Calpain Activation and Secretion Promote Glomerular Injury in Experimental Glomerulonephritis: Evidence from Calpastatin-Transgenic Mice

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Glomerular injury and albuminuria in acute glomerulonephritis are related to the severity of inflammatory process. Calpain, a calcium-activated cysteine protease, has been shown to participate in the development of the inflammatory process. Therefore, for determination of the role of calpain in the pathophysiology of acute glomerulonephritis, transgenic mice that constitutively express high levels of calpastatin, a calpain-specific inhibitor protein, were generated. Wild-type mice that were subjected to anti–glomerular basement membrane nephritis exhibited elevated levels of calpain activity in kidney cortex at the heterologous phase of the disease. This was associated with the appearance in urine of calpain activity, which originated potentially from inflammatory cells, abnormal transglomerular passage of plasma proteins, and tubular secretion. In comparison with nephritic wild-type mice, nephritic calpastatin-transgenic mice exhibited limited activation of calpain in kidney cortex and limited secretion of calpain activity in urine. This was associated with less severe glomerular injury (including capillary thrombi and neutrophil activity) and proteinuria. There was a reduction in NF-κB activation, suggesting that calpain may participate in inflammatory lesions through NF-κB activation. There also was a reduction in nephrin disappearance from the surface of podocytes, indicating that calpain activity would enhance proteinuria by affecting nephrin expression. Exposure of cultured podocytes to calpain decreased nephrin expression, and, conversely, exposure of these cells to calpastatin prevented TNF-α from decreasing nephrin expression, demonstrating a role for the secreted form of calpain. Thus, both activation and secretion of calpains participate in the development of immune glomerular injury.


Inflammatory damages to the glomerulus characterize acute glomerulonephritis, an immune-mediated disorder that is a major cause of renal failure. The experimental model of anti–glomerular basement membrane (anti-GBM) nephritis has been used widely to analyze the underlying mechanisms of this inflammatory process (1). Injection of heterologous anti-GBM antiserum to rabbit, rat, or mouse causes an immediate and transient, complement-dependent influx of polymorphonuclear neutrophils into glomerular capillaries that is responsible directly or indirectly for both proteinuria and glomerular fibrin deposition (heterologous phase) (2). Immune responses against the heterologous Ig leads later to mononuclear cell recruitment and subsequent proteinuria (autologous phase).

Calpains are calcium-activated neutral cysteine proteases (3,4). Two major isoforms, calpain μ, or I, which requires micromolar Ca2+ concentrations for activity and calpain m, or II, which requires millimolar Ca2+ concentrations, are ubiquitously expressed, whereas the other isoforms are tissue-specific forms. Every calpain isoform is present in the cytosol as an inactive proenzyme. Binding of Ca2+ to μ- or m-calpain induces the release of constraints that are imposed by domain interactions and results in a two-stage activation process, with first the release of an approximately 30-kD regulatory subunit and second the rearrangement of the active site cleft in an approximately 80-kD catalytic subunit (5). Calpain activity is tightly controlled by calpastatin, a specific endogenous inhibitor that contains four equivalent inhibitory domains (3).

Calpains play an important role in the inflammatory process. First, they are involved in the activation of NF-κB and, thereby, in the NF-κB–dependent expression of proinflammatory cytokines and adhesion molecules. Underlying mechanisms include degradation of the PEST (proline, glutamate, serine, threonine) sequence in the inhibitor IkBα, a key step in nuclear translocation of NF-κB (6). Second, calpains are critical for inflammatory cell adhesion and chemotaxis and inflammatory mediator processing (7,8). Third, calpains are implicated in the cleavage of the heat-shock protein 90, which is required to maintain glucocorticoid receptor in a ligand-binding conformation (9). As a consequence, binding and anti-inflammatory efficacy of glu-
corticoid are limited in inflammatory cells. Finally, calpains are externalized during the inflammatory process and play a role in the microenvironment of inflammatory cells (10), for instance by facilitating their recruitment (11). Thus, both intracellular and externalized calpains would strengthen inflammatory process. The observation that calpain inhibitor administration decreases inflammatory lesions in lung, joint, and kidney supports this hypothesis (12,13).

Whether calpains are involved in the course of acute glomerulonephritis still is unknown. Therefore, the aim of this study was to measure the activity of calpains in kidney tissue and urine of C57BL/6 mice with anti-GBM nephritis and to characterize the pathogenic role of these enzymes. Different approaches could be considered to hamper calpain activation: First a pharmacologic approach. Second, a disruption of calpain gene; but it is a complex approach because different forms of calpains have been identified. In addition, disruption of calpain 4 gene, a common regulatory subunit that is shared by different forms of calpains, completely blunts calpain activity and results in a lethal phenotype (14). Therefore, we opted for a third approach: To create mice that are transgenic for calpastatin. Its overexpression affects potentially the different forms of calpains, the activity of which is blunted rather than suppressed.

Materials and Methods

Cells

Human proximal tubular epithelial cells (HK-2 cell line; American Type Culture Collection, Manassas, VA) were cultured at 37°C in a serum-free keratinocyte medium supplemented with human recombinant EGF and pituitary bovine extract (Life Technologies, Cergy Pontoise, France) under a 5% CO₂ and 95% air atmosphere. Primary cultures of human podocytes were established and characterized as described previously (15). Established lines of differentiated podocytes were cultured at 37°C in a serum-free keratinocyte medium supplemented with human recombinant EGF and pituitary bovine extract (Life Technologies) and random hexamer primers. Total RNA (2 μg) was diluted in RNase-free water to 10 μl. First-strand cDNA synthesis was performed with the superscript RNase H− reverse transcriptase kit (Life Technologies) and random hexamer primers. Total RNA (2 μg) was diluted in RNase-free water to 10 μl. First-strand buffer (5×; 4 μl), dithiothreitol (0.1 M, 2 μl), dNTP (10 mM, 1 μl), dNTP (100 μg/ml, 0.5 μl; Amersham, Orsay, France), oligo(dT) 12 to 18 (0.5 μg/μl, 0.5 μl; Invitrogen, Cergy Pontoise, France), Rnasin (0.2 μl; Promega, Madison, WI), and RNase-free water (0.8 μl) were added to each sample of diluted RNA. One microliter of RT enzyme or RNase-free water were added to the RT+ and control samples, respectively. The reaction was allowed to proceed for 10 min at 21°C and 60 min at 42°C, followed by 5 min at 95°C and 1 min at 98°C for inactivation. The template cDNA obtained was analyzed with PCR as detailed in the previous paragraph.

Induction of Nephritis

Glomerulonephritis was induced in female C57BL/6 mice with a single injection of 200 μl of sheep anti-mouse GBM serum into the tail vein, as described previously (16). Age-matched mice were used as controls. After 6 to 24 h, mice were anesthetized by intraperitoneal administration of sodium pentobarbital, urine samples were collected, and kidneys were removed for morphologic analyses and assays for both calpain and myeloperoxidase (MPO) activities. Urine samples were centrifuged immediately at 1800 rpm during 10 min at 4°C, and supernatants were frozen at −20°C. Kidneys were snap-frozen or fixed in 4% paraformaldehyde and processed for paraffin embedding.

Assessment of Albuminuria

Urine albumin concentration was determined by an ELISA assay (Albuwell, Exocell, Philadelphia, PA), and albuminuria was reported to creatininuria as measured by a colorimetric method.

Histologic and Immunofluorescence Preparations

Renal fragments that were embedded in paraffin were cut into 3-μm sections and stained with Masson’s trichrome for histologic analysis. Calpastatin expression was assessed using a polyclonal primary antibody (Affinity BioReagents, Golden, CO; 1:200). Samples were revealed with Envision System (Dako, Glostrup, Denmark) and counterstained with hematoxylin. No primary antibody was used for negative control. Fibrin deposition in glomeruli was counted on a minimum of 50 glomeruli by two independent examiners who were unaware of the genotypes of the mice. Fibrin deposition also was detected by immunofluorescence on cryosections that were fixed for 10 min in 4% paraformaldehyde and stained for 30 min in FITC-conjugated goat anti-mouse fibrin (Nordisk Immunoal, Paris la Défense, France; 1:50). Polymorphonuclear neutrophils that infiltrated glomeruli were stained...
with hematoxylin and naphthol AS-D chloroacetate esterase (Sigma Chemical Co., St. Louis, MO) to be counted.

To analyze nephrin expression, 4-μm-thick cryostat sections were fixed in 3.5% paraformaldehyde for 15 min and washed in PBS. The sections were incubated with an anti-nephrin antibody that specifically recognizes the extracellular fibronectin domain of nephrin (GP-N1; Progen Biotechnic, Heidelberg, Germany; 1:50) for 1 h at room temperature, washed in PBS, and incubated with the appropriate FITC-conjugated secondary antibody (Sigma) for 30 min at room temperature. The number of glomeruli that were available on each section for analysis of nephrin expression ranged between five and 10.

To analyze nephrin expression on cultured podocytes, immunofluorescence experiments were performed as described previously (17). Briefly, podocytes were plated in eight-well Permanox slide at density of 40,000 cells per well in DMEM plus 10% FCS. For determination of whether nephrin expression was modified by exogenous μ-calpain, cells were incubated with variable concentrations of μ-calpain or vehicle alone (0.1% BSA) for 30 min before fixation with 3.5% paraformaldehyde that contained 2% sucrose for 15 min at room temperature. Nonpermeabilized cells then were blocked for 30 min with 3% BSA and stained for 60 min at 37°C with anti-nephrin antibody (GP-N1, 1:50) before incubation for 30 min at room temperature with the appropriate FITC-conjugated secondary antibody (Sigma). The slides then were mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA) and examined. Control experiments included incubation of sections or cells with nonimmune isotypic control antibodies or the omission of primary antibodies followed by the appropriate labeled secondary antibodies.

Nephrin expression was analyzed semiquantitatively by measuring fluorescence intensity by digital image analysis (Windows Microlmage, version 3.4; CASTI Imaging, Venice, Italy) on images that were obtained using a low-light video camera (Leica DC100, Rueil Malmaison, France) with a 180-μm diameter field. The results are expressed as relative fluorescence intensity on a scale from 0 (fluorescence of background of tissue) to 255 (fluorescence of standard filter). For each experimental point on cultured podocytes, a minimum of five microscopic fields were examined.

**Calpain Activity Assay**

For measuring calpain activity in urine and plasma, 10 μl of either fluid sample was diluted one sixth in KRB solution (pH 7.4) supplemented with CaCl₂ (2 mM final concentration) in wells of a 96-well plate. These samples were exposed at 37°C to the substrate N-succinyl-Leu-Tyr-AMC 50 μM together with or without the calpain inhibitor calpeptin (100 μM; Calbiochem, VWR International, Fontenay sous Bois, France). After a 90-min incubation period, fluorescence was detected at 360 nm excitation and 460 nm emission, using the FLX800 spectrofluorometer (Bio-Tek Instruments, Winoski, UT). Calpain activity was determined as the difference between fluorescences measured without and with calpeptin and expressed as μM AMC using a standard curve (0 to 25 μM) constructed for each assay.

For measuring calpain activity in cell supernatant, cells were plated in 12-well tissue culture dishes in appropriate medium. After the indicated culture period, the medium was replaced with KRB solution (pH 7.4) that contained 2 mM CaCl₂ and 20 μM N-succinyl-Leu-Leu-Val-Tyr-AMC together with or without calpeptin 100 μM. Calpain activity was determined in supernatants as indicated in the previous paragraph.

**MPO Assay**

Snap-frozen kidney samples were thawed, added to ice-cold 50 mM PBS (pH 6.0) supplemented with 0.5% hexadecyltrimethylammonium bromide up to concentrations of 0.1 g renal tissue/ml, and homogenized with 20 strokes in a glass homogenizer. The lysates then were freeze-thawed three times and centrifuged at 20,000 × g for 1 h at 4°C. Supernatants were assayed for MPO, as described previously (18). Results were expressed as OD/min.

**NF-κB Activation Assay**

Nuclear proteins were extracted from fresh kidney samples, and the amounts of activated NF-κB p65 subunit that was contained in these proteins were measured with commercial kits (Nuclear Extract Kit and TransAM; Active Motif, Rixensart, Belgium), according to the manufacturer’s instructions.

**Western Blotting**

Half-kidneys were homogenized in 400 μl of radioimmunoprecipitation assay buffer, as described previously (19). Protease inhibitor cocktail (1 μg/ml; Sigma) was added to the radioimmunoprecipitation assay buffer just before use. After homogenization, the lysate was centrifuged at 1000 × g for 1 h, and the supernatant was frozen at −80°C. Protein concentration in supernatant was measured using the Bradford method. Twenty-five micrograms of protein was separated by electrophoresis on a Bis-Tris gel 4 to 12% (Invitrogen). After transfer of the proteins for 3 h onto nitrocellulose membrane, the membrane was incubated for 2 h at room temperature with anti-spectrin mAb (1:5000; Chemicon International, Temecula, CA). Thereafter, the membrane was incubated for 2 h at room temperature with a secondary anti-mouse IgG antibody (1:4000) and developed with the ECL detection reagent (Amersham Pharmacia Biotech). Comparative densitometry was performed on the 145/150-kD spectrin breakdown products (BDP).

The same protocol was used for assessing m-calpain expression in urine (2 μl) and cell supernatant (10 μl). First and secondary antibodies used were goat anti-m-calpain polyclonal antibody (1:3000; Santa Cruz Biotechnology) and anti-goat IgG antibody (1:10,000, respectively).

**Statistical Analyses**

Values are expressed as mean ± SEM. Comparisons between two groups of values were made with the t test. Multiple group comparisons were performed using the ANOVA with Bonferroni post test. P < 0.05 was considered statistically significant.

**Results**

**Calpain Activity Was Increased in Kidney Cortex and Released in Urine at the Heterologous Phase of Anti-GBM Nephritis in Wild-Type Mice**

To assess initially the role of calpains in renal inflammation, we induced an anti-GBM nephritis in C57BL/6 mice. Kidneys were removed 24 h after injection of anti-GBM antiserum, and calpain activity was determined in the renal cortex by measurement of the accumulation of 145/150-kD spectrin BDP that is known to be generated specifically by calpains. The renal level of BDP was increased significantly in nephritic mice (Figure 1). The upregulation was evident from 0.5 h after injection and sustained to 48 h (i.e., during all of the heterologous phase of nephritis; data not shown). Because several groups showed that calpains may be released from cells into the extracellular environment and therefore may have an extracellular role, we also determined the appearance of calpain activity in urine by measuring the calpain-specific cleavage of fluorescence AMC substrate. In most cases, urine calpain activity was undetectable in saline-treated mice, whereas it was markedly increased in ne-
phritic mice (Figure 2A). This apparent release of calpain in urine of nephritic mice was confirmed by Western blot analysis using an antibody that was directed against m-calpain (Figure 2B).

Urine Calpain Activity Originated from Multiple Potential Sources

There are at least three different ways in which the appearance of calpain in urine of nephritic mice might happen: The release of calpain from inflammatory cells that infiltrate the glomerulus, the abnormal transglomerular passage of plasma proteins (including calpain) as a result of increased permeability of glomerular capillary wall, and the secretion of calpain by tubular epithelial cells that are exposed to proteinuria. First, to test the possibility that urinary calpain originates from inflammatory cells, we determined in vitro the appearance of calpain activity in the microenvironment of polymorphonuclear neutrophils (10⁵/ml) that were isolated from the blood of C57BL/6 mice. After 1 h of incubation without and with 10 ng/ml TNF-α, extracellular calpain activity reached 0.10 ± 0.06 and 4.70 ± 1.22 μM AMC, respectively (n = 4). Second, to address the role of alterations in the glomerular filtration barrier, we analyzed calpain activity in the urine of podocin-deficient (Nphs 2⁻/⁻) mice, which develop massive proteinuria in the absence of glomerular inflammation. Elevations in urine calpain activity were similar in those mice and in mice with anti-GBM nephritis, suggesting that calpain appearance in the urine of mice with anti-GBM nephritis could result from the abnormal transglomerular passage of plasma proteins, irrespective of inflammatory process (Figure 2A). Accordingly, calpain activity was detectable in plasma of both control and nephritic mice (4.41 ± 0.90 and 4.78 ± 0.52 μM AMC, respectively; n = 7 to 14). Third, we analyzed in vitro the leakage of calpain activity from tubular epithelial cells. We found that HK-2 cells that were exposed to albumin released calpain activity in a dose-dependent manner, as determined by measuring the calpain-specific cleavage of fluorescein AMC substrate (Figure 3). These data suggested that urine calpain also might result, at least partially, from tubular secretion.

Calpastatin-Transgenic Mice Developed Less Severe Glomerular Inflammation Than Wild-Type Mice

To determine the exact role of both intracellular and externalized calpain activities in the development of renal inflammation, we induced an anti-GBM nephritis in mice that were transgenic for calpastatin and compared them with wild-type mice from the same genetic background. The presence and the expression of the transgene were identified in homozygous
calpastatin-transgenic mice by PCR and RT-PCR analysis, respectively (Figure 4A). These mice showed normal development, fertility, morphology, and life span as compared with wild-type mice. To localize the transgene product in the kidney, we compared the expression of calpastatin in wild-type and calpastatin-transgenic mice. As assessed by immunohistochemistry, only a few tubules showed calpastatin staining in wild-type mice (Figure 4B). In calpastatin-transgenic mice, the intensity of the staining and the number of calpastatin-positive tubules increased markedly. By contrast, calpastatin expression was barely detectable in glomeruli, in both wild-type and calpastatin-transgenic mice.

Whereas calpastatin overexpression did not affect calpain activity in control mice, it prevented completely calpain activation in the kidney of mice with anti-GBM nephritis (Figure 1). In those mice, calpain release in the urine was reduced, albeit not significantly (from 0.159 ± 0.037 to 0.090 ± 0.012 μM AMC/mM creatinine; n = 7 and 5, respectively). The decreased activity of both intracellular and externalized calpains was accompanied by a lessening in the severity of glomerular injury. Despite an equivalent deposition of heterologous sheep antibody (data not shown), glomerular fibrin deposition was markedly attenuated in calpastatin-transgenic mice, as reflected by the decrease in fibrin immunoreactivity (Figure 5). Anti-GBM–induced loss of nephrin expression was limited significantly in calpastatin-transgenic mice. Therefore, we tested the hypothesis that urinary calpain activity would participate in the development of proteinuria by altering nephrin expression. To this aim, we analyzed the effect of externalized calpains on nephrin expression by podocytes in vitro. When tested by indirect immunofluorescence, antibody that was specific for the extracellular domain of nephrin bound to both human podocytes in primary culture and human immortalized podocytes,

**Calpain-Transgenic Mice Developed Less Severe Albuminuria Than Wild-Type Mice**

Development of albuminuria, which peaked at 24 h (heterologous phase) and again at 15 d (autologous phase), was reduced significantly in calpastatin-transgenic mice at these two phases (Figure 7). Because nephrin is a critical component of the barrier to protein permeation, we analyzed nephrin expression. In wild-type control mice, nephrin exhibited a glomerular epithelial pattern with a punctate/linear distribution. In glomeruli of wild-type mice with an anti-GBM nephritis, a more granular pattern or a loss of staining of nephrin was observed 24 h after induction of the disease (Figure 8). Anti-GBM–induced loss of nephrin expression was limited significantly in calpastatin-transgenic mice. Therefore, we tested the hypothesis that urinary calpain activity would participate in the development of proteinuria by altering nephrin expression. To this aim, we analyzed the effect of externalized calpains on nephrin expression by podocytes in vitro. When tested by indirect immunofluorescence, antibody that was specific for the extracellular domain of nephrin bound to both human podocytes,
suggesting a surface expression of nephrin (data not shown). Exposure of those cells to calpain promoted the disappearance of nephrin from the surface, as indicated by a significant decrease in immunofluorescence staining (Figure 9A). Similarly, the appearance of calpain activity in the culture medium of podocytes that were exposed to TNF-α/H9251 for 1 h (1.76 ± 0.20 nM AMC; n = 3) was concomitant with a significant decrease in nephrin expression (Figure 9B). Under these conditions, blocking extracellular calpain activity by adding the non–cell-permeant calpastatin prevented completely nephrin disappearance from the cell surface, confirming a role for extracellular calpain activity in this process.

Discussion

Calpains are intracellular proteases that potentially are involved in the pathophysiology of inflammation. According to in vitro experiments, they are responsible primarily for the activation of NF-κB, a transcription factor with a pivotal role in inflammation (6). They play a key role in inflammatory cell adhesion and migration, proinflammatory mediator release, and anti-inflammatory hormone resistance as well (7–9). In addition, because they are precursors of chemotactic factors, their release in the microenvironment of inflammatory cells would amplify neutrophil recruitment (11). Studies of a variety of experimental models of inflammation have confirmed the relevance of these processes in vivo. However, attempts to intervene in calpain activation in these models have been made by using synthetic peptidic and nonpeptidic calpain inhibitors that have problems in terms of specificity and stability. To overcome these drawbacks and to gain new insights into the pathophysiologic roles of calpains in glomerular inflammation, we induced an anti-GBM nephritis in mice that were transgenic for calpastatin, the specific endogenous inhibitor of calpain, and we compared them with wild-type mice from the same genetic background. Whereas RT-PCR studies confirmed calpastatin transgene expression in homozygous calpastatin-transgenic mice, there was no evidence of calpain inactivation in the kidney cortex of those mice under normal conditions, as determined by measuring the accumulation of calpain-specific spectrin BDP. Recently, Takano et al. (20) also observed that basal calpain activity remained unchanged in the brain of calpastatin-transgenic mice as compared with wild-type controls. Altogether, these results support the idea that calpastatin levels are physiologically sufficient to control fully calpain activity under normal conditions and/or that calpastatin controls calpain activity only under pathologic conditions. In line with the latter hypothesis, these studies demonstrated that renal injury in the early heterologous phase of anti-GBM nephritis was associated with an increase in the renal activity of calpain in wild-type mice but not in calpastatin-transgenic mice. The only published reports of enhanced calpain activity in kidney disease were so far limited to models of ischemia-reperfusion (19).

Our study was the first demonstration that calpain secretion in urine paralleled calpain activation in renal cortex during the
early course of anti-GBM nephritis. The peak expression in urine occurred at 24 h, when albuminuria was the most abundant, suggesting that lesions of the glomerular filtration barrier played a major role. Consistent with this hypothesis, we detected high amounts of calpain activity in the urine of Nphs2/H11002/H11002 mice, which develop massive proteinuria in the absence of glomerular inflammation (21). These events suggest that calpain is present in plasma and is filtered through the injured glomerular filtration barrier. Accordingly, calpain activity was evidenced in plasma of mice with anti-GBM nephritis, as previously shown in plasma of rats with CCl4-induced hepatitis (22). Another possible mechanism of calpain appearance in urine of anti-GBM nephritic mice is its secretion by tubular epithelial cells. Indeed, as other intracellular enzymes, calpains may leak out from injured and dying cells such as hepatocytes that are exposed to toxic chemicals (22) or tubular epithelial cells that are submitted to hypoxia (23). Our finding that epithelial cells of proximal tubule (HK-2 cells) that were exposed to albumin released calpain in the extracellular milieu supports this hypothesis. It is interesting that exposure of renal proximal tubular cells to albumin was shown recently to induce endoplasmic reticulum stress and calpain activation, in turn responsible for caspase activation and apoptosis (24). It is possible, therefore, that calpain activation and release are linked in tubular epithelial cells. Although the mechanisms of calpain release from HK-2 cells that were exposed to albumin were not examined in our study, the observation that calpain release from osteoblasts and parathyroid cells is due to the shedding of membrane vesicles (25,26) suggests possible pathways of secretion to explore.

The results reported here showed that calpain inactivation in calpastatin-transgenic mice was effective in reducing the severity of glomerular inflammation. The marked reduction in the incidence of glomerular capillary thrombi may be due to interference with the TNF-α-induced expression of procoagulant activity by endothelial and mesangial cells. Indeed, tissue factor expression is responsible for glomerular fibrin deposition in experimental models of anti-GBM nephritis (27), and TNF-α plays a critical role in tissue factor production by glomerular cells (28). As NF-κB is the main factor involved in the transcriptional expression of TNF-α, calpain inactivation in calpastatin-transgenic mice would prevent NF-κB activation and thereby limit TNF-α–dependent expression of tissue factor. The progressive activation of NF-κB that is observed at the onset of anti-GBM nephritis (29) and its marked limitation in calpastatin-transgenic mice support this possibility.

These studies demonstrated also a decrease in glomerular injury and albuminuria in nephritic calpastatin-transgenic mice as compared with nephritic wild-type mice. This protection was associated with the persistence of nephrin expression, suggesting a role for calpain in nephrin redistribution. There are a number of different ways in which calpain activity might affect nephrin expression. First, calpain is known to cleave kinases and phosphatases as well as many of the cytoskeletal proteins that are involved in cytoskeletal-plasma membrane interactions (3). Therefore, it is conceivable that intracellular calpain activity promotes a slit diaphragm disruption through loss of the nephrin–actin connection, either directly or indirectly through alterations in the phosphorylation state of nephrin (30). Second, TNF-α has been shown to induce the shedding of nephrin from podocytes in culture (17). This suggests that calpain activity might amplify TNF-α–dependent loss of nephrin expression.

Figure 7. Albuminuria is decreased in nephritic TG mice as compared with nephritic WT mice. WT mice (□) and TG mice (■) received an injection of anti-GBM antiserum. Urine albumin concentration was determined by ELISA. *P < 0.05 versus WT mice (n = 5).

Figure 8. Nephrin expression in vivo. (A) Immunofluorescence staining for nephrin in glomeruli of control and nephritic mice 24 h after injection of anti-GBM antiserum. (B) Semiquantitative analysis of nephrin expression in glomeruli of nephritic WT mice (□) as compared with nephritic TG mice (■). *P < 0.05 versus WT mice (n = 3). Magnification, ×400.
indirectly through NF-κB–dependent TNF-α gene transcription. Nevertheless, these two mechanisms are insufficient to explain our in vitro results, because nephrin expression by podocytes was affected by extracellular rather than intracellular calpain. Therefore, the most attractive explanation is that extracellular calpain cleaves directly the extracellular domain of nephrin and thereby promotes its shedding.

Conclusion
These results are in agreement with our hypothesis that tissue calpain activity plays a critical role in glomerular inflammation and injury in experimental glomerulonephritis and that its inhibition may have therapeutic benefits. They also are the first to show that urinary calpain activity participates in nephrin redistribution, thereby strongly indicating its role as a marker and also a mediator of renal injury.

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