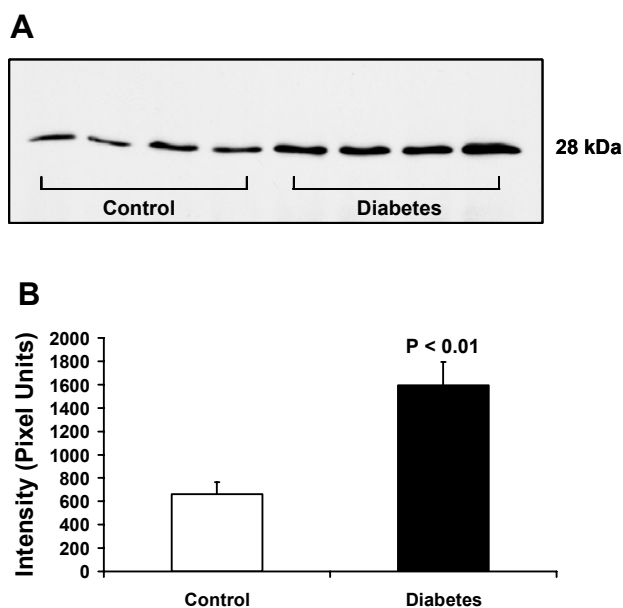


Figure 2. Western blot analysis for renal calbindin-D28k. **A:** Renal proteins derived from the whole kidney of OVE26 diabetic and FVB background mice ($n = 4$ in each group) were separated by 1-D PAGE and transferred onto a nitrocellulose membrane. The membrane was treated with mouse monoclonal anti-calbindin-D28k and immunoreactive protein was detected by autoradiography using horseradish peroxidase-conjugated antibody and chemiluminescent substrate. Only a single band at 28 kDa, the expected molecular size, was detected in each lane. Densitometric intensity analysis revealed the 2.6-fold increase of the calbindin-D28k level in diabetic kidney (**B**).

connecting tubules and CD [23, 24], and co-localizes with epithelial Ca^{2+} channel, plasma membrane Ca^{2+} -ATPase, and $\text{Na}^{+}/\text{Ca}^{2+}$ exchanger along $1,25(\text{OH})_2\text{D}_3$ -responsive nephron segments [25]. These proteins regulate tubular Ca^{2+} reabsorption via an active transcellular Ca^{2+} transport process in the distal nephron [26]. The study by Christakos and colleagues [27] indicates that renal calbindin-D28k expression is probably the best marker for the $1,25(\text{OH})_2\text{D}_3$ activity. Renal calbindin-D28k appears to enhance tubular Ca^{2+} reabsorption, and a reduction of renal calbindin-D28k is associated with hypercalciuria [28-32]. Findings from a recent *in vivo* study using a gene-knockout model confirm the critical role of calbindin-D28k in maintaining renal calcium homeostasis [33]. Hypercalciuria is commonly associated with diabetes [4-9]. However, the number of studies addressing the pathophysiology of altered Ca^{2+} homeostasis during diabetes [7-9, 34] is limited. The results from these studies clearly indicate that diabetic animals have hypercalciuria, decreased

circulating $1,25(\text{OH})_2\text{D}_3$, and reduced bone mass. While hypercalciuria occurs, plasma levels of ionized and total Ca^{2+} can be maintained at normal levels in several study models [7-9, 34]. These data implicate that a compensatory mechanism for diabetes-induced renal Ca^{2+} loss exists. In the present study, we show that the increased calbindin-D28k expression was prominent in the DCT and CD ($1,25(\text{OH})_2\text{D}_3$ -responsive nephron segments) of OVE26 diabetic mice. Considered together, these data suggest the hypothesis that the increase in renal calbindin-D28k in diabetic kidney may be one of the compensatory mechanisms responsive to diabetes-induced abnormal Ca^{2+} homeostasis [4-9].

Interestingly, the increase in renal calbindin-D28k expression localized not only in the distal nephron, where calbindin-D28k facilitates active Ca^{2+} reabsorption, but also in PCT, in which a role for calbindin-D28k is not clear. A recent study by Wu and colleagues [35] demonstrated that transfection of calbindin-D28k gene into murine proximal tubular epithelial cells provided protective effects against chemical hypoxic injury. Several other lines of inquiry indicate that calbindin-D28k has a cytoprotective role in preventing various cell types from cellular degeneration and apoptosis via a Ca^{2+} -buffer mechanism [36-39]. Therefore, we speculate that calbindin-D28k may have another potential role in preventing apoptotic tubular cell death induced by diabetes.

There have been few previous studies that examined renal calbindin-D28k levels in diabetic models using primarily immunoblot methodology [7-9, 40]. The results in those studies, however, were not conclusive and did not provide information about localization of changes. Ward and colleagues [9] recently reported that calbindin-D28k level in the whole kidney did not change in diabetic rats made diabetic with streptozotocin for 2 weeks. In the present study, we report a 6.7-fold increase in renal calbindin-D28k expression in 120-day-old OVE26 diabetic mice. The increase in renal calbindin-D28k was observed in tubular epithelial cells of DCT, CD and PCT, as determined by immunohistochemical analysis. At this age, the OVE26 mice had been diabetic for approximately 16 weeks. The greater duration of diabetes in our model may explain the difference in our results versus those reported by Ward *et al.* [9]. We recently performed proteomic analysis of the whole kidney from 120-day-old *db/db* diabetic mice. The data clearly confirmed that long-term diabetes causes increased renal calbindin-D28k expression (2.8-fold increase was observed in a *db/db* model of Type 2 diabetes).

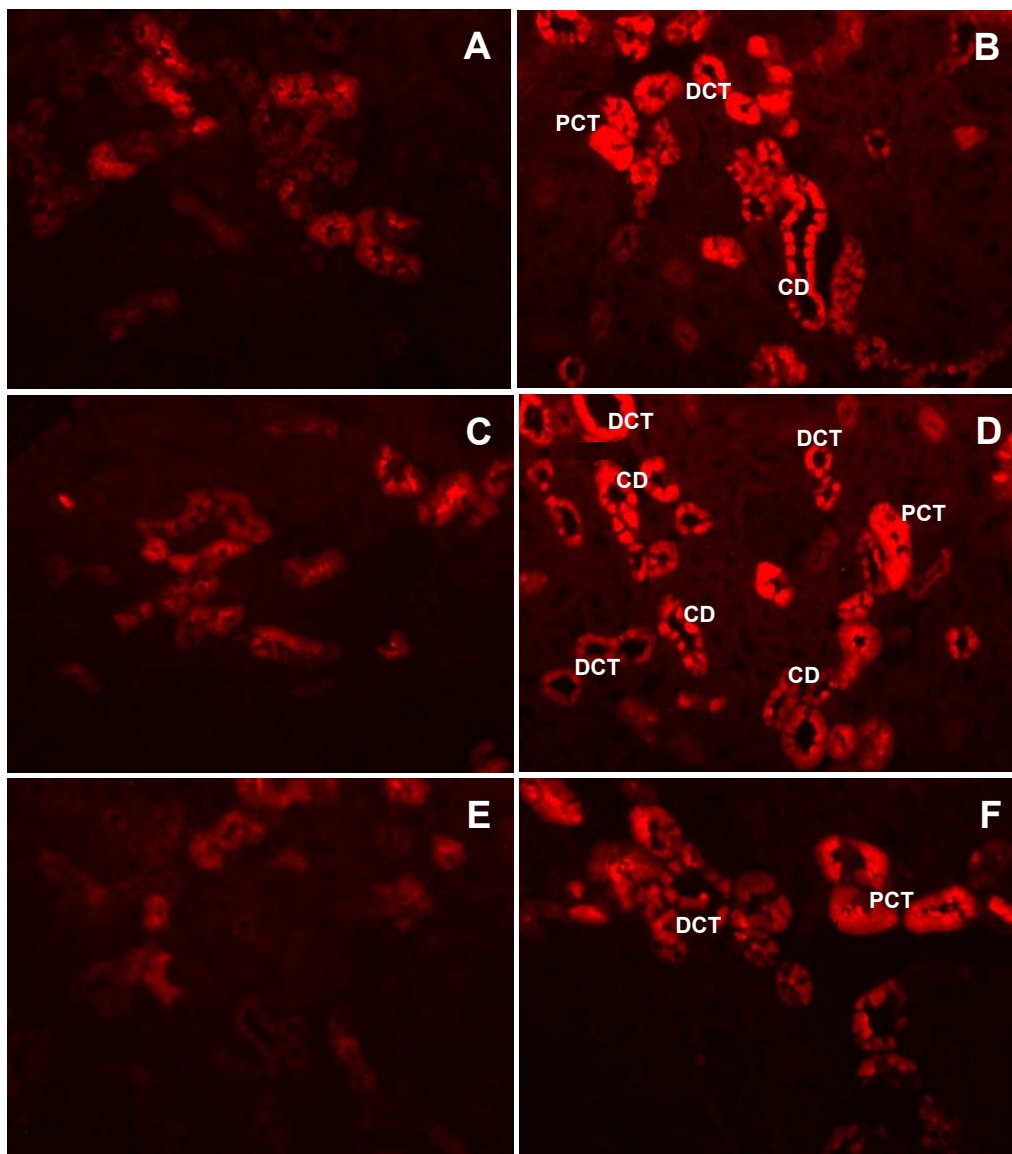


Figure 3. Immunolocalization of increased calbindin-D28k expression in diabetic kidney. After de-paraffinization, re-hydration, digestion and blocking, 5- μ m-thick kidney sections were incubated with rabbit polyclonal anti-calbindin-D28k followed by incubation with CyTM3-conjugated AffiniPure donkey anti-rabbit IgG. Immunoreactive protein was observed under fluorescence microscopy. Panels **A**, **C**, and **E** were from FVB control mice ($n = 3$), whereas panels **B**, **D**, and **F** were from OVE26 diabetic mice ($n = 3$). Renal calbindin-D28k expression was markedly increased in tubular epithelial cells of distal convoluted tubules (DCT), collecting ducts (CD), and proximal convoluted tubules (PCT). Magnification power was 20X in all panels.

In summary, we applied expression proteomics to define alterations in renal protein expression during diabetes. The greatest magnitude of change among differentially expressed proteins was for calbindin-D28k. Immunolocalization demonstrated that renal calbindin-D28k expression was increased in tubular epithelial cells along distal nephron segments and proximal convoluted tubules. These data may indicate a compensatory mechanism to normalize tubular calcium reabsorption in diabetes. Further systematic functional study, perhaps with a calbindin - D28k - transgenic and / or knockout model, is required to address our hypothesis.

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