[Original Article]

[Title]

Urinary neutrophil gelatinase-associated lipocalin levels reflect damage in glomeruli, proximal and distal nephrons

T Kuwabara[1], K Mori[1], M Mukoyama[1], M Kasahara[1,2], H Yokoi[1], Y Saito[1], T Yoshioka[1], Y Ogawa[1], H Imamaki[1], T Kusakabe[1], K Ebihara[1], M Omata[3], N Satoh[4], A Sugawara[5], J Barasch[6] and K Nakao[1].

[1]Department of Medicine and Clinical Science, Kyoto University Graduate School of Medicine, Kyoto, Japan;
[2]Division of Nephrology, Kobe City General Hospital, Kobe, Japan;
[3]Biomedical Research Laboratories, Asubio Pharma Co. Ltd., Osaka, Japan;
[4]Clinical Research Institute for Endocrine Metabolic Diseases, National Hospital Organization, Kyoto Medical Center, Kyoto, Japan;
[5]Department of Nephrology, Kyoto Medical Center, Kyoto, Japan;
[6]Department of Medicine, College of Physicians and Surgeons of Columbia University, New York, NY, USA.

Correspondence: *K Mori, Department of Medicine and Clinical Science, Kyoto University Graduate School of Medicine; 54 Shogoin Kawahara-cho, Sakyo-ku, Kyoto 606-8507, Japan.*
E-mail: keyem@kuhp.kyoto-u.ac.jp

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[Running Headline]

Source and changes of urinary Ngal in renal injury

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ABSTRACT

Urinary (u) neutrophil gelatinase-associated lipocalin (Ngal or lipocalin 2) is a very early and sensitive biomarker for kidney injury but its source and time course during treatment remain elusive. In the present study we show that uNgal concentrations were markedly elevated in A-ZIP/F-1 lipoatrophic diabetes and streptozotocin-induced diabetic (STZ) mice. In STZ mice, reabsorption of Ngal in the proximal tubule was severely reduced but upregulation of Ngal mRNA and protein expression in the kidney was little compared to non-diabetic control mice, indicating that increased uNgal excretion derived mainly from impaired renal reabsorption. In mouse kidneys treated with unilateral ureteral obstruction, Ngal protein was highly accumulated in thick ascending limbs of Henle and in the urine pooled in pelvis. In 5 patients with nephrotic syndrome or interstitial nephritis, uNgal levels were elevated markedly, and were decreased in response to treatment including steroids. Treatment of STZ mice with angiotensin receptor blocker candesartan dramatically reduced uNgal excretion. These findings indicate that uNgal level is a unique biomarker of renal injury incorporating information of glomerular filtration defect, tubular reabsorption dysfunction and distal nephron damage, and may be useful in monitoring disease activity and treatment efficacy in various forms of renal injury.

INTRODUCTION

Neutrophil gelatinase-associated lipocalin (Ngal) is a differentiation inducer for epithelia in embryonic kidney, whose expression is dramatically increased in acute kidney injury (AKI).[1-5] Ngal exerts a spectrum of iron-dependent biological activities,[1-4,6] and administration of Ngal protein mitigates renal injury in mice, suggesting that functional consequence of Ngal upregulation is renoprotection.[2] Ngal mRNA levels in the kidney are increased as much as by 1000-fold during renal ischemia-reperfusion injury in mice.[2,7] Ngal protein starts to accumulate within a few hours in the blood and urine during AKI.[8-11] These characteristics of Ngal have made it a promising biomarker of AKI that is found in the blood and urine.[1,7,12-15] Furthermore, several studies reported that serum and urinary Ngal levels are elevated proportionally to the extent of renal damage in chronic kidney disease (CKD),[16,17] but the source and the time course of urinary Ngal concentrations are largely unknown. In this study, we investigated urinary Ngal levels in four types of renal damage caused by distinct mechanism: nephrotic syndrome caused by glomerular disorders, diabetic nephropathy, obstructive nephropathy and interstitial nephritis. We also examined whether measurement of urinary Ngal is useful for the monitoring of renal damage in mice or humans during the treatment course.

RESULTS

Urinary Ngal excretion is proportional to albumin excretion in mouse models of diabetic nephropathy

As a model of diabetic nephropathy, we first examined urinary Ngal concentrations in A-ZIP/F-1 transgenic mice, which are characterized with lipoatrophic diabetes, fatty liver, hyperlipidemia, severe insulin resistance and massive proteinuria.[18-20] Urinary Ngal excretion in A-ZIP/F-1 mice at 10 months of age was much larger than that in control FVB/N mice (Figure 1). By Western blot, we observed 30 and 25 kDa bands with Ngal immunoreactivity, and the larger band was found only in the urine from A-ZIP/F-1 mice and not in the urine or tissues from normal, diabetic or obstructed kidneys of mice with C57BL/6 background (see below). The larger protein may have heavier glycosylation than the smaller protein.[21] When the amounts of two proteins were added, mice with larger urinary Ngal levels tended to have larger urinary albumin levels.

Next, we studied streptozotocin (STZ)-induced diabetes, which manifests with insulin deficiency and microalbuminuria. In STZ mice, urinary albumin excretion increased gradually and, after 8 weeks, reached 7.8-fold of the level before STZ injection (Figure 2). On the other hand, urinary Ngal levels were elevated by 77-fold at 8 weeks. The extent of Ngal and albumin excretion was highly variable among different mice, but urinary albumin levels and log transformation of Ngal levels showed a close linear correlation throughout the course of 12 week observation period.

Elevation of urinary Ngal excretion in STZ mice is not caused by renal synthesis but by reabsorption defect, and treatment with angiotensin receptor blocker reduces urinary Ngal levels

To examine whether local expression of Ngal is increased in STZ mouse kidneys, we studied expression levels of Ngal protein in the whole kidney preparation of STZ and non-STZ control mice and found no significant difference at 8 weeks after STZ treatment (Figure 3). We did not find significant alteration of Ngal protein expression in the livers, either. Of note, serum Ngal levels in STZ mice were significantly lower than those in non-STZ mice (23±5 versus 111±22 ng ml^{-1}, n=3-4, $P<0.01$). We measured Ngal mRNA expression levels in the kidneys and livers of STZ mice, but they were increased only marginally compared to control mice. As a positive control, Ngal mRNA expression was increased by 100 fold in obstructed kidneys after 1 day of ureter ligation ($P<0.001$). These findings suggested that dramatic (nearly 80 fold) increase of urinary Ngal excretion in STZ mice cannot be explained by augmentation of Ngal protein synthesis in the kidney, and led us to investigate tubular reabsorption of Ngal. Injection of histidine-tagged or Alexa Fluor 546-labeled Ngal (21 kDa in size) in the peritoneum of non-STZ mice resulted in glomerular filtration and efficient reabsorption of Ngal at the proximal tubules from the apical side, thus no exogenous Ngal was detected in the urine (Figure 4). In STZ mice, on the other hand, substantial amount of exogenous Ngal was

excreted in the urine and reabsorption of labeled Ngal was reduced by 47 % ($P<0.01$).

Since treatment of diabetic nephropathy with angiotensin receptor blocker (ARB) reduces proteinuria and ameliorates renal injury,[22] we gave the ARB candesartan to STZ mice through drinking water at 10 mg per kg per day (Figure 5). After one week, urinary albumin levels were decreased by 14 % ($P<0.05$) and urinary Ngal levels were decreased by 77 % ($P<0.05$). Serum Ngal levels were not altered by candesartan (20±6 ng ml^{-1}). The dose of candesartan used was a subdepressor dose, and did not significantly affect body weights, blood glucose, urea nitrogen and creatinine levels (Table 1). Through these findings, we conclude that increased urinary Ngal excretion in STZ mice was caused mainly by reabsorption defect and treatment with candesartan partially normalized urinary Ngal levels.

Urinary Ngal levels are highly elevated in human cases of nephrotic syndrome and are decreased in response to treatment

As cases of glomerular disorders with nephrotic syndrome, we investigated the clinical courses and changes in serum and urinary Ngal levels in human subjects. Case 1 was a 68-year-old female with biopsy-proven minimal change disease. She had nephrotic range proteinuria (14 g day^{-1}) and gained body weight by 15 kg (from 62 to 77 kg) within 3 weeks. She was treated with iv steroid pulse (methyl prednisolone 1 g x 3 days), followed with oral prednisolone (beginning with 35 mg per day), and with three courses of hemodialysis on days 8, 9 and 12 after admission (Figure 6). Her proteinuria, edema and azotemia resolved gradually. Concomitantly, urinary and serum Ngal levels decreased during the treatment, but reduction was much faster for urinary Ngal levels. There was a temporal elevation of urinary Ngal levels on day 21, which might reflect the reappearance of oligouria or proteinuria.

Case 2 was a 26-year-old female, who was diagnosed to have membranous-type lupus nephritis (ISN/RPS class V). She was treated with two courses of iv steroid pulse, followed with oral prednisolone (Figure 6). Her urinary levels of protein and Ngal were decreased sharply within 17 days.

Case 3 was a 55-year-old female with clinically-diagnosed lupus nephritis (no biopsy). She had been treated with two courses of iv steroid pulse (1 g x 3 days) and oral prednisolone had been tapered from 50 to 35 mg per day, before changing the hospitals to ours. She was given iv cyclophosphamide pulse (0.4 g, once, 6 days after admission) and the immunosuppressant mizoribine (Asahi Kasei Pharma, Osaka, Japan; 100 mg per day). Urinary excretion of protein and Ngal decreased slowly (Supplementary Figure S1). Serum creatinine levels were constant throughout the course (0.4-0.5 mg per 100 ml).

Case 4 was a 69-year-old male, who suffered from rapidly-progressing, crescentic glomerulonephritis accompanied with moderate tubulointerstitial damage. His serum contained myeloperoxidase-type anti-neutrophil cytoplasmic antibody

(MPO-ANCA, 138 EU). The maximum serum creatinine level was 5.6 mg per 100 ml, and the proteinuria and azotemia responded slowly to treatment containing oral and iv steroid and iv cyclophosphamide (Supplementary Figure S1). Macrohematuria was observed, peaking at 5 days after cyclophosphamide administration. After 10 months, he showed signs of recurrence which were worsening in proteinuria, hematuria, azotemia and MPO-ANCA titer (from <10 to 37 EU). We observed re-elevation of urinary Ngal levels during acute worsening of nephritis. Cyclosporine A (75 mg day^{-1} before breakfast) was added at 13 months, which appeared to support reduction of above mentioned signs.

To understand renal localization of Ngal protein in nephrotic patients, we carried out immunofluorescence study of renal biopsy samples and found close co-localization of signals of Ngal and albumin at the apical side of tubules. The results for Case 1 are shown in Figure 7. These findings are consistent with those in animal experiments shown above (Figure 4), indicating highly active reabsorption of Ngal from glomerular filtrate.

In mice with obstructive nephropathy, Ngal protein is specifically located in the distal nephrons in the obstructed side by local synthesis, whereas it is confined to the proximal tubules in the contra-lateral side of the kidneys by reabsorption

As a model of post-renal kidney injury, we investigated mice with unilateral ureteral obstruction (UUO), in which distal nephrons are primarily affected, and studied the changes of Ngal protein levels in the obstructed and non-obstructed sides of the kidneys, serum, and urine (Figures 7, 8). We also determined the renal distribution of Ngal by immunohistochemistry along with nephron segment markers: aquaporin (AQP) 1 for proximal tubules, Tamm-Horsfall protein (THP) for thick ascending limbs of Henle, and AQP 2 for collecting ducts (Figure 7, Supplementary Figures S2-S4). In the UUO kidneys, after 1 day of ureter ligation, Ngal protein was expressed exclusively in thick ascending limbs of Henle, which was also expressing THP and was prominently dilated, suggesting that Ngal was synthesized in damaged epithelia. By striking contrast, in the contra-lateral kidneys, Ngal protein was confined to the apical side of aquaporin 1^+ proximal tubules. Ngal protein levels in the obstructed kidneys and in the urine from the dilated pelvis were continuously elevated for 2 weeks, whereas those in the non-obstructed kidneys and in the serum peaked at day 1 and decreased gradually. A smaller (17 kDa) fragment detected in the kidneys (but not in the urine and serum) using polyclonal anti-Ngal antibody may have been generated by lysosomal proteolysis of Ngal in the tubules.[2] Ngal mRNA expression was elevated by 100-fold in the obstructed kidneys (Figure 3), but it was only elevated by 3-fold in the contra-lateral kidneys at day 1 (data not shown). These findings indicate that Ngal was synthesized *de novo* in distal nephrons of obstructed kidneys, while it was highly but transiently

accumulated in the serum, filtrated and reabsorbed in the contra-lateral kidneys.

In a case with interstitial nephritis, urinary Ngal levels decreased more rapidly than classic markers of tubular injury.

Next, we investigated whether urinary Ngal is useful for evaluation of renal disorder with low level proteinuria. Case 5 was a 25-year-old male, who was admitted to our hospital, presenting with general malaise, proteinuria (0.4 g day^{-1}), renal glucosuria and mild azotemia. He had taken an over-the-counter cold medicine 6 weeks earlier, and was positive in lymphocyte stimulation test for the drug. Renal biopsy revealed subacute interstitial nephritis with minor glomerular lesions. His signs and symptoms resolved by oral and iv prednisolone treatment. Table 2 and Supplementary Figure S5 summarize the clinical course and changes in urinary biomarkers. The time to 50% reduction of urinary markers was in the order of Ngal ≤ (total) protein < α1- and β2-microglobulins (classic markers of tubular proteinuria) < N-acetyl-beta-D-glucosaminidase (NAG). The fold-change during treatment was also largest in urinary Ngal, suggesting that urinary Ngal may be useful in monitoring the activity of non-glomerular renal disorders.

DISCUSSION

In the present study, we investigated urinary Ngal concentrations in patients with nephrotic syndrome (caused by acute and severe glomerular disorders) and interstitial nephritis and in mouse models of diabetic or obstructive nephropathy and found that the levels were unequivocally elevated (over 10 fold of control). In diabetic mice induced by STZ (as a model of slowly-progressive CKD), urinary Ngal appeared to derive mostly from impaired reabsorption in proximal tubules. In obstructed kidneys (as a model of post-renal AKI), Ngal was highly expressed in distal nephrons and accumulated in the urine collected from the pelvis. Therefore, STZ-diabetic and obstructed kidneys are the two extreme examples in which the primary source of urinary Ngal is glomerular filtrate and renal synthesis, respectively. In human renal disorders, urinary Ngal should be a mixture of these two major components. These findings indicate that in a variety of kidney diseases, urinary Ngal is a biomarker that can reflect damage in glomeruli, proximal tubules and distal nephrons.

Cross-sectional studies published so far have elucidated that urinary Ngal levels show certain correlation with urinary protein levels.[16,23] To our knowledge, this is the first human report to show very rapid and simultaneous reduction of urinary Ngal and protein concentrations by medical intervention. Surprisingly, the time course of urinary Ngal levels was associated to that of urinary protein levels not only in diabetic nephropathy and minimal change disease but also in crescentic glomerulonephritis and interstitial nephritis. In the latter disorders, treatment with steroid and immunosuppressant may have ameliorated Ngal reabsorption impairment and epithelial Ngal synthesis at the same time. The present findings suggest that urinary Ngal may be useful in the monitoring of disease activity and treatment efficacy. Of note, we cannot over-generalize the findings in this study to all renal disorders, especially because we did not examine patients with severe, acute tubular necrosis, for instance, caused by renal ischemia or nephrotoxins (in which Ngal is abundantly synthesized by renal epithelia).[2]

In diabetic nephropathy, albumin excretion is increased by leakage from glomerular filtration barrier.[24] On the other hand, a number of reports elucidated the involvement of tubular dysfunction as a cause of albuminuria.[24-27] The size of Ngal protein (25 kDa) is smaller than albumin and, in normal conditions, Ngal is rapidly filtered by glomeruli and reabsorbed very efficiently by proximal tubules, leaving only 0.1-0.2% in the urine.[2] By analyzing renal handling of exogenously injected Ngal, the present study emphasized the existence of tubular reabsorption impairment in diabetic nephropathy.[28] In reverse, cellular stress in the proximal tubules may cause deterioration in diabetic nephropathy, since it was reported that transgenic overexpression of catalase in the proximal tubules of mice attenuated development of hypertension and albuminuria associating diabetic nephropathy.[29]

Treatment of STZ-diabetic mice with ARB significantly reduced urinary

excretion of both Ngal and albumin. Two major scenarios can be proposed. First, ARB directly improved reabsorption efficiency in the proximal tubules, which may be mediated by increased peritubular capillary blood flow[30] and by amelioration of oxidative stress.[31] Second, ARB reduced intraglomerular hypertension and hyperfiltration,[32] reduced albumin leakage from glomeruli, and the total amount of the ligands for the common scavenger receptor megalin was decreased in the tubular lumen. This may increase the ratio of Ngal and albumin endocytosed at proximal tubules.

Here we used high-dose STZ to induce a model of diabetic nephropathy. Direct toxicity of STZ to proximal tubules may have exaggerated the elevation of urinary Ngal levels in these mice (which is 77-fold of control mice).[33] Hyperfiltration is reported in rodents given STZ.[32] In a previous report, 3-fold elevation in creatinine clearance was observed in C57BL/6 mice after treatment with STZ, but serum creatinine levels were not significantly decreased, likely due to concomitant osmotic diuresis and dehydration.[34] In the present study, hyperfiltration may have similarly occurred in STZ-diabetic mice. Growth arrest and leanness of STZ-diabetic mice may be partly related to selective reduction of serum Ngal levels (but not serum creatinine and BUN levels) in the present study, since obesity has been shown to be associated with elevated circulating Ngal levels.[35]

We also observed abundant urinary Ngal excretion in 4 patients with nephrotic syndrome. The mechanism may involve direct competition between Ngal and albumin at the surface of megalin molecule for receptor-mediated endocytosis[26,36] and also general malfunctioning of proximal tubules because of protein overload. Furthermore, a fraction of urinary Ngal may originate from renal synthesis in addition to reabsorption defect, but Ngal gene expression in human samples was not investigated in this study. The time required for urinary Ngal reduction was variable among cases: ranging from 2 weeks (cases 1 and 2) to more than a month (cases 3 and 4).

In a case with interstitial nephritis, urinary Ngal showed the largest fold increase and the quickest response to steroid treatment in comparison to other urinary biomarkers: total protein, α1- and β2-microglobulins and NAG. These findings suggest that urinary Ngal might be particularly useful in the evaluation of kidney recovery in patients with low grade proteinuria.

To summarize, urinary Ngal is a rapid biomarker of kidney injury and recovery showing a large fold-increase or decrease during clinical course of various renal disorders. Proteinuria seems to be one of important factors affecting Ngal'uria.

MATERIALS AND METHODS

Animal experiments

All animal experiments were conducted in accordance with our institutional guidelines for animal research. Male A-ZIP/F-1 heterozygous transgenic mice and control FVB/N littermates were used at 10 month of age, when A-ZIP/F-1 mice exhibit diabetic nephropathy with massive proteinuria.[18-20] Other animal experiments were carried out with male C57BL/6J mice (Japan Clea, Tokyo, Japan) starting at 8 week of age, when they weighted 21-23 g. Diabetes was induced by intraperitoneal injection of STZ (180 mg kg^{-1} of body weight; Sigma, St. Louis, MO, USA) in citrate buffer (pH 4.6) and control mice received only citrate buffer. Blood pressure was measured by the indirect tail-cuff method with MK-2000ST (Muromachi Kikai, Tokyo, Japan). Urine samples were collected with metabolic cages. Urinary albumin was measured with murine albumin ELISA (Exocell; Philadelphia, PA, USA). Serum and urinary creatinine levels were assayed by the enzymatic method (SRL, Tokyo, Japan). This method gives reliable measurement when compared to high-performance liquid chromatography method, even in low concentration materials, and performs much better than Jaffe's colorimetric method.[37] Blood glucose and HbA1c levels were determined in tail vein blood at ad libitum-fed conditions using Glutest Ace (Sanwa Kagaku, Nagoya, Japan) and DCA2000+ Analyzer (Bayer Medical, Tokyo, Japan), respectively. Mice were killed under pentobarbital anesthesia before organ collection. Prodrug of candesartan, candesartan cilexetil (TCV-116; Takeda Chemical Industries, Osaka, Japan), was initially dissolved at 10 mg ml^{-1} in a solution containing 16% polyethylene glycol #300 (vol/vol; Nacalai, Kyoto, Japan), 16% ethanol (Nacalai) and 0.7 M Na_2CO_3, and further diluted in drinking water to be given at a final dose of 10 mg per kg per day. This treatment method[38] gave less blood pressure lowering effects compared to gavage administration as previously described.[39] For UUO, mice were anesthetized with pentobarbital, the left kidney was exposed by midline incision, and the left ureter was ligated with 4-0 silk at two points.[40] Mice were sacrificed 1-14 days after the operation.

Recombinant Ngal injection and detection

To investigate renal reabsorption of Ngal, 200 μg of 6×histidine-tagged (at the carboxyl terminus) or 60 μg of Alexa Fluor 546 (Molecular Probes, Eugene, OR, USA)-labeled recombinant mouse Ngal (expressed in BL21 strain of E. coli)[2] was injected into the peritoneum of mice. Urine samples were collected for 12 hours after His-tagged Ngal injection. Urinary excretion of administered His-tagged Ngal was evaluated by Western blot analysis described below with anti-His antibody (MBL, Nagoya, Japan) or with goat polyclonal anti-mouse Ngal antibody (R&D Systems, Minneapolis, MN, USA). Kidneys were harvested 30 minutes after Alexa Fluor 546-labeled Ngal injection, snap frozen, sliced at 10 μm thickness and examined by a fluorescence microscope (IX81-PAFM; Olympus, Tokyo, Japan). The signal positive areas were measured using MetaMorph 7.5 software (Molecular Devices, Downingtown, PA, USA)

Patients and measurement of human Ngal

Patients who admitted to Kyoto University Hospital for the diagnosis and treatment of renal disorders were enrolled under informed consent. This study was approved by the ethical committee on human research of Kyoto University Graduate School of Medicine. Ngal concentrations in the human serum and urine were determined by sandwich ELISA (AntibodyShop, Gentofte, Denmark) usually after 1000 and 250-fold dilution, respectively.

Western blot analysis

Urine, serum and proteins extracted from organs were separated by SDS-PAGE, transferred onto PVDF membranes, incubated with primary antibody and detected with peroxidase-conjugated secondary antibody and chemiluminescence. Serum was passed through 100-kDa cut-off membrane (Microcon YM-100, Millipore, Bedford, MA, USA) to remove immunoglobulins before analysis.[2] The amount of Ngal protein was measured by densitometry. Known amounts of recombinant mouse Ngal protein were used as standards.[2]

Real-time reverse transcription PCR

Total RNA was extracted from mouse kidneys and livers with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and cDNA in each sample was synthesized by High capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). The mRNA levels of Ngal were determined using Premix Ex Taq (Takara Bio, Otsu, Japan) and ABI Prism 7300 Sequence Detector with the following primers and probe: Ngal forward, 5'- ggcagctttacgatgtacagca-3'; Ngal reverse, 5'- tctgatccagtagcgacagcc-3'; Ngal probe, 5'-FAM-catcctggtcagggaccaggaccag-TAMRA-3'. Expression levels of Ngal were normalized by GAPDH (internal control) levels, whose primer-probe set was purchased from Applied Biosystems.[41] Standard curve was made by serial dilution of cDNA from UUO kidneys.

Immunofluorescence of Ngal and albumin

Snap frozen human biopsy samples were sliced at 3 μm thickness, fixed with acetone, and incubated with a solution containing both FITC-labeled rabbit anti-human albumin antibody (Dako, Glostrup, Denmark) and goat polyclonal anti-human Ngal antibody (R&D Systems), followed by incubation with TexasRed-labeled anti-goat IgG (Jackson Immunoresearch, West Grove, PA, USA). The spillover of each signal was negligible.

Immunohistochemistry of Ngal and nephron markers

Mouse kidneys were fixed in 4% paraformaldehyde at 4oC for 12 hours and embedded in paraffin. Renal sections of 4 μm were deparaffined, hydrated and incubated with 0.3% hydrogen peroxide. Antigen retrieval was performed by 0.05 mol L^{-1} citrate buffer (pH 6.0) for 10min in a water bath heated at 100 oC (for Ngal, AQP1 and AQP2), or in a microwave oven (for THP). After cooling, the sections were incubated with 10% normal donkey or goat serum, followed by goat anti-mouse Ngal (1:300, R&D Systems), rabbit anti-AQP1 and AQP2 (1:200, Chemicon International, Temecula, CA, USA), or rabbit

anti-THP antibodies (1:200, Biomedical Technologies, Stoughton, MA, USA). Primary antibodies were visualized with horseradish peroxidase-conjugated secondary antibodies and 3,3-diaminobenzidine tetrahydrochloride. Nuclei were counterstained with hematoxylin.

Statistical analysis

Results are expressed as mean±s.e. Student's t-test was carried out to compare two groups. Statistical significance was defined as $P<0.05$.

DISCLOSURE

The authors declare that they have no competing interests.

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SUPPLEMENTARY MATERIALS

Figure S1

Clinical course of cases with (a) lupus nephritis (Case 3, LN) and (b) crescentic glomerulonephritis (Case 4, CrescGN).

Figure S2

Low power fields showing expression of Ngal and nephron markers in unilateral ureteral obstruction (UUO) and contra-lateral kidneys.

Figure S3

High power fields of cortex showing expression of Ngal and nephron markers in UUO and contra-lateral kidneys.

Figure S4

High power fields of medulla showing expression of Ngal and nephron markers in UUO and contra-lateral kidneys**.**

Figure S5

Clinical course of a case with drug-induced interstitial nephritis (Case 5, IntN).

REFERENCES

1. Mori K, Nakao K. Neutrophil gelatinase-associated lipocalin as the real-time indicator of active kidney damage. *Kidney Int* 2007; **71:** 967-970.

2. Mori K, Lee HT, Rapoport D *et al.* Endocytic delivery of lipocalin-siderophore-iron complex rescues the kidney from ischemia-reperfusion injury. *J Clin Invest* 2005; **115:** 610-621.

3. Barasch J, Mori K. Cell biology: iron thievery. *Nature* 2004; **432:** 811-813.

4. Yang J, Goetz D, Li JY *et al.* An iron delivery pathway mediated by a lipocalin. *Mol Cell* 2002; **10:** 1045-1056.

5. Mori K, Yang J, Barasch J. Ureteric bud controls multiple steps in the conversion of mesenchyme to epithelia. *Semin Cell Dev Biol* 2003; **14:** 209-216.

6. Flo TH, Smith KD, Sato S *et al.* Lipocalin 2 mediates an innate immune response to bacterial infection by sequestrating iron. *Nature* 2004; **432:** 917-921.

7. Schmidt-Ott KM, Mori K, Li JY *et al.* Dual action of neutrophil gelatinase-associated lipocalin. *J Am Soc Nephrol* 2007; **18:** 407-413.

8. Mishra J, Dent C, Tarabishi R *et al.* Neutrophil gelatinase-associated lipocalin (NGAL) as a biomarker for acute renal injury after cardiac surgery. *Lancet* 2005; **365:** 1231-1238.

9. Mishra J, Ma Q, Prada A *et al.* Identification of neutrophil gelatinase-associated lipocalin as a novel early urinary biomarker for ischemic renal injury. *J Am Soc Nephrol* 2003; **14:** 2534-2543.

10. Wagener G, Jan M, Kim M *et al.* Association between increases in urinary neutrophil gelatinase-associated lipocalin and acute renal dysfunction after adult cardiac surgery. *Anesthesiology* 2006; **105:** 485-491.

11. Bennett M, Dent CL, Ma Q *et al.* Urine NGAL Predicts Severity of Acute Kidney Injury After Cardiac Surgery: A Prospective Study. *Clin J Am Soc Nephrol* 2008; Epub ahead of print

12. Devarajan P. Emerging biomarkers of acute kidney injury. *Contrib Nephrol* 2007; **156:** 203-212.

13. Coca SG, Yalavarthy R, Concato J, Parikh CR. Biomarkers for the diagnosis and risk stratification of acute kidney injury: A systematic review. *Kidney Int* 2008; Epub ahead of print.

14. Waikar SS, Bonventre JV. Biomarkers for the diagnosis of acute kidney injury. *Curr Opin Nephrol Hypertens* 2007; **16:** 557-564.

15. Nickolas TL, O'Rourke MJ, Yang J et al. Sensitivity and specificity of a single emergency department measurement of urinary neutrophil gelatinase-associated lipocalin for diagnosing acute kidney injury. *Ann Intern Med* 2008; **148:** 810-819.

16. Ding H, He Y, Li K *et al.* Urinary neutrophil gelatinase-associated lipocalin (NGAL) is an early biomarker for renal tubulointerstitial injury in IgA nephropathy. *Clin Immunol* 2007; **123:** 227-234.

17. Mitsnefes MM, Kathman TS, Mishra J *et al.* Serum neutrophil gelatinase-associated lipocalin as a marker of renal function in children with chronic kidney disease. Pediatr Nephrol 2007; **22:** 101-108.

18. Suganami T, Mukoyama M, Mori K *et al.* Prevention and reversal of renal injury by leptin in a new mouse model of diabetic nephropathy. *FASEB J* 2005; **19:** 127-129.

19. Moitra J, Mason MM, Olive M *et al.* Life without white fat: a transgenic mouse. *Genes Dev* 1998; **12:** 3168-3181.

20. Ebihara K, Ogawa Y, Masuzaki H *et al.* Transgenic overexpression of leptin rescues insulin resistance and diabetes in a mouse model of lipoatrophic diabetes. *Diabetes* 2001; **50:** 1440-1448.

21. Kjeldsen L, Johnsen AH, Sengeløv H *et al.* Isolation and primary structure of NGAL, a novel protein associated with human neutrophil gelatinase. *J Biol Chem* 1993; **268:** 10425-10432.

22. Brenner BM, Cooper ME, de Zeeuw D *et al.* RENAAL Study Investigators. Effects of losartan on renal and cardiovascular outcomes in patients with type 2 diabetes and nephropathy. *N Engl J Med* 2001; **345:** 861-869.

23. Bolignano D, Coppolino G, Campo S *et al.* Urinary Neutrophil Gelatinase-Associated Lipocalin (NGAL) is associated with severity of renal disease in proteinuric patients. *Nephrol Dial Transplant* 2008; **23:** 414-416.

24. Tucker BJ, Rasch R, Blantz RC. Glomerular filtration and tubular reabsorption of albumin in preproteinuric and proteinuric diabetic rats. *J Clin Invest* 1993; **92:** 686-694.

25. Tojo A, Onozato ML, Kurihara H *et al.* Angiotensin II blockade restores albumin reabsorption in the proximal tubules of diabetic rats. *Hypertens Res* 2003; **26:** 413-419.

26. Birn H, Christensen EI. Renal albumin absorption in physiology and pathology. *Kidney Int* 2006; **69:** 440-449.

27. Osicka TM, Houlihan CA, Chan JG *et al.* Albuminuria in patients with type 1 diabetes is directly linked to changes in the lysosome-mediated degradation of albumin during renal passage. *Diabetes* 2000; **49:** 1579-1584.

28. Chouinard S, Viau C. Reversibility of renal tubular dysfunction in streptozotocin-induced diabetes in the rat. *Can J Physiol Pharmacol* 1992; **70:** 977-982.

29. Brezniceanu ML, Liu F, Wei CC *et al.* Attenuation of interstitial fibrosis and tubular apoptosis in db/db transgenic mice overexpressing catalase in renal proximal tubular cells. *Diabetes* 2008; **57:** 451-459.

30. Manotham K, Tanaka T, Matsumoto M *et al.* Evidence of tubular hypoxia in the early phase in the remnant kidney model. *J Am Soc Nephrol* 2004; **15:** 1277-1288.

31. Onozato ML, Tojo A, Goto A *et al.* Oxidative stress and nitric oxide synthase in rat diabetic nephropathy: effects of ACEI and ARB. *Kidney Int* 2002; **61:** 186-194.

32. Zatz R, Dunn BR, Meyer TW *et al.* Prevention of diabetic glomerulopathy by pharmacological amelioration of glomerular capillary hypertension. *J Clin Invest* 1986; **77:** 1925-1930.

33. Breyer MD, Böttinger E, Brosius FC 3rd et al. Mouse models of diabetic nephropathy. *J Am Soc Nephrol* 2005; **16:** 27-45.

34. Ichinose K, Maeshima Y, Yamamoto Y et al. Antiangiogenic endostatin peptide ameliorates renal alterations in the early stage of a type 1 diabetic nephropathy model. *Diabetes* 2005; **54:** 2891-2903.

35. Yan QW, Yang Q, Mody N et al. The adipokine lipocalin 2 is regulated by obesity and promotes insulin resistance. *Diabetes* 2007; **56:** 2533-2540.

36. Hvidberg V, Jacobsen C, Strong RK *et al.* The endocytic receptor megalin binds the iron transporting neutrophil-gelatinase-associated lipocalin with high affinity and mediates its cellular uptake. *FEBS Lett* 2005; **579:** 773-777.

37. Keppler A, Gretz N, Schmidt R et al. Plasma creatinine determination in mice and rats: an enzymatic method compares favorably with a high-performance liquid chromatography assay. *Kidney Int* 2007; **71:** 74-78.

38. Kosugi R, Shioi T, Watanabe-Maeda K *et al.* Angiotensin II receptor antagonist attenuates expression of aging markers in diabetic mouse heart. *Circ J* 2006; **70:** 482-488.

39. Shao J, Iwashita N, Ikeda F *et al.* Beneficial effects of candesartan, an angiotensin II type 1 receptor blocker, on beta-cell function and morphology in db/db mice. *Biochem Biophys Res Commun* 2006; **344:** 1224-1233.

40. Nagae T, Mori K, Mukoyama M *et al.* Adrenomedullin inhibits connective tissue growth factor expression, extracellular signal-regulated kinase activation and renal fibrosis. *Kidney Int* 2008; **74:** 70-80.

41. Yokoi H, Mukoyama M, Mori K *et al.* Overexpression of connective tissue growth factor in podocytes deteriorates diabetic nephropathy in mice. *Kidney Int* 2008; **73:** 446-455.

Figure Legends

Figure 1

Urinary Ngal and albumin excretion in (a-c) A-ZIP/F-1 diabetic mice at 10 months of age and (d, e) diabetic mice at 8 weeks after streptozotocin (STZ) injection.

(**a, d**) Western blot of urine (25 μl each) in individual mice and (**b, c, e**) urinary levels of Ngal and albumin (Alb) normalized by creatinine (Cr) are shown. (**b**) A-ZIP/F-1 mice including #1 excreted much larger volumes of urine than control FVB/N mice. In 3 control FVB/N mice, mean urinary Ngal/Cr ratio (±s.e.) was 42±24 ng per mgCr. uNgal, urinary Ngal; rNgal, recombinant Ngal.

Figure 2

(a) Time course and (b) correlation of urinary Ngal and albumin excretion in streptozotocin (STZ)-induced diabetic mice.

(**a**) Urinary Ngal (uNgal) and albumin (uAlb) levels normalized by urinary creatinine (uCr) were examined before and at 4, 8, 12 weeks after STZ injection (mean±s.e.). #, $P<0.05$ versus pre-STZ. Elevation of urinary Ngal levels was significant at 4, 8, 12 weeks if analyzed after log transformation. (**b**) Correlation between uNgal/uCr and uAlb/uCr. $r=0.86$, $P<0.001$, $n=19$.

Figure 3

Ngal protein and mRNA expression in the serum, kidney and liver of STZ mice.

(**a**) Western blot of serum, whole kidney and liver at 8 weeks after induction of diabetes. Mice without STZ injection served as control (non-STZ). Equal amounts of serum (15 μl), and protein (30 μg) of whole kidney and liver were separated by electrophoresis. rNgal; recombinant Ngal. (**b**) Ngal mRNA expression levels were measured using real-time PCR and normalized by GAPDH expression (n=4). The mean Ngal/GAPDH level in non-STZ whole kidney was arbitrary defined as 1.0. The whole kidneys at one day after unilateral uretereral obstruction (UUO) were also examined as a positive control. N.S., not significant.

Figure 4

Urinary excretion and tubular reabsorption of exogenously administered Ngal in STZ mice.

(**a**) Western blot of urinary Ngal detected either with anti-Ngal or with anti-His-tag antibody at 8 weeks after induction of diabetes. Urine samples were collected for 12 hours after His-tagged Ngal injection (i.p.) and 25 μl aliquots of them were separated by electrophoresis. Of note, STZ mice excreted much more diluted urine

compared to non-STZ control mice. 1, endogenous Ngal (25 kDa, glycosylated); 2, His-tagged Ngal (21 kDa, unglycosylated). (**b**) Alexa Fluor 546-labeled Ngal was injected in STZ mice and kidneys were examined 30 min later. Arrows indicate Ngal protein distribution (in orange) which was homogeneous in non-STZ but was irregular and sparse in STZ mice. Top right panel shows selected area (in green) of positive fluorescence by a computer software. G, glomuruli. Magnification, x 20. (**c**) Quantitation of exogenous Ngal uptake in kidneys of STZ and non-STZ mice (n=4).

Figure 5

Reduction of urinary Ngal and albumin excretion by candesartan in STZ mice.

(**a**) Urinary Ngal (uNgal) and (**b**) albumin (uAlb) levels at 4 and 8 weeks after STZ injection and after one more week with candesartan (10 mg per kg per day, orally) or vehicle treatment (n=4).

Figure 6

Clinical course of cases with (a) minimal change disease (Case 1, MCD) and (b) lupus nephritis (Case 2, LN).

mPSL, methyl prednisolone; PSL, prednisolone; uProt, urinary protein excretion; s and uNgal, serum and urinary Ngal; BUN, blood urea nitrogen; sCr, serum creatinine; uNgal/uCr, urinary Ngal normalized by urinary creatinine; Ab, antibody.

Figure 7

Localization of Ngal protein expression in human and mouse kidneys.

Immunofluorescence of albumin (**a**, green) and Ngal (**b**, red) in a patient with minimal change disease (MCD, magnification 40 x). Arrows, co-localization of signals; asterisk, tubular lumen. Immunohistochemistry of Ngal (**c, d**), aquaporin 1 (**e**, AQP1) and Tamm-Horsfall protein (**f**, THP) in the mouse kidney treated with unilateral ureteral obstruction (UUO; **d, f**) and in the contra-lateral kidney (UUN; **c, e**) at 1 day after operation (20 x). Serial sections were analyzed.

Figure 8

Ngal protein levels in UUO, UUN, serum and urine in mice with kidney obstruction.

(**a**) Western blot analysis of Ngal protein. N.D., not determined. (**b**) The mean Ngal concentrations in UUO, UUN, serum and urine (collected from dilated pelvis of the UUO side using a needle and syringe). Elevation of Ngal concentrations were statistically significant at day 1 in UUO or UUN kidneys and serum, and at

day 3 in urine ($P<0.01$ by unpaired t test, $n=3$) compared to day 0 (no operation).

Table 1

Blood glucose, urea nitrogen, creatinine levels, body weight and blood pressure in STZ diabetic mice before and after candesartan treatment

	Vehicle		Candesartan	
	Before	After	Before	After
Blood Glucose (mg per 100 ml)	598±2	593±6	600±3	591±5
HbA1c (%)	11.7±0.5	N.D.	12.5±0.2	N.D.
Blood Urea Nitrogen (mg per 100 ml)	N.D.	50±2	N.D.	54±1
Serum Creatinine (mg per 100ml)	N.D.	0.13±0.01	N.D.	0.11±0.01
Body Weight (g)	23.8±0.9	23.5±0.7	22.4±0.5	22.8±0.5
Systolic Blood Pressure (mmHg)	104±2	105±0.9	103±1	100±1
Diastolic Blood Pressure (mmHg)	55±2	56±1	50±1	49±2

Treatment with candesartan did not significantly alter these parameters. Blood urea nitrogen and serum creatinine levels in non-STZ control mice were 23±3 and 0.09±0.02 mg per 100 ml, respectively. Blood was drawn when mice were fed ad libitum. N.D., not determined.

Table 2

Changes of urinary biomarker levels in case 5, who had interstitial nephritis

Days after Admission	9	37	Fold Difference[a]	Normal Value
uNgal/uCr (μg per gCr)	256.6	25.4	10.1	[b]<10 μg L^{-1}
uProt/uCr (g per gCr)	0.381	0.052	7.3	<0.15 g day^{-1}
uβ2MG/uCr (mg per gCr)	18.2	2.8	6.5	<0.3 mg L^{-1}
uα1MG/uCr (mg per gCr)	46.4	17.2	2.7	<15 mg L^{-1}
sNgal (μg L^{-1})	224	90	2.5	[b]<106 μg L^{-1}
uNAG/uCr (U per gCr)	21.1	8.7	2.4	<7 U L^{-1}
BUN (mg per 100 ml)	15	11	1.4	<22 mg per 100 ml
sCr (mg per 100 ml)	1.2	1.0	1.2	[c]<1.1 mg per 100 ml

u and sNgal, urinary and serum Ngal; uCr, urinary creatinine; Prot, protein; MG, microglobulin; NAG, N-acetyl-beta-D-glucosaminidase.

[a]Fold difference between days 9 and 37.

[b]Value suggested by ELISA kit supplier.

[c]<1.1 for male and <0.8 for female.

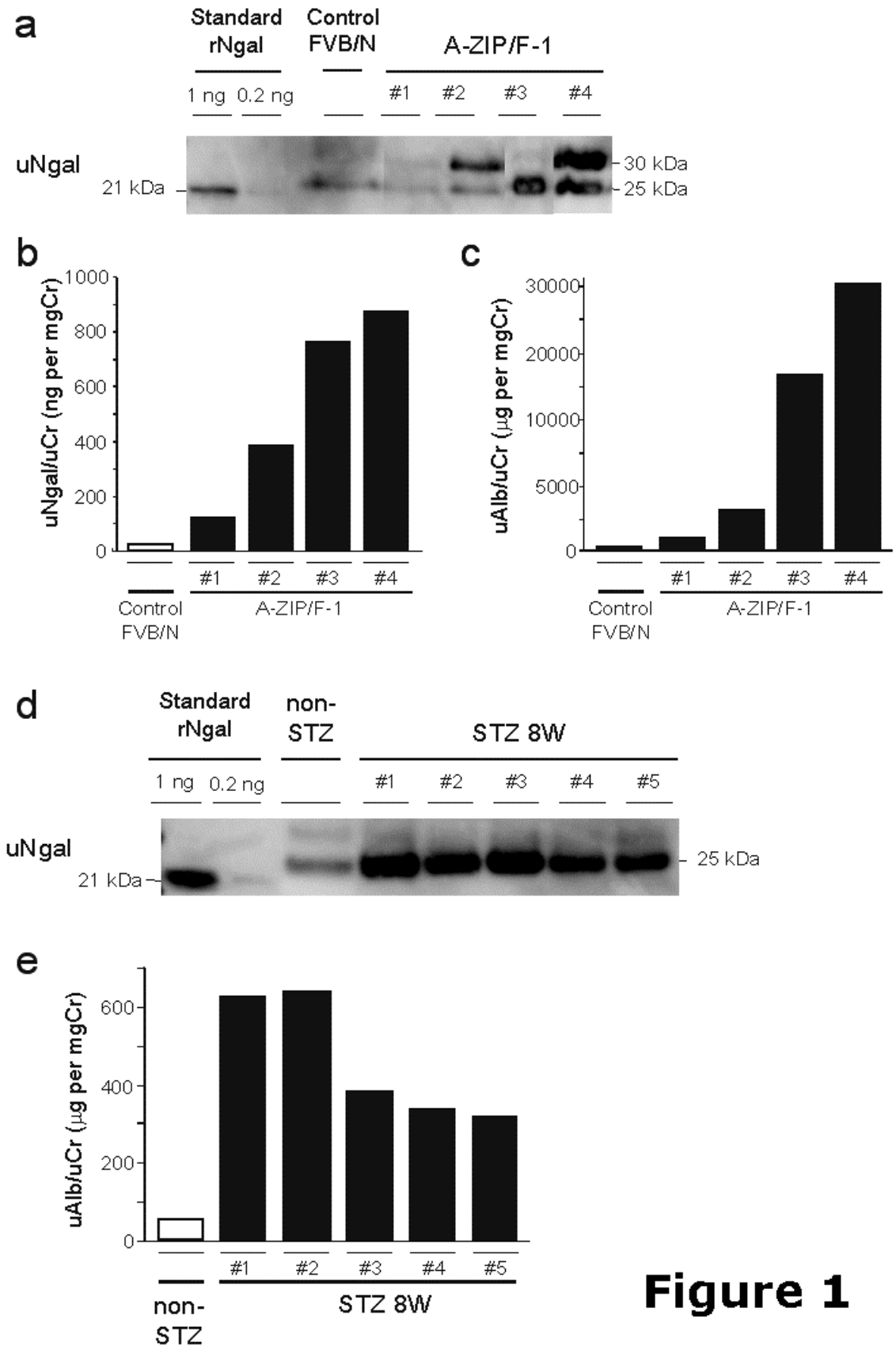
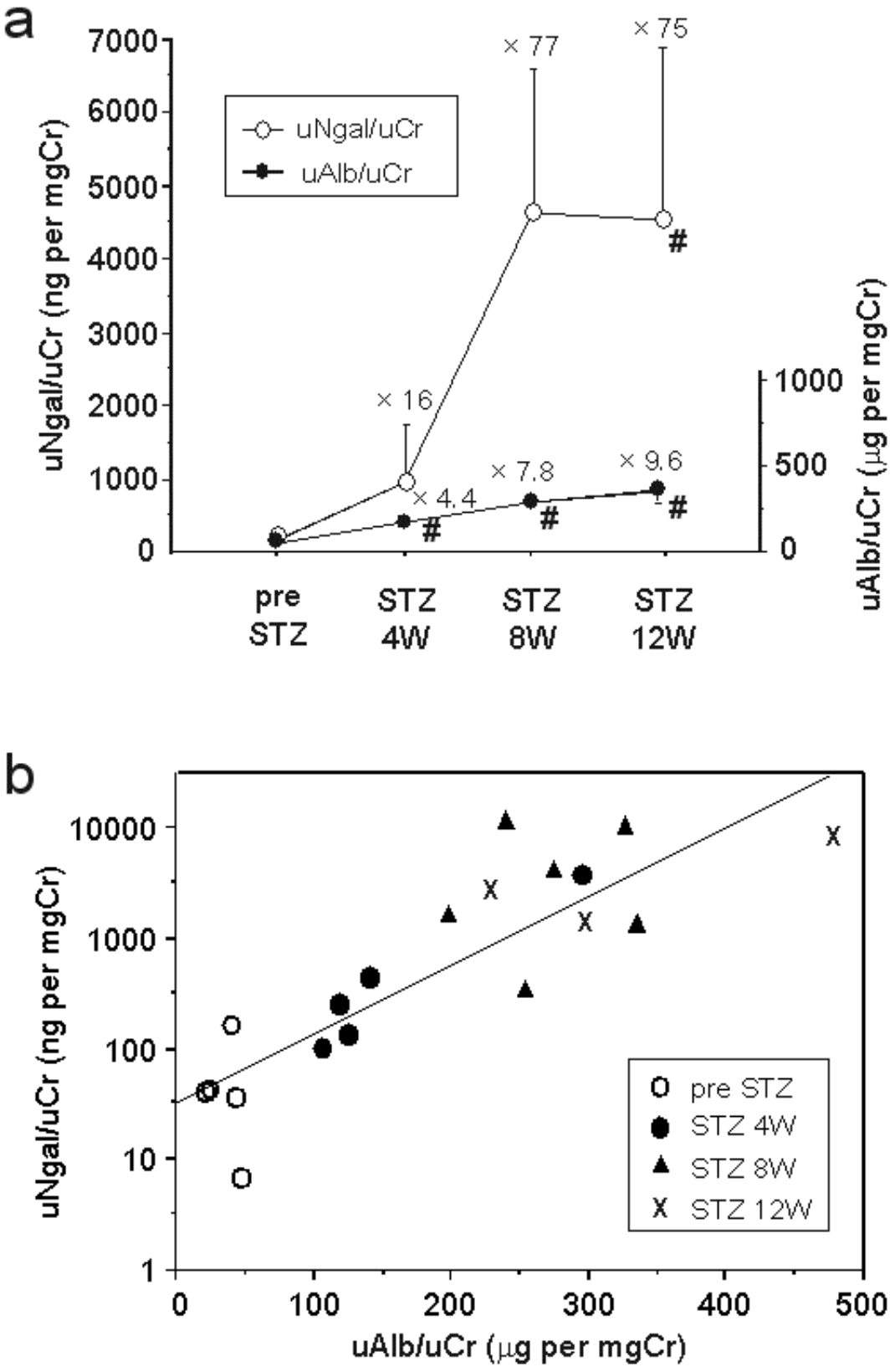
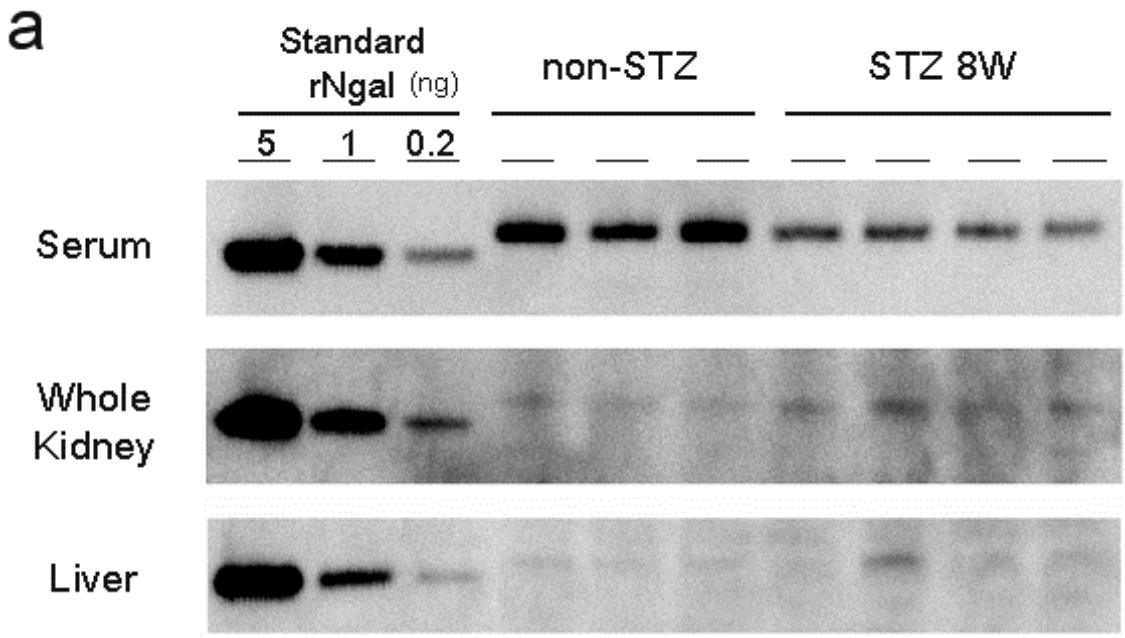

a
Standard rNgal
Control FVB/N
A-ZIP/F-1
1 ng
0.2 ng
#1
#2
#3
#4
uNgal
21 kDa
30 kDa
25 kDa
b
uNgal/uCr (ng per mgCr)
1000
800
600
400
200
0
#1
#2
#3
#4
Control FVB/N
A-ZIP/F-1
c
uAlb/uCr (μg per mgCr)
30000
20000
10000
5000
0
#1
#2
#3
#4
Control FVB/N
A-ZIP/F-1
d
Standard rNgal
non-STZ
STZ 8W
1 ng
0.2 ng
#1
#2
#3
#4
#5
uNgal
21 kDa
25 kDa
e
uAlb/uCr (μg per mgCr)
600
400
200
0
#1
#2
#3
#4
#5
non-STZ
STZ 8W

Figure 1

Figure 2


a
7000
6000
5000
4000
3000
2000
1000
0
uNgal/uCr (ng per mgCr)
uNgal/uCr
uAlb/uCr
× 77
× 75
× 16
× 7.8
× 9.6
× 4.4
#
1000
500
0
uAlb/uCr (μg per mgCr)
pre STZ
STZ 4W
STZ 8W
STZ 12W
b
10000
1000
100
10
1
uNgal/uCr (ng per mgCr)
pre STZ
STZ 4W
STZ 8W
STZ 12W
0
100
200
300
400
500
uAlb/uCr (μg per mgCr)


a
Standard
rNgal (ng)
5
1
0.2
non-STZ
STZ 8W
Serum
Whole
Kidney
Liver

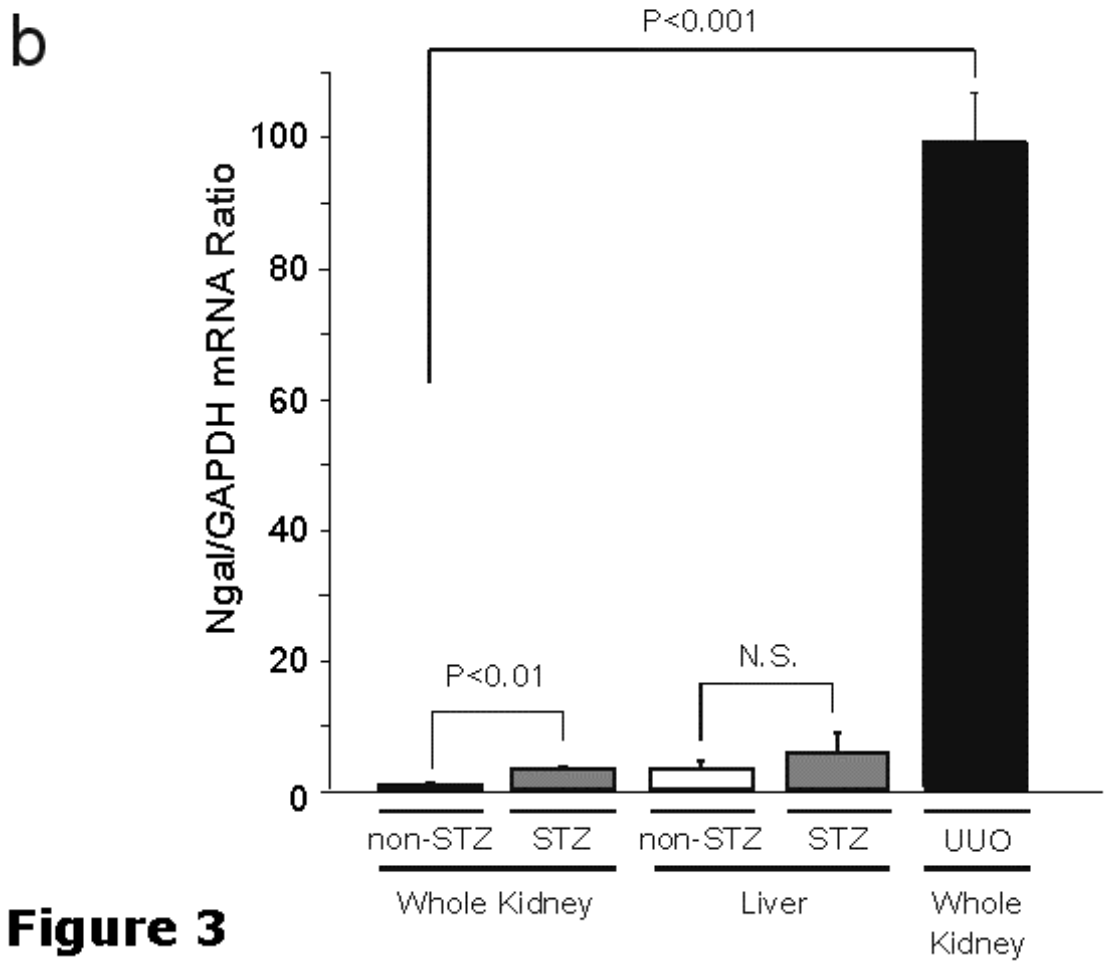

b
P<0.001
Ngal/GAPDH mRNA Ratio
100
80
60
40
20
0
P<0.01
N.S.
non-STZ
STZ
non-STZ
STZ
UUO
Whole Kidney
Liver
Whole
Kidney

Figure 3

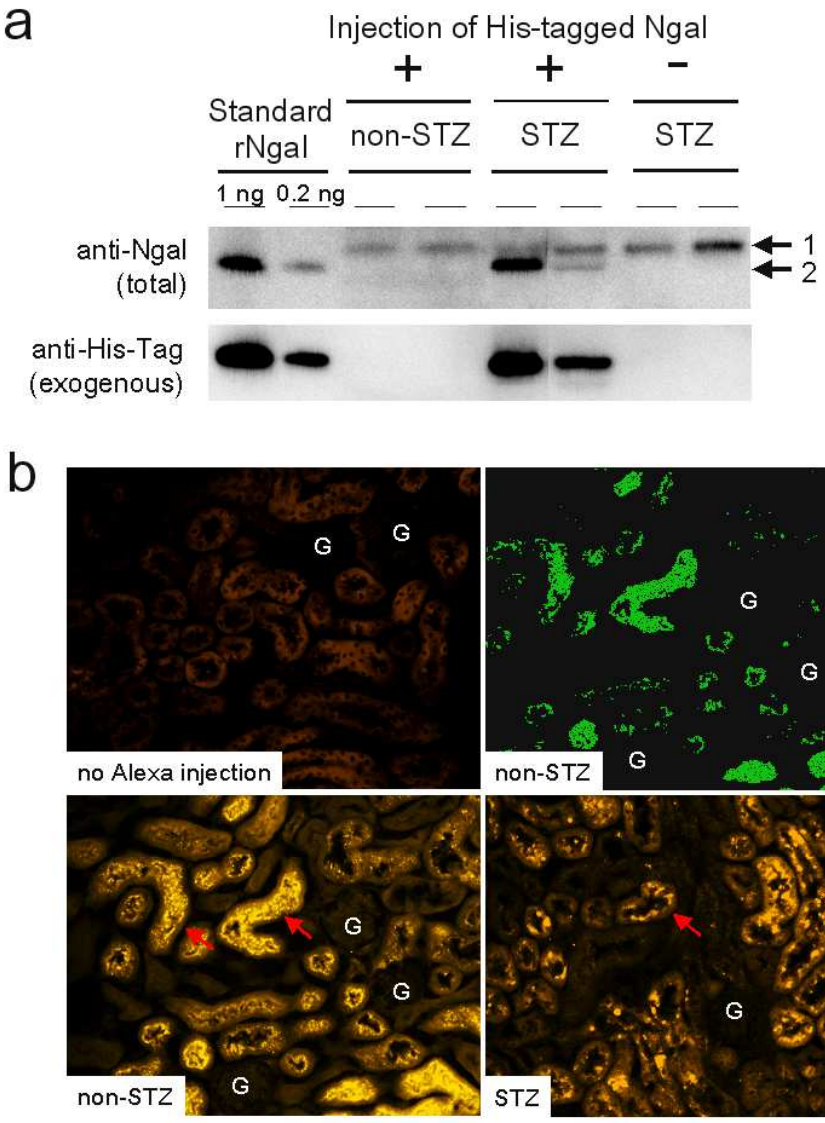
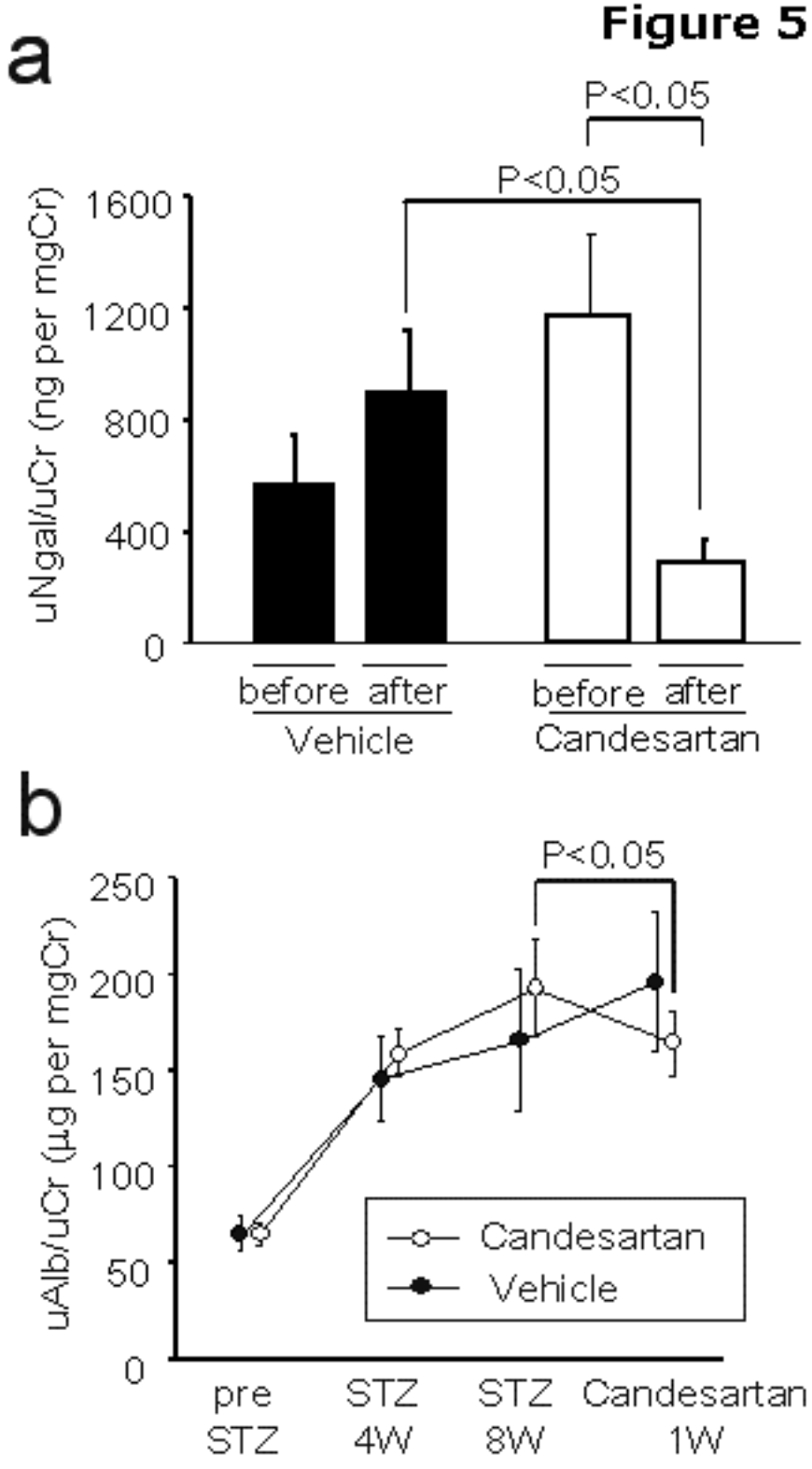

a
Injection of His-tagged Ngal
+
+
-
Standard rNgal
non-STZ
STZ
STZ
1 ng
0.2 ng
anti-Ngal (total)
anti-His-Tag (exogenous)
1
2
b
G
no Alexa injection
non-STZ
STZ
c
P<0.01
Positive area of Alexa 546 (% of non-STZ)
100
50
0
no Alexa
non-STZ
STZ

Figure 4

Figure 5


a
P<0.05
P<0.05
uNgal/uCr (ng per mgCr)
1600
1200
800
400
0
before after
Vehicle
before after
Candesartan
b
P<0.05
uAlb/uCr (µg per mgCr)
250
200
150
100
50
0
Candesartan
Vehicle
pre STZ
STZ 4W
STZ 8W
Candesartan 1W

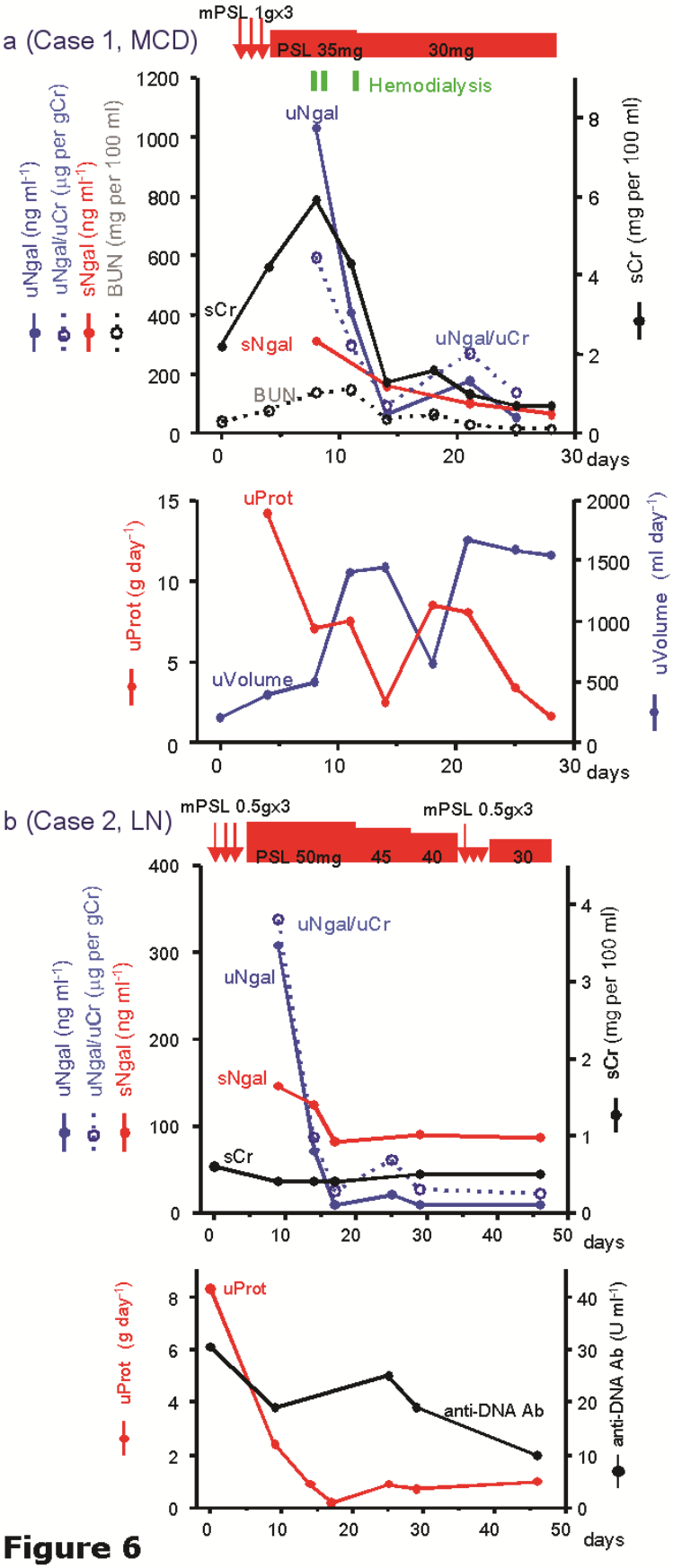

a (Case 1, MCD)
mPSL 1gx3
PSL 35mg
30mg
Hemodialysis
uNgal (ng ml-1)
uNgal/uCr (μg per gCr)
sNgal (ng ml-1)
BUN (mg per 100 ml)
sCr (mg per 100 ml)
uNgal
sCr
sNgal
uNgal/uCr
BUN
days
uProt
uVolume
uProt (g day-1)
uVolume (ml day-1)
b (Case 2, LN)
mPSL 0.5gx3
mPSL 0.5gx3
PSL 50mg
45
40
30
uNgal/uCr
uNgal
sNgal
sCr
anti-DNA Ab
anti-DNA Ab (U ml-1)

Figure 6

Figure 7

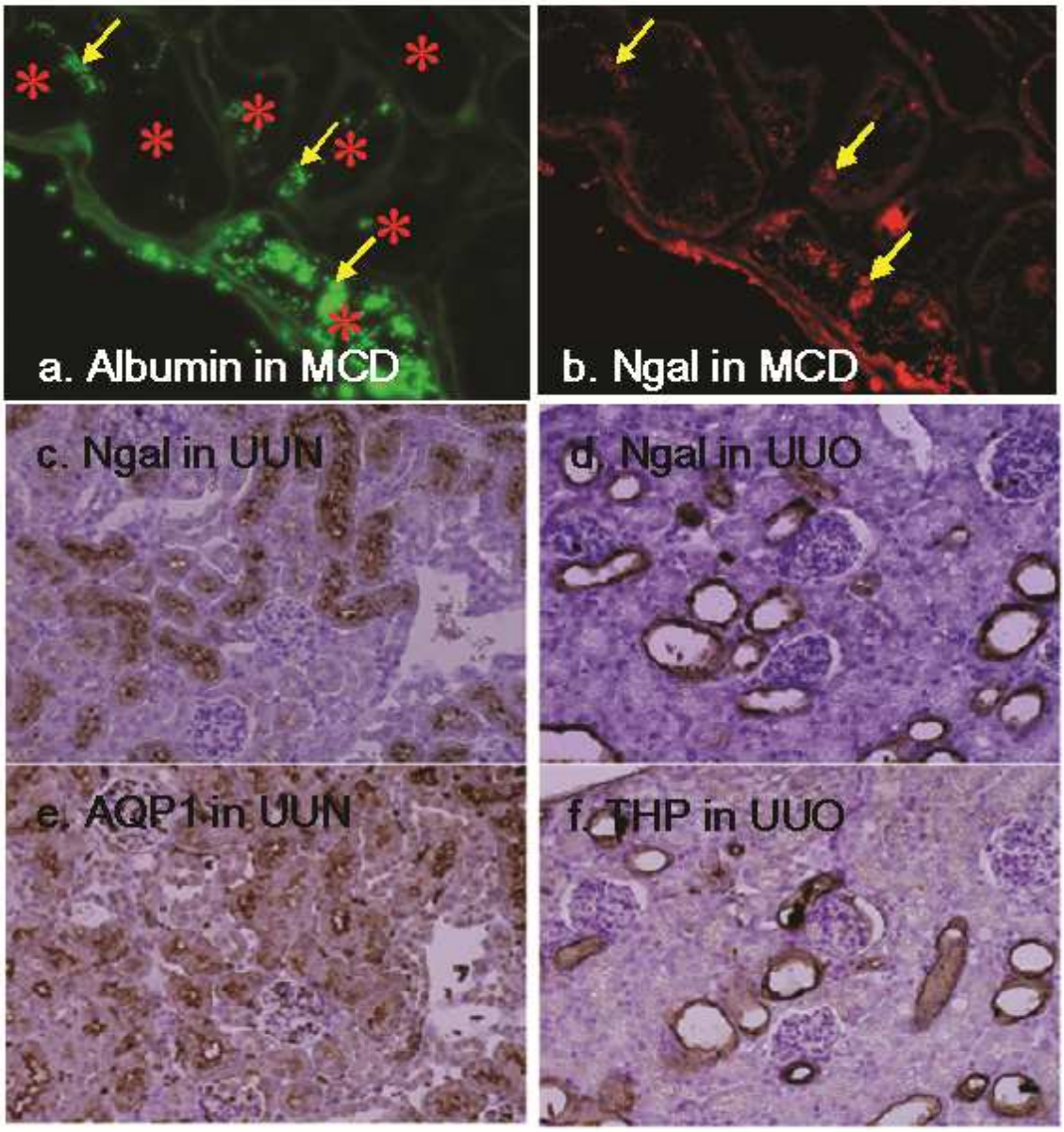
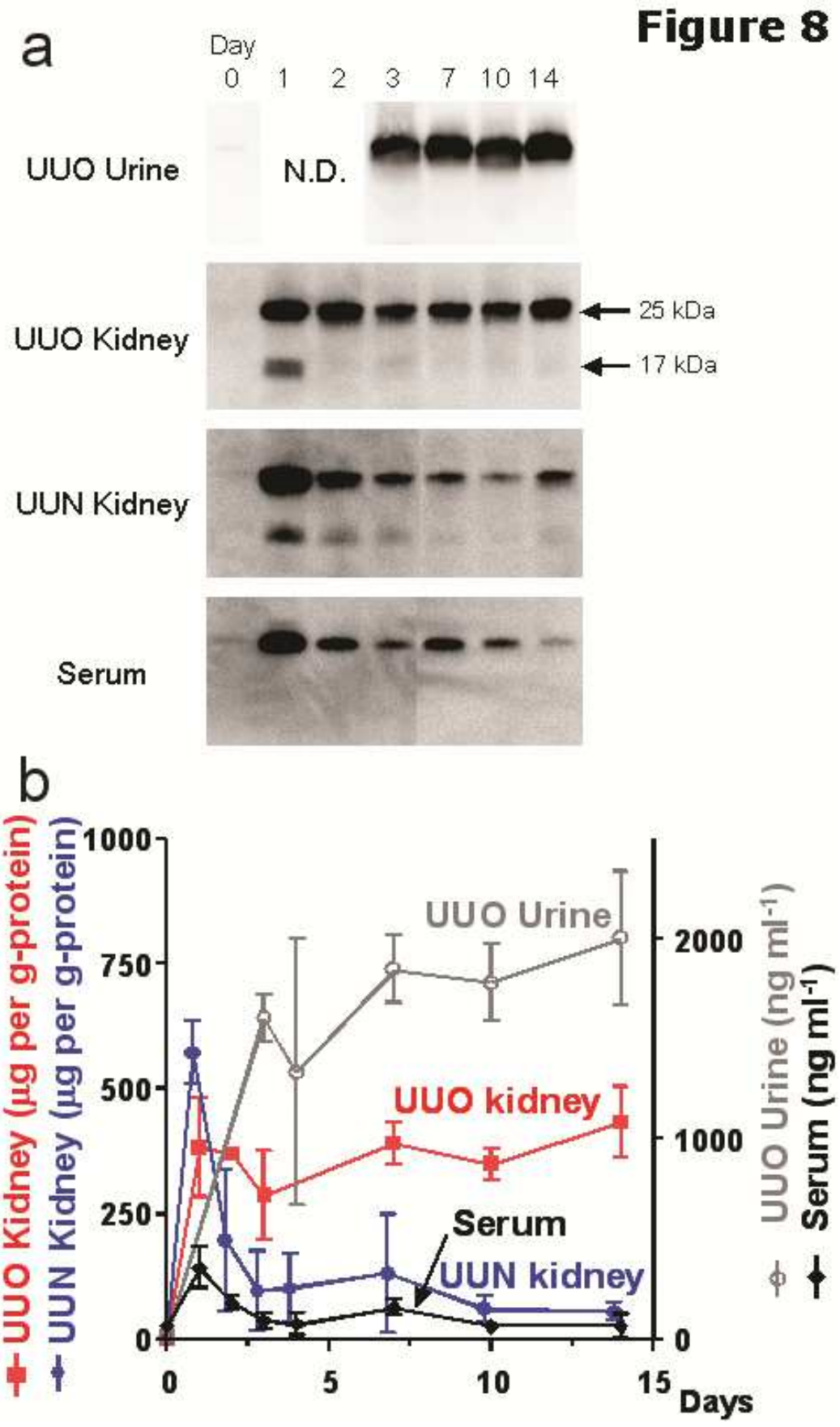

a. Albumin in MCD
b. Ngal in MCD
c. Ngal in UUN
d. Ngal in UUO
e. AQP1 in UUN
f. THP in UUO

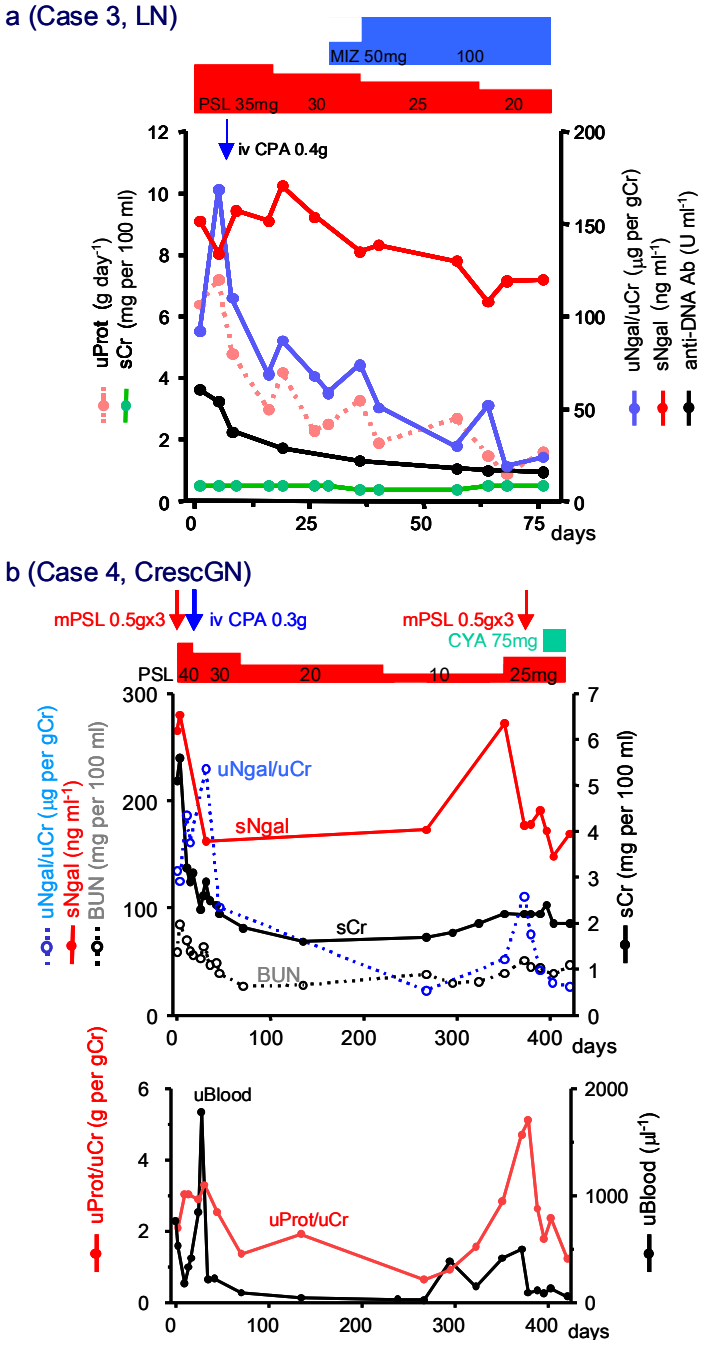
Figure 8


a
Day
0 1 2 3 7 10 14
UUO Urine
N.D.
UUO Kidney
25 kDa
17 kDa
UUN Kidney
Serum
b
UUO Kidney (µg per g-protein)
UUN Kidney (µg per g-protein)
1000
750
500
250
0
UUO Urine
UUO kidney
Serum
UUN kidney
2000
1000
0
UUO Urine (ng ml-1)
Serum (ng ml-1)
0
5
10
15
Days

Supplementary Figure S1

Clinical course of cases with (a) lupus nephritis (Case 3, LN) and (b) crescentic glomerulonephritis (Case 4, CrescGN).

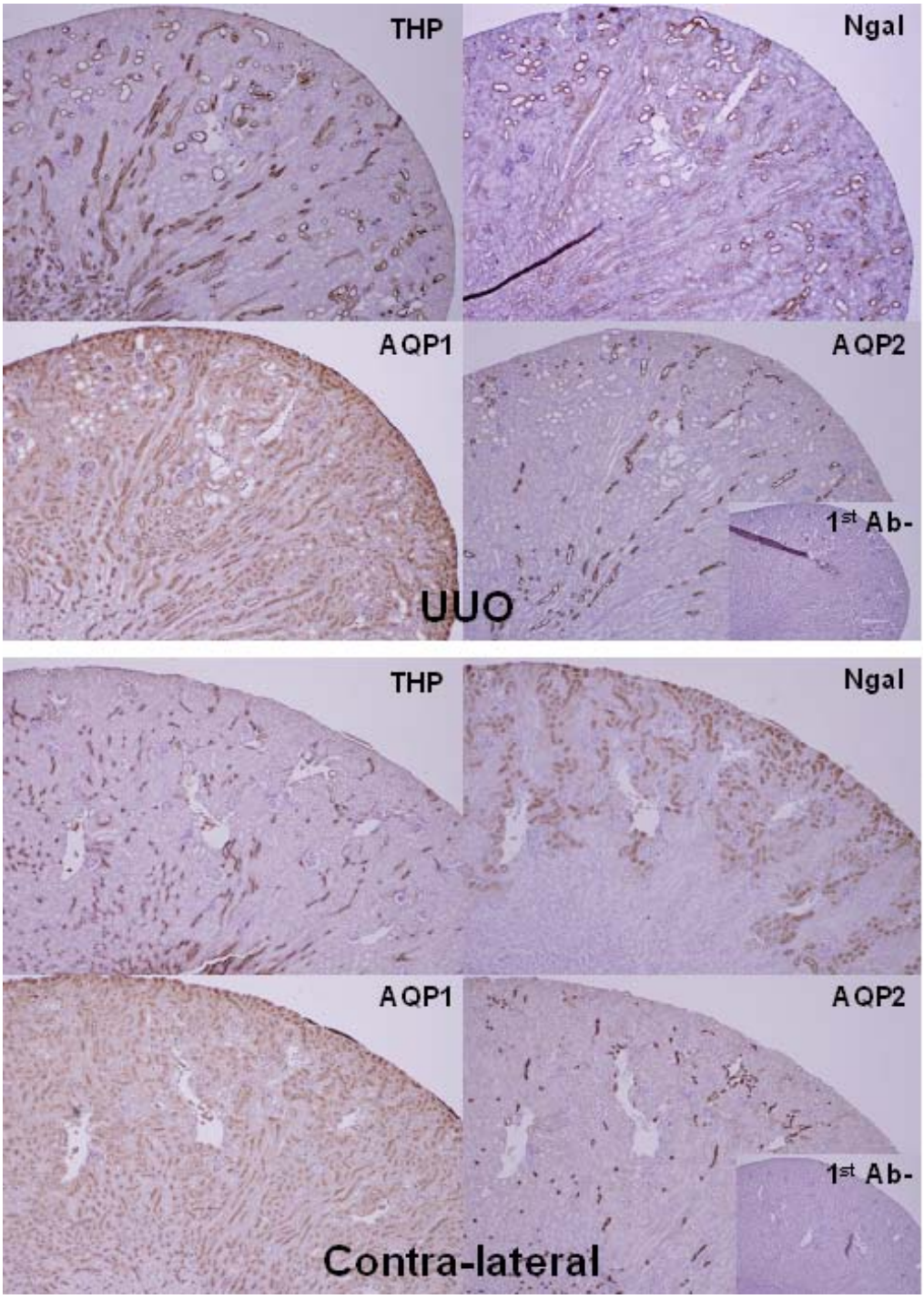
mPSL, methyl prednisolone; PSL, prednisolone; uProt, urinary protein excretion; s and uNgal, serum and urinary Ngal; BUN, blood urea nitrogen; sCr, serum creatinine; uNgal/uCr, urinary Ngal normalized by urinary creatinine; Ab, antibody. CPA, cyclophosphamide; MIZ, mizoribine; CYA, cyclosporine A; uBlood, extent of hematuria determined by flowcytometer.



Supplementary Figure S2

<u>Low power</u> fields (4 x) showing expression of Ngal and nephron markers in unilateral ureteral obstruction (UUO, top) and contra-lateral kidneys (bottom)(in brown) after 1 day of ureter ligation.

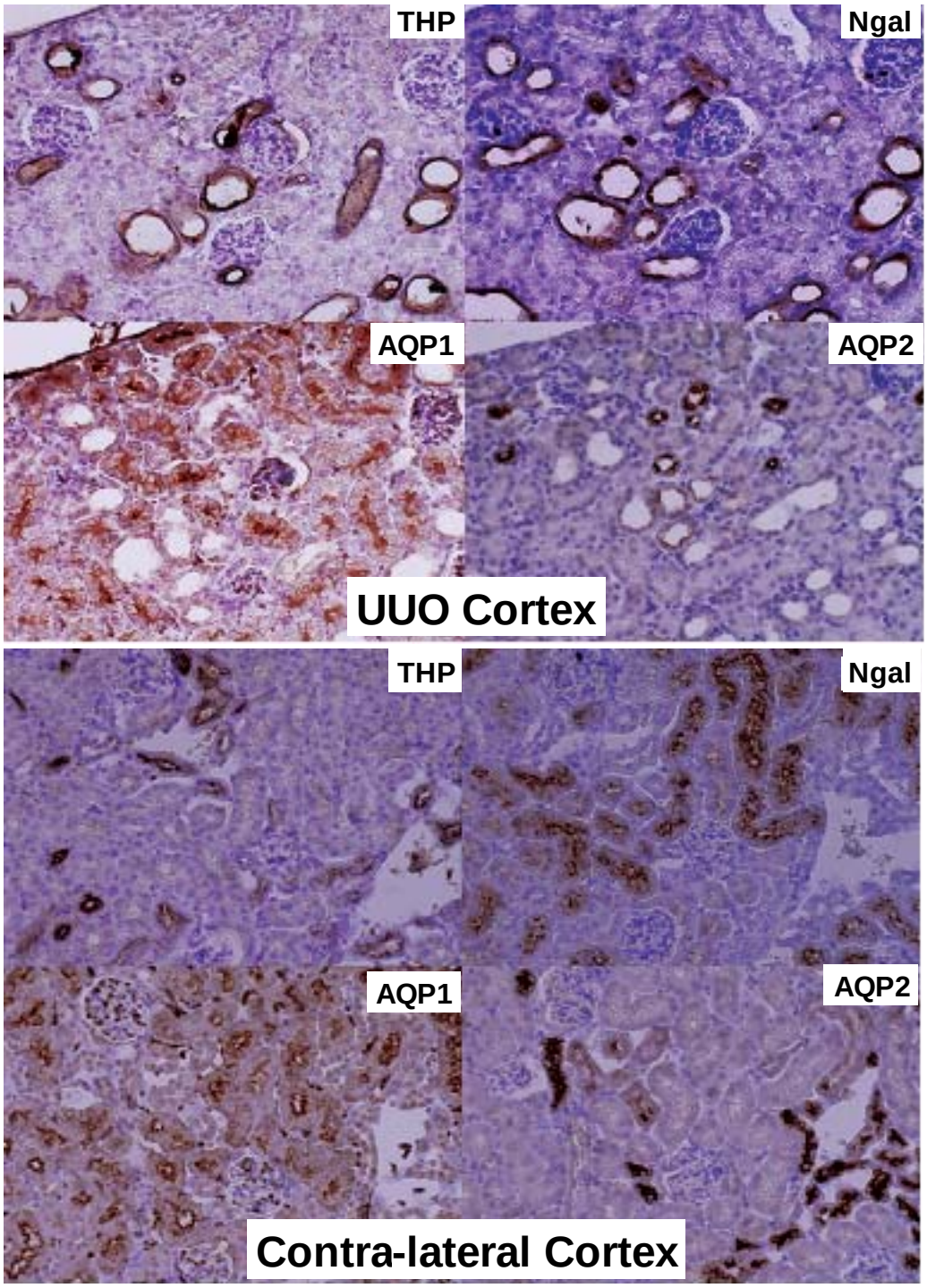
AQP, Aquaporin; THP, Tamm-Horsfall protein. 1st Ab-, without 1st antibody (negative control for immunohistochemistry, inset).



Supplementary Figure S3

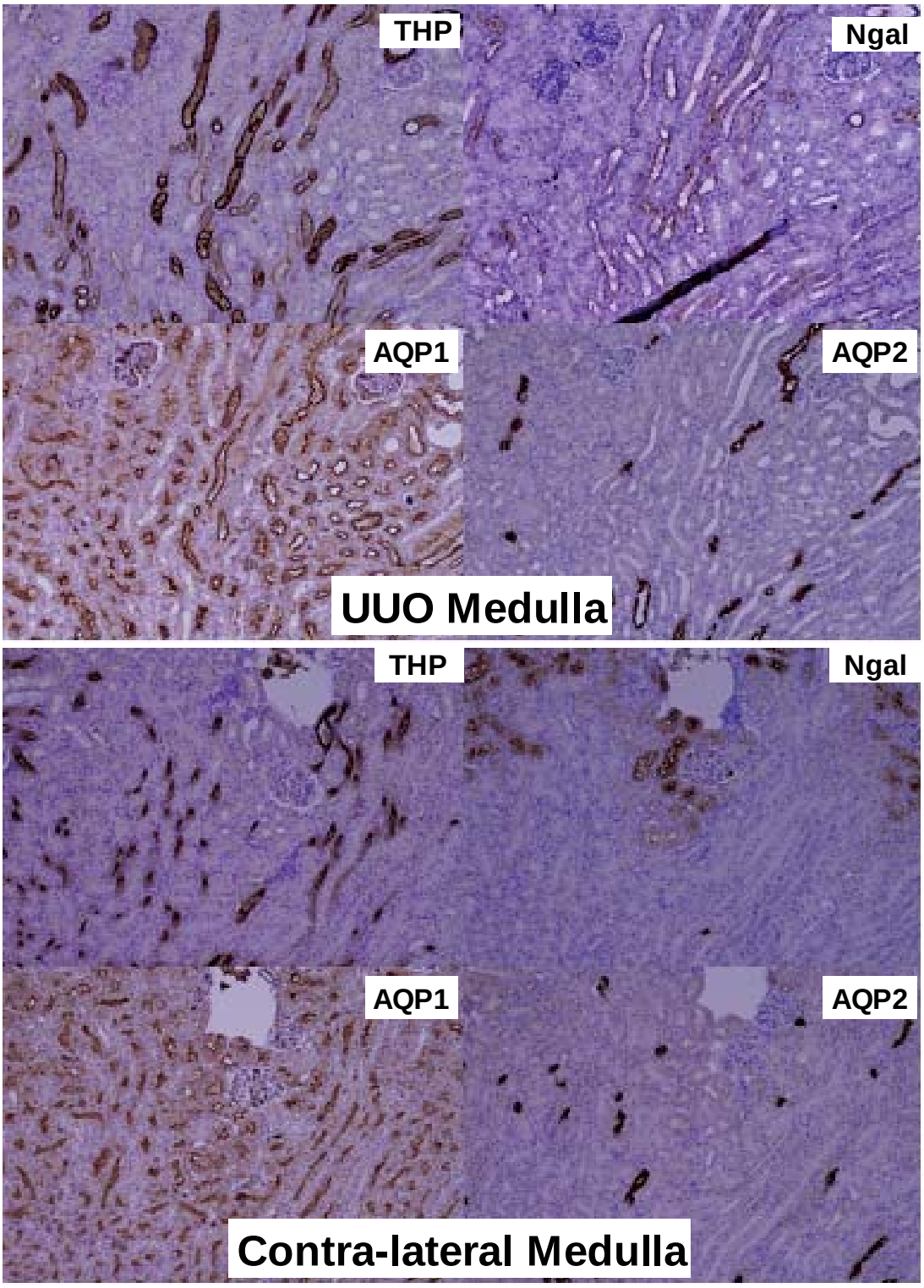
High power fields of cortex (20 x) showing expression of Ngal and nephron markers in UUO and contra-lateral kidneys.

Distribution of Ngal co-localized well with THP in UUO kidneys, whereas with AQP1 in contra-lateral kidneys.



Supplementary Figure S4

High power fields of <u>medulla</u> (10 x) showing expression of Ngal and nephron markers in UUO and contra-lateral kidneys.



Supplementary Figure S5

Clinical course of a case with drug-induced interstitial nephritis (Case 5, IntN).

Changes of urinary biomarkers are shown in percentage by assigning 100% and 0% for the levels on days 9 and 37, respectively. mPSL, methyl prednisolone; PSL, prednisolone; uProt, urinary protein excretion; s and uNgal, serum and urinary Ngal; BUN, blood urea nitrogen; sCr, serum creatinine; uNgal/uCr, urinary Ngal normalized by urinary creatinine; MG, microglobulin; NAG, N-acetyl-beta-D-glucosaminidase