

**Identification of Urinary Activin A as a Novel Biomarker
Reflecting the Severity of Acute Kidney Injury**

尿中アクチビン[®]は急性腎障害の重症度を反映する新たなバイオマーカーである

指導教員 腎臓・リウマチ内科学 廣村 桂樹 准教授
平成29年12月28日

群馬大学大学院医学系研究科
平成26年入学
内科学講座 腎臓・リウマチ内科学分野
高橋 駿介

発表予定論文

**Identification of Urinary Activin A as a Novel Biomarker
Reflecting the Severity of Acute Kidney Injury**

Scientific Reports (投稿中、平成29年11月22日投稿受付)

Shunsuke Takahashi, Masao Nakasatomi, Yoshinori Takei,
Hidekazu Ikeuchi, Toru Sakairi, Yoriaki Kaneko,
Keiju Hiromura, Yoshihisa Nojima, Akito Maeshima

Introduction

Acute kidney injury (AKI) represents a very important and potentially devastating disorder in clinical medicine. The incidence of AKI is increasing to epidemic proportions. AKI is associated with prolonged hospital stay, increased healthcare costs and high mortality in critically ill patients. Serum creatinine does not function well to predict AKI early enough to do early intervention, which is the primary impetus for the discovery of novel AKI biomarkers. The goal of most AKI biomarker research has been the discovery of a sensitive and specific early biomarker of renal injury.

Recently, several new AKI biomarkers have been developed to facilitate early detection, differential diagnosis, and prognosis, and which include neutrophil gelatinase-associated lipocalin (NGAL)¹⁻⁴, kidney injury molecule 1 (KIM-1)⁵⁻⁷, interleukin 18 (IL-18)^{8,9} and liver-type fatty acid-binding protein (L-FABP)¹⁰⁻¹².

Activin is a multifunctional cytokine belonging to the TGF- β superfamily that regulates the growth and differentiation of cells in various organs¹³. Its action is modulated by an endogenous activin antagonist, follistatin¹⁴. Activin signals are mediated by two types of cell surface serine/threonine receptors. Activin first binds to the type II receptor (ActRII or ActRIIB), which leads to recruitment and phosphorylation of the type I receptor (ActRI or ActRIB) and formation of receptor complexes. The activated type I receptor then phosphorylates Smad proteins, which are subsequently translocated to the nucleus and regulate target gene expression¹⁵. Activin A is expressed in the developing stage of the kidney¹⁶. Organ culture experiments demonstrated that activin A inhibits branching morphogenesis of ureteric buds¹⁷⁻¹⁹ as well as ureteric bud budding from the Wolffian duct²⁰. In an in vitro tubulogenesis model using Madin-Darby canine kidney (MDCK) cells, activin A tonically inhibited branching tubulogenesis. On the other hand, blockade of endogenous activin A action by follistatin induced branching tubulogenesis²¹, suggesting that activin A negatively regulates tubulogenesis during kidney organogenesis^{22,23}.

Previously, we demonstrated that activin A expression was significantly increased in tubular cells of the kidney after renal ischemia in rats. Furthermore, intravenously

administered follistatin, which binds to activins and block their actions, improved renal dysfunction and histological changes after renal ischemia^{24,25}. We also demonstrated that activin A acts as an autocrine inhibitor of cell growth as well as an inducer of apoptosis in cultured proximal tubular cells²⁶, suggesting that activin A negatively regulates tubular repair of the kidney after AKI.

In the present study, we demonstrated the presence of activin A in the urine of mice with renal ischemia-reperfusion injury. Activin A expression was markedly upregulated in proximal tubular cells of ischemic kidneys. Urinary activin A level was correlated with the degree of tubular damage. Urinary activin A was also significantly increased in patients with renal AKI, but not with pre-renal AKI, suggesting that urinary activin A might serve as a sensitive biomarker reflecting AKI severity.

Materials and Methods

Experimental Protocols

Eight or twelve-week-old male C57BL/6j mice (Japan Charles River, Yokohama, Japan) were housed under specific pathogen-free conditions and provided with autoclaved food and sterile water ad libitum. Ischemia reperfusion injury and volume depletion were induced in mice as described below. All animal experiments were performed in accordance with Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions and were approved by the Ethics Review Committee for Animal Experimentation of Gunma University (approval number 12-067).

Ischemia reperfusion injury

Ischemia reperfusion injury was induced in mice as described below. Briefly, the mice were anesthetized with pentobarbital sodium (50 mg/kg) by intraperitoneal injection. Shortly thereafter, buprenorphine (50 µg/kg) was administered subcutaneously to control pain and distress. After deep anesthesia, the abdominal cavity was exposed via a midline incision. Renal ischemia was induced by clamping both renal arteries for 15, 22, 25 or 30 min using a non-traumatic vascular clamp. Core body temperature was maintained at 37°C by placing the animal on a homoeothermic table. After the ischemia periods indicated above, the clamps were removed and reperfusion of the kidneys was confirmed visually. The mice were subsequently sacrificed after various post-ischemic periods. The kidneys were removed and frozen for RNA extraction or fixed in 10% formalin for routine paraffin embedding and sectioning for histologic analysis. Urine was collected from individual mice housed in metabolic cages. After centrifugation at 10,000 rpm for 5 min, all urine samples were stored at -80°C until the day of analysis.

Volume depletion models

To mimic pre-renal AKI, simple volume depletion was induced in mice by water

intake restriction. The degree of dehydration was evaluated by measurement of body weight recorded every 24 hr. Urine and blood samples were collected at 48 hr after initiation of water restriction and then mice were sacrificed.

Materials

Primary antibodies used in this study were as follows: rabbit anti-inhibin beta A antibody (ab97705) (Abcam, Cambridge, UK), mouse anti-neutrophil gelatinase-associated lipocalin (NGAL) antibody (AF1857), mouse anti-kidney injury molecule-1 (KIM-1) antibody (AF 1817) (R&D Systems, Minneapolis, MN), goat anti-aquaporin 2 antibody (sc-9882), goat anti-Tamm-Horsfall-protein antibody (sc-19554), mouse anti-E-cadherin antibody (sc-8426), goat anti-proliferating cell nuclear antigen (PCNA) antibody (sc-9857) (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-cleaved caspase-3 antibody (#9661; Cell Signaling Technology, Danvers, MA). Fluorescein lotus tetragonolobus lectin (LTL) (FL-1321) was from VECTOR Laboratories (Burlingame, CA).

In Situ Hybridization

In situ hybridization was performed using a InHyb In Situ Hybridization Kit (BioChain Institute Inc., Newark, CA). Hybridization probes were obtained from Genostaff Co. Ltd (Tokyo, Japan). After deparaffinization and rehydration, sections were fixed in 4% paraformaldehyde in DEPC-PBS at room temperature for 20 min. After digestion with 10 µg/ml proteinase K at 37°C for 20 min, sections were postfixed in 4% paraformaldehyde in DEPC-PBS at room temperature for 15 min. Sections were incubated with pre-hybridization solution for 3 hr at 50°C. Hybridization was performed with sense or antisense probes (2.5 ng/µl) at 50°C for 16 hr. After hybridization, sections were washed once in 2 × SSC at 45°C for 10 min, 1.5 × SSC 45°C for 10 min, and washed twice in 0.2 × SSC at 37°C for 20 min. Sections were incubated in 1× blocking solution for 60 min at room temperature and then incubated in a 1:200 diluted solution of AP-conjugated anti-digoxigenin antibody for 1 hr, before

washing and detection of the label with nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyphosphate.

Real-time PCR

Tissues were homogenized using a microhomogenizer and total RNA was extracted using RNAiso (Takara). First-strand cDNA was made from total RNA using SuperScript III First-strand (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Real-time PCR was performed using the ABI 7300 Real-time PCR System (Applied Biosystems, Foster City, CA). Reactions included 5 µl of a SYBR Green Real-time PCR Master Mix (TOYOBO, Osaka, Japan), 0.2 µl of 3' primer, 0.2 µl of 5' primer, and 1 µl of cDNA. Samples were incubated at 50°C for 2 min, then at 95°C for 1 min, followed by 35 cycles of 15 sec at 95°C, 15 sec at 62°C and 60 sec at 72°C. The expression of each gene was quantified in separate tubes with the following primers; mouse inhibin βA (143 bp) sense 5'-GATCATCACCTTTGCCGAGT-3', antisense 5'-TGGTCCTGGTTCTGTTAGCC-3'; and mouse GAPDH (460 bp) sense, 5'-TGCTGAGTATGTCGTGGAGTCTA-3%', antisense 5'-AGTGGGAGTTGCTGTTGAAATC-3'. Data was expressed as the relative expression normalized to GAPDH. Agarose gel electrophoresis of the PCR products after quantification by real-time PCR showed single bands of the expected size (data not shown).

Renal Function and Urinary Protein

Serum or urinary creatinine and blood urea nitrogen (BUN) levels were assessed using a Hitachi 7180 autoanalyzer (Hitachi High-Technologies, Tokyo, Japan). Urinary protein was measured using a BCA assay kit (Pierce, Rockford, IL, USA).

Immunohistochemical Analysis

Immunostaining was performed using a VECTASTAIN ABC-kit (Vector Laboratories) as described previously²⁴. Briefly, paraffin-embedded sections (4 µm)

were deparaffinized, hydrated according to standard methods, soaked in blocking serum, and incubated with primary antibody overnight at 4°C. After washing with phosphate-buffered saline (PBS), sections were incubated with peroxidase-conjugated secondary antibody followed by diaminobenzidine and were counterstained with periodic acid-Schiff (PAS). Indirect fluorescent immunostaining was performed as follows²⁵. Briefly, sections were incubated with fluorescein-labeled secondary antibodies (Alexa; Molecular Probes, Eugene, OR) and 4',6'-diamidino-2'-phenylindole dihydrochloride (DAPI). Fluorescent images were recorded with the BZ-X700 all-in-one fluorescence microscope (KEYENCE, Osaka, Japan). For the immunostaining control, the primary antibody was replaced with PBS, which did not show positive staining, confirming specificity. Primary antibodies used in this study were shown in above.

Quantification of activin A-positive areas was performed by measurement of positive area in five randomly selected fields of the outer medulla at ×200 magnification using Image J software (National Institutes of Health, Bethesda, MD).

Histological Examination

PAS-stained sections were microscopically examined at the indicated periods after reperfusion. The changes observed were limited to the outer medulla, where tubular damage is most obvious, and were graded as follows: 0, normal; 1, areas of tubular dilation, necrosis, hemorrhage, and cell desquamation involving <20% of the fields; 2, similar changes involving >20% but <40% of the fields; 3, similar changes involving >40% but <60% of the fields; 4, similar changes involving >60% of the fields. Five sections per mouse were used for analysis.

Terminal Deoxynucleotidyl Transferase-Mediated dUTP-Nick-End-Labeling

For identification of nuclei with DNA strand breaks at the cellular level, the terminal deoxynucleotidyl transferase-mediated dUTP-nick-end-labeling (TUNEL) method was performed using an apoptosis in situ detection kit (Takara, Tokyo, Japan)

according to the manufacturer's instructions.

ELISA

Urinary and serum human/mouse activin A, urinary human/mouse KIM-1 (R&D Systems), and urinary mouse NGAL (BIOPORTO, Hellerup, Denmark) was measured by ELISA according to the manufacturer's instructions.

Patients

Patients who were admitted to Gunma University Hospital for the diagnosis and treatment of AKI (n=18) were enrolled. Informed consent was obtained from all participants. This study was approved by the ethical committee on human research of Gunma University Graduate School of Medicine (approved number 855). Urine and serum were collected from patients with renal AKI (n=13, drug-induced, hypercalcemia, rhabdomyolysis, and cast nephropathy) and pre-renal AKI (n=5, dehydration, sepsis, and shock) when the diagnosis was made or following recovery from AKI.

Statistical analysis

Statistical analysis was performed using SPSS Statistics 24 (Chicago, IL). The significance of differences between means was compared using a t-test. When comparing the means of more than two variables, data were analyzed using Kruskal-Wallis test followed by the Mann-Whitney U test using Bonferroni correlation to adjust the probability. Correlation was analyzed with Spearman's rank correlation test coefficients. $P < 0.05$ was considered significant.

Results

Expression of β A subunit for Activin A in the Kidneys of Mice after Renal Ischemia

To examine whether activin A is expressed in ischemic kidneys, we first analyzed the mRNA expression of β A subunit for activin A in the kidneys of mice after renal ischemia for 25 min using real-time PCR (Fig. 1A). The expression of β A subunit mRNA, which was almost undetectable in normal kidneys, was significantly increased in ischemic kidneys and peaked at 24 hr after reperfusion. Next, we examined the localization of β A subunit mRNA in normal and ischemic kidneys by in situ hybridization (Fig. 1B). Hybridization signals were not observed in normal kidneys and ischemic kidneys at 3 hr after reperfusion. In contrast, strong hybridization signals for β A subunit mRNA was observed, mainly in tubular cells of the outer medulla of the ischemic kidneys, at 6 hr after reperfusion and thereafter. There were no hybridization signals in the glomeruli of the ischemic kidneys. A control experiment using a sense probe showed no hybridization signal.

Localization of activin A was also examined by immunostaining (Fig. 1C). Activin A was detected in tubular cells of the cortex and outer medulla of ischemic kidneys, but not in normal kidneys. Activin A protein was localized in the cytoplasm of LTL-positive proximal tubular cells at 6 hr after reperfusion (Fig. 1D). At the later phase of AKI (120 hr after reperfusion), there were many activin A-positive casts in the lumen of renal tubules (Fig. 1D). Double-staining with several nephron markers showed that activin A was localized in LTL-positive proximal tubular cells and the THP-positive ascending limb of loop of Henle in ischemic kidneys at 48 hr after reperfusion, but not in E-cadherin-positive distal tubular cells and AQP2-positive collecting ducts (Fig. 1E).

We further compared the localization of activin A and other AKI biomarkers, such as NGAL and KIM-1, in the kidney after renal ischemia for 25 min. NGAL was present

in tubular cells of the ischemic kidneys at 6 to 72 hr after reperfusion. Activin A was partly co-localized with NGAL in the ischemic kidneys (Fig. 2A). KIM-1 was also present in tubular cells of the ischemic kidneys. Co-localization of activin A and KIM-1 was observed in the ischemic kidneys at 24 hr after reperfusion and thereafter (Fig. 2B).

To characterize activin A-producing cells, we examined the localization of PCNA-positive proliferating cells in the kidney after renal ischemia (Fig. 2C). Many PCNA-positive cells were observed in the ischemic kidneys at 48 hr after reperfusion, but none were co-localized with activin A. There were many apoptotic cells positive for TUNEL or active caspase-3 in the ischemic kidneys. However, activin A-positive cells were TUNEL-negative (Fig. 2D) or active caspase-3-negative (Fig. 2E). These results suggest that the regulation of activin A expression was not associated with cell proliferation or apoptosis during tubular regeneration after injury.

Detection of Activin A in the Urine of Mice with Ischemia-Reperfusion Injury

We next examined whether urinary activin A was detectable in mice with AKI. Renal ischemia for 22 min was induced in mice and urine was collected for analysis at the indicated periods after reperfusion. Urinary activin A was measured by ELISA. Activin A was absent in the urine of normal mice; in contrast, activin A was detected in the urine of ischemic mice and a bimodal peak was observed at 3 and 48 hr after renal ischemia (Fig. 3A).

Next, we examined the correlation of urinary activin A with other parameters, such as serum creatinine (Fig. 3B), serum activin A (Fig. 3C), urinary NGAL (Fig. 3D), and urinary KIM-1 (Fig. 3E). Urinary activin A was not correlated with any of these parameters. Moreover, there was no significant correlation between serum activin A and serum creatinine (Fig. 3F).

Correlation of Urinary Activin A Level with Severity of AKI

We then examined the correlation of urinary activin A with the severity of kidney

damage. Mild (15 min), moderate (22 min), and severe (30 min) renal ischemia was induced in C57BL/6j mice. Serum, urine and kidney tissues were collected at 48 hr after reperfusion. Immunostaining showed that activin A was detected in the ischemic kidneys regardless of ischemic periods (Fig. 4A). BUN, serum creatinine and ATN score significantly increased in mice with renal ischemia for 22 and 30 min, but not for 15 min (Fig. 4B-4D). Quantitative analysis demonstrated that activin A-positive area significantly increased in the kidneys of mice with renal ischemia for 22 and 30 min, but not for 15 min (Fig. 4E). Urinary activin A level was significantly increased in mice with renal ischemia at 48 hr after reperfusion regardless of ischemic period (Fig. 4F).

We examined the correlation between ATN score, activin A-positive area, and urinary activin A. There was no significant correlation between ATN score and activin A-positive area (Fig. 4G). Urinary activin A was significantly correlated with activin A-positive area (Fig. 4H), but there was no correlation between urinary activin A and ATN score (Fig. 4I).

Urinary Activin A Level in Patients with Acute Kidney Injury

We also analyzed urinary activin A in eighteen patients with renal AKI and pre-renal AKI. The baseline characteristics of patients are shown in Table 1. Similar to the mouse AKI model described above, urinary activin A was significantly increased in renal AKI patients, but was not detected in healthy controls or in pre-renal AKI patients (Fig. 5A). Urinary KIM-1 also increased significantly in patients with renal AKI, but not with pre-renal AKI (Fig. 5B). To predict renal AKI patients from non-AKI, the area under the receiver-operating characteristic curve was 0.923, sensitivity was 0.846, specificity was 1.00, positive predictive value was 1.00 and negative predictive value was 0.800 for a cutoff value of 2.23 pg/ml. We examined the correlation of urinary activin A with urinary KIM-1, urinary protein level, serum creatinine, and urinary NAG. None of these parameters were correlated with urinary activin A (Fig. 5C-5F).

A significant increase in urinary activin A was observed at the acute phase of AKI,

but became almost undetectable at the recovery phase of AKI (Fig. 5G). In one case of drug-induced AKI, urinary activin A increased in parallel with serum creatinine and rapidly decreased before the normalization of serum creatinine level (Fig. 5H).

Urinary Activin A Level in Volume Depletion Model Mice

To confirm that urinary activin A increases in renal AKI, we examined whether urinary activin A was detectable in mice with volume depletion that mimics pre-renal AKI. Volume depletion significantly induced body weight reduction (Fig. 6A) and increased BUN levels (Fig. 6B), but not serum creatinine levels (Fig. 6C). In contrast to the ischemic injury model, activin A was not present in tubular cells of the kidneys in volume depletion model mice (Fig. 6D). Urinary activin A was slightly detected in mice with volume depletion, which was significantly lower than that in mice with AKI (Fig. 6E).

Discussion

In the present study, we demonstrated that urinary activin A was significantly increased in both ischemic mice (Fig. 3A) and AKI patients (Fig. 5A). We also observed that urinary activin A was detectable in rats with ischemia-reperfusion injury (unpublished observation). There are several mechanisms by which activin A could appear in the urine in AKI. The first is that urinary activin A is derived from glomerular filtered activin A. The molecular weight of activin A is 25 kDa, a size that can be theoretically filtered by glomeruli. Similar to other urinary biomarkers, including NGAL²⁷ or L-FABP²⁸, glomerular filtered activin A might be reabsorbed by renal tubules through endocytosis in the normal kidney. Dysfunction of tubular reabsorption might lead to the presence of activin A in the urine. To support this idea, urinary activin A significantly increased in mice with renal ischemia at 3 hr after reperfusion, at which time β A subunit mRNA was not detected in the ischemic kidneys by in situ hybridization (Fig.1B, Fig.3A). The second possible mechanism is that urinary activin A originates from tubular cells of the ischemic kidneys. In situ hybridization revealed that β A subunit mRNA for activin A was present in tubular cells of the ischemic kidneys at 6 hr after reperfusion and thereafter (Fig. 1B). Consistent with this data, activin A protein was localized in the cytoplasm of LTL-positive tubular cells at 6 hr after reperfusion (Fig. 1D). At the later phase of AKI (120 hr after reperfusion), there were many activin A-positive casts in the lumen of renal tubules (Fig. 1D). Since activin A-producing tubular cells are not proliferating (Fig. 2C) or apoptotic (Fig. 2D, 2E), the mechanism by which activin A expression is regulated is unknown. Nevertheless, these results suggest that activin A appears in the urine by tubular cell dysfunction or tubular damage in AKI.

Measurement of urinary activin A has several advantages for the early diagnosis and treatment of AKI in the clinical setting. We demonstrated here that activin A was present in proximal tubular cells of the ischemic kidneys (Fig 1C, D, E), but not in normal kidneys (Fig. 1C). Activin A-positive area in ischemic kidneys increased according to the ischemic period (Fig. 4E). Urinary activin A level was positively

correlated with the degree of activin A-positive area (Fig. 4H). On the other hand, renal ischemia for 15 min did not induce the elevation of serum creatinine level (Fig. 4C), but significantly increased urinary activin A (Fig. 4F). In one patient with drug-induced AKI, urinary activin A increased in parallel with serum creatinine level and rapidly decreased before the normalization of serum creatinine level (Fig. 5H).

These data suggest that urinary activin A may serve as a sensitive biomarker reflecting the severity of tubular damage. Urinary activin A also appears to be valuable in distinguishing renal and pre-renal AKI. Similar to mice with AKI, urinary activin A was present in the urine of patients with renal AKI, but not in patients with pre-renal AKI (Fig. 5A). Although a slight increase in urinary activin A level was found in volume depletion model mice, its absolute value was significantly lower than that in the AKI mouse model (Fig. 6E). The expression of β A subunit for activin A was not detected in pre-renal AKI model mice by immunostaining (Fig. 6D). Collectively, urinary activin A might be a beneficial marker to distinguish between renal and pre-renal AKI according to its absolute values.

The transition of AKI to chronic kidney disease (CKD) has major clinical significance. Recent research has provided insights into the pathologic basis for failed recovery from AKI, such as tubular atrophy and renal fibrosis²⁹. Late upregulation of NGAL and KIM-1 could be a useful marker for sustained renal injury after AKI³⁰. Activin A acts as a potent inducer of renal fibrosis^{31,32}, and the activin receptor type IIA ligand trap protects against vascular disease and renal fibrosis in mice with CKD³³. Given that activin A inhibits tubular regeneration of the kidney after renal ischemia²⁴⁻²⁶, activin A produced by tubular cells inhibits tubular regeneration in a paracrine manner and induces incomplete recovery from AKI. In the present study, we could not clarify if the urinary activin A level reflects renal prognosis in AKI patients. Sustained activin A expression in the ischemic kidneys might be involved in the transition from AKI to CKD.

There are several limitations in this study. First, we have no data regarding urinary activin A in children with AKI. AKI in children is associated with increased

mortality and prolonged hospital stay and may also be associated with long-term CKD development. It has been reported that urinary activin A in infants who developed intraventricular hemorrhage was significantly higher than in controls at all monitoring time-points³⁴. Urinary activin A seems to be a promising tool for identifying preterm infants at risk of intraventricular hemorrhage. Previous studies also demonstrated that urinary activin A could be detected in the urine of pregnant females with pre-eclampsia³⁵. Normal pregnancy urine samples had very low levels of activin A. In contrast, patients with pre-eclampsia had significantly higher levels of activin A compared to controls. Therefore, the advantage of urinary activin A is limited to patients except for infants and pregnant women. We measured urinary activin A in AKI, but not in other kidney diseases. Given that urinary activin A was increased in lupus-prone MRL-lpr mice³⁶, urinary activin A might be increased in patients with other kidney diseases including lupus nephritis. Further investigation will be needed to address this issue.

Summary

Acute kidney injury (AKI) is a common but complex condition that is associated with increased morbidity and mortality. In the present study, we examined whether urinary activin A, a member of the TGF-beta superfamily, is present in mice with ischemia-reperfusion injury and in humans with AKI, as well as its potential as a biomarker for AKI. Expression of activin A was markedly increased in ischemic mouse kidneys. In situ hybridization demonstrated that activin mRNA was expressed in tubular cells of ischemic kidneys but not of normal kidneys. Immunoreactive activin A, which was absent in normal kidneys, was detected in the cytoplasm of proximal tubular cells in ischemic kidneys. Activin A was undetectable in the urine of normal mice. In contrast, activin A was significantly increased in the urine of ischemic mice at 3 h after reperfusion. Urinary activin A levels increased according to the period of ischemia. In humans, urinary activin A was almost undetectable in healthy volunteers and in patients with pre-renal AKI, but was significantly increased in patients with renal AKI. There was no significant correlation between urinary activin A and serum activin A. Collectively, urinary activin A might be a useful biomarker reflecting the severity of AKI.

Acknowledgements

This research was supported in part by Grants-in-Aid for Scientific Research (C)(Grant Numbers 23591181, 26461212) from the Ministry of Education, Culture, Sports, Science and Technology of Japan (MEXT) and a Grant-in-aid for Takeda Science Foundation.

We would like to thank Rumiko Koitabashi and Noriko Kagami for their expert technical assistance.

Competing Financial Interests

This study was partly funded by Astellas Pharma Inc.

Figure Legends

Figure 1 Expression of β A Subunit for Activin in the Kidneys after Renal Ischemia

A: Expression of β A subunit for activin in the kidneys of mice after renal ischemia for 25 min was examined by real-time PCR. Values (relative expression ratio to GAPDH) are means \pm S.E. (n=5-8). * p <0.05, ** p <0.01 vs. 0 hr. B: Localization of β A subunit mRNA in the kidneys after renal ischemia was examined by in situ hybridization. Cortex: CO, Outer medulla: OM, Glomerular:G, Hybridization signals (blue). AS, anti-sense probe, S, sense probe. Magnification: \times 100 (upper panels) and \times 1000 (middle, lower panels). C: Localization of activin A in the kidneys after renal ischemia was examined by immunostaining. β A subunit for activin (brown), Arrows indicate activin A positive renal tubules. Magnification: \times 1000. D: Double-staining of activin A with nephron marker in the kidneys after renal ischemia (Upper panels are IR 6 hr, lower panels are IR 120 hr). β A subunit for activin (red), LTL (green), DAPI (blue). Magnification, \times 1000. Arrows indicate casts in the lumens of renal tubules. E: Double-staining of activin A with several nephron markers in the kidneys after renal ischemia (IR 48 hr). LTL (green), THP (red), E-cadherin (red) and aquaporin 2 (AQP2; red), and DAPI (blue). Magnification, \times 400

Figure 2 Localization of β A subunit for Activin, NGAL, and KIM-1 in the Kidneys after Renal Ischemia

A: Localization of β A subunit for activin and NGAL in the kidneys after renal ischemia for 25 min was examined by immunostaining. β A subunit for activin (green), NGAL (red). Magnification: \times 400. B: Localization of β A subunit for activin and KIM-1 in the kidneys after renal ischemia was examined by immunostaining. β A subunit for activin (green), KIM-1 (red). Magnification: \times 1000. C: Localization of β A subunit for activin and PCNA in the ischemic kidneys at 48 hr after reperfusion. β A subunit for activin (red), PCNA (green), and DAPI (blue). Magnification: \times 400. D: Localization of β A subunit for activin and TUNEL-positive cells in the ischemic kidneys at 48 hr after

reperfusion using serial sections. Positive signals (brown). Magnification: $\times 1000$. E: Localization of β A subunit for activin and caspase 3-positive cells in the ischemic kidneys at 48 hr after reperfusion using serial sections. Positive signals (brown), Magnification: $\times 1000$.

Figure 3 Urinary Activin A Level in Mice with Ischemia-reperfusion Injury

A: Urinary activin A in mice with renal ischemia for 22 min was measured by ELISA. Values are means \pm S.E. (n=5-11). B, C: Correlation between urinary activin A and serum creatinine (B) or serum activin A (C) at 48 hr after reperfusion. D, E: Correlation between urinary activin A and urinary NGAL (D) or urinary KIM-1 (E) at 48 hr after reperfusion. F: Correlation between serum creatinine and serum activin A at 48 hr after reperfusion.

Figure 4 Correlation of Urinary Activin A with the Severity of Kidney Damage

A: Localization of β A subunit for activin in the kidneys of mice with renal ischemia for 15, 22, or 30 min. The kidneys were removed for analysis at 48 hr after reperfusion. β A subunit for activin (red), DAPI (blue). Magnification: $\times 100$. B, C: BUN (B) and serum creatinine (C) in mice with renal ischemia at 48 hr after reperfusion. * $p < 0.05$, ** $p < 0.01$ vs. 0 hr. D: Semiquantitative analysis of the histologic changes induced by renal ischemia. ATN score was quantified as described in the Methods. * $p < 0.05$, ** $p < 0.01$ vs. 15 min ischemia. E: Quantitative analysis of positive area of β A subunit for activin. Five randomly selected fields of the kidneys were assessed at $\times 200$ magnification. Activin A-positive area was measured using Image J software. Values are means \pm S.E. (n=6). ** $p < 0.01$ vs. 15 min ischemia. F: Urinary activin A in mice with renal ischemia for 15, 22, or 30 min. Urine was collected at 48 hr after reperfusion and urinary activin A was measured by ELISA. Values are means \pm S.E. (n=6). * $p < 0.05$ vs. 0 hr. G, H: Correlation between activin A-positive area and ATN score (G) or urinary activin A (H) at 48 hr after reperfusion. I: Correlation between ATN score and urinary activin A level at 48 hr after reperfusion.

Figure 5 Urinary Activin A Level in Patients with Acute Kidney Injury.

A: Urinary activin A in healthy controls (n=8), renal AKI patients (n=13) and pre-renal AKI patients (n=5). *p<0.05 vs. healthy controls, #p<0.05 vs pre-renal AKI patients. B: Urinary KIM-1 in healthy controls (n=7), renal AKI patients (n=13) and pre-renal AKI patients (n=5). *p<0.05 vs. healthy controls. C-H: Correlation between urinary activin A and urinary KIM-1 (C), urinary protein level (D), serum creatinine (E) or urinary NAG (F). G: Urinary activin A level at the acute and recovery phases of AKI (n=3). Data at the initial visit (acute phase) and before discharge (recovery phase) were shown. H: Time course changes of urinary activin A and serum creatinine in patient with drug-induced AKI.

Figure 6 Urinary Activin A Level in Mice with Volume Depletion

A: Changes of body weight in the volume depletion model. Simple volume depletion was induced in mice by water intake restriction to mimic pre-renal AKI. Values are mean \pm S.E. **p<0.01 vs. 0 hr. B, C: Blood urea nitrogen (BUN) (B) and serum creatinine (C) in mice before and after 48 hr volume depletion. Values are mean \pm S.E. *p<0.05 vs. control (n=6). N.S., not significant. D: Localization of β A subunit for activin (red) and LTL (green) in the kidneys of volume depletion model mice was examined by immunostaining. DAPI (blue). Magnification: \times 1000. E: Urinary activin A level in normal mice, volume depletion (VD) model mice and I/R model mice (at 48 hr after reperfusion, 22 min ischemia). *p<0.05 vs. control. #p<0.05 vs. IR.

Table 1 Baseline Characteristics of AKI Patients.

Age, sex, urinary protein level, serum creatinine, BUN, hemoglobin, WBC, platelet, sodium, potassium, chloride, and urinary NAG of patients with renal AKI (n=13, drug-induced, hypercalcemia, rhabdomyolysis, and cast nephropathy) and pre-renal AKI (n=5, dehydration, sepsis, and shock) at the initial visit are shown.

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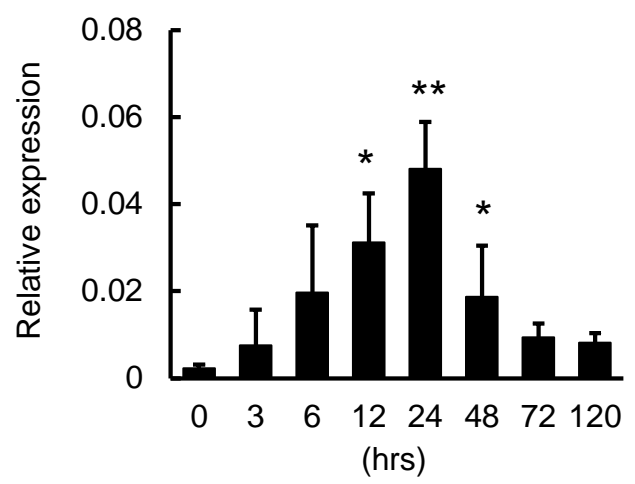
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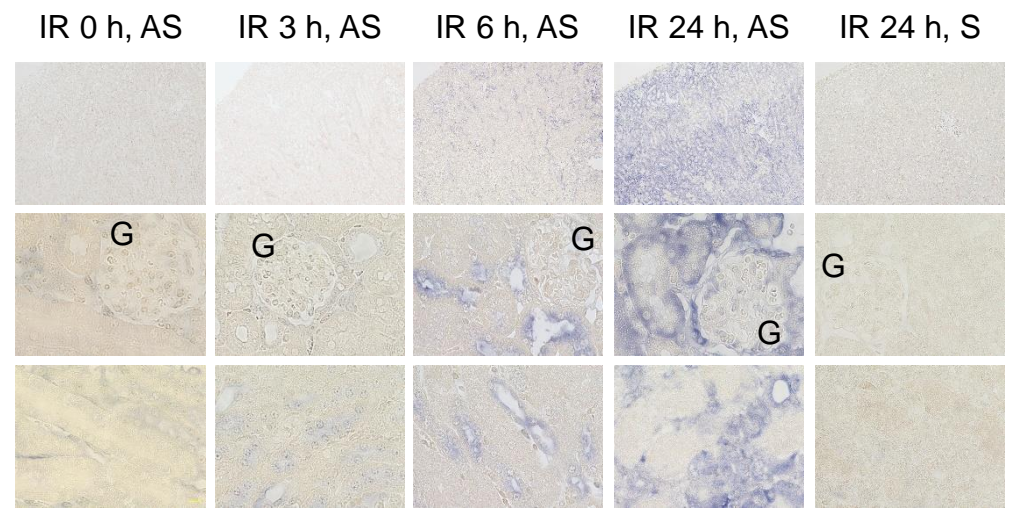
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Figure 1

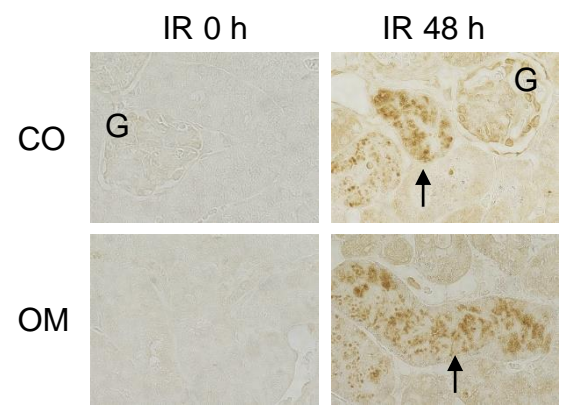
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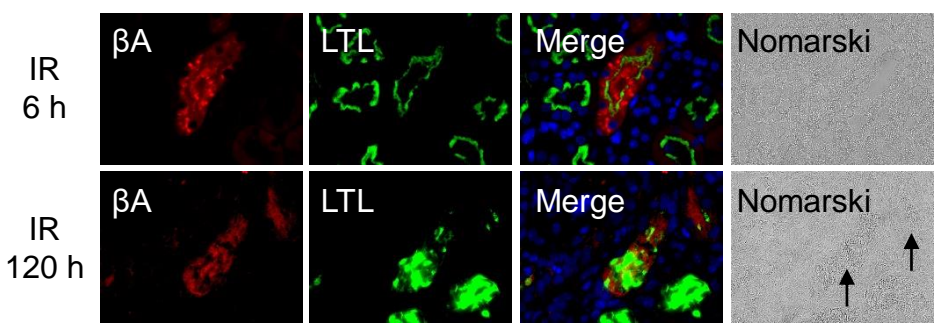
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C



D



E

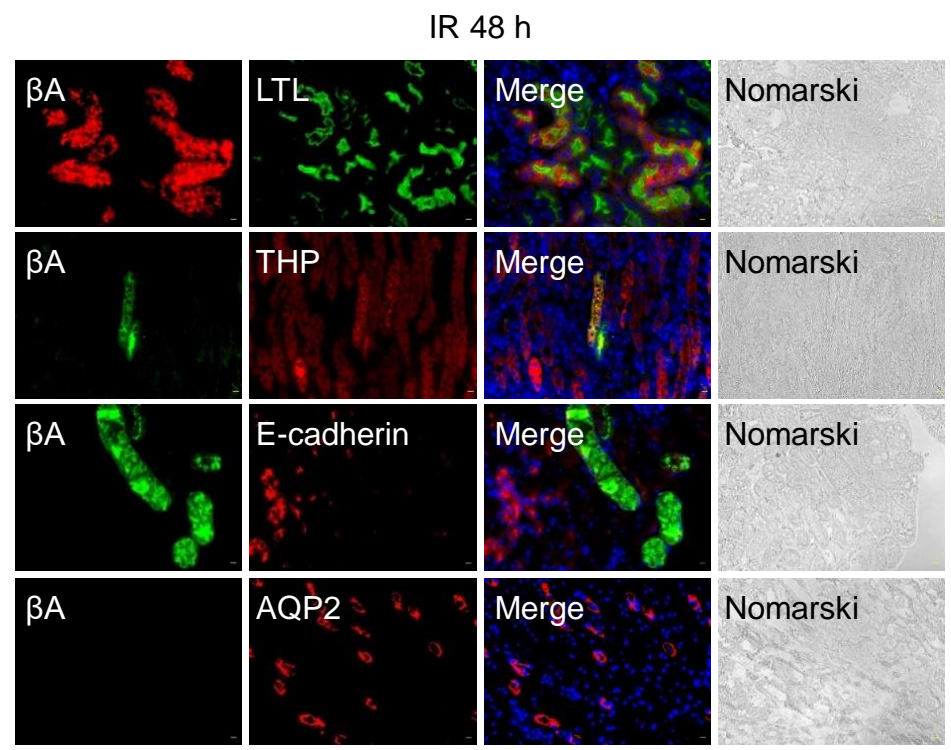


Figure 2

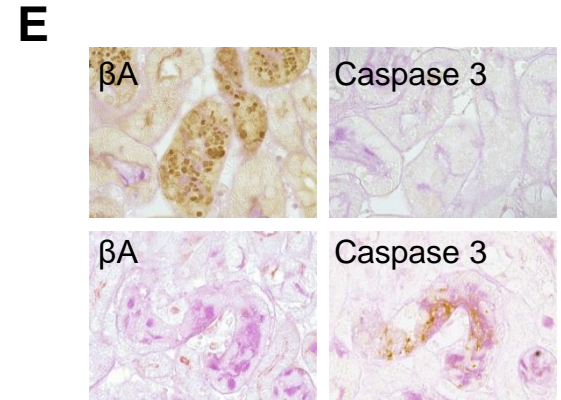
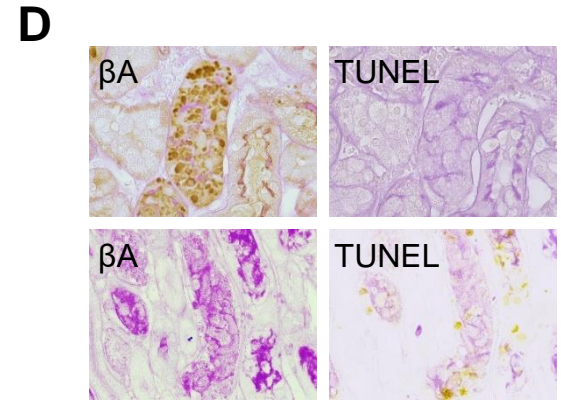
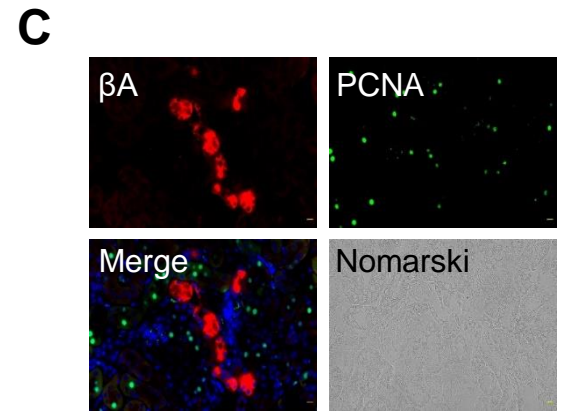
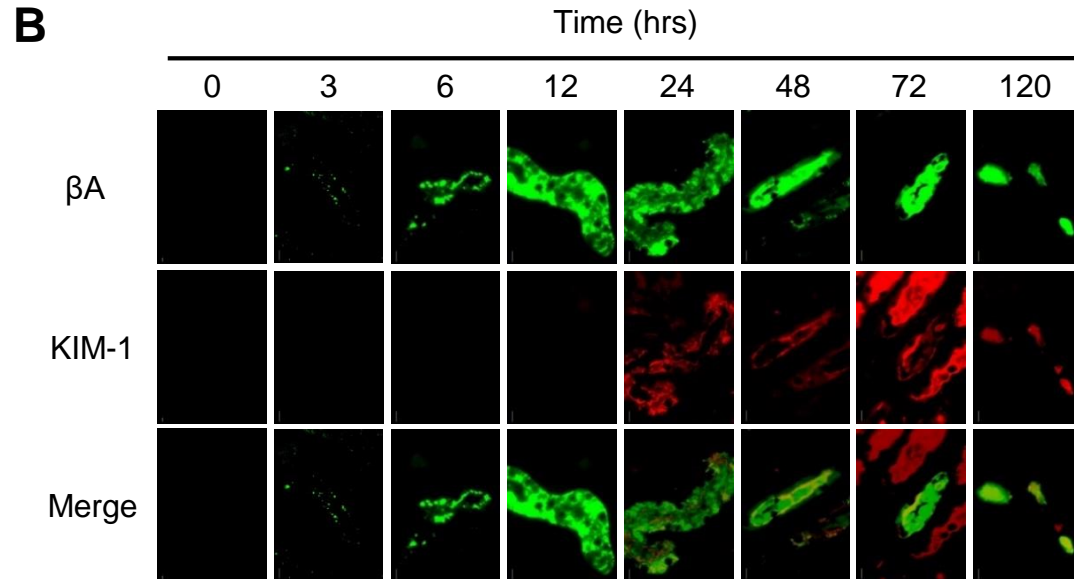
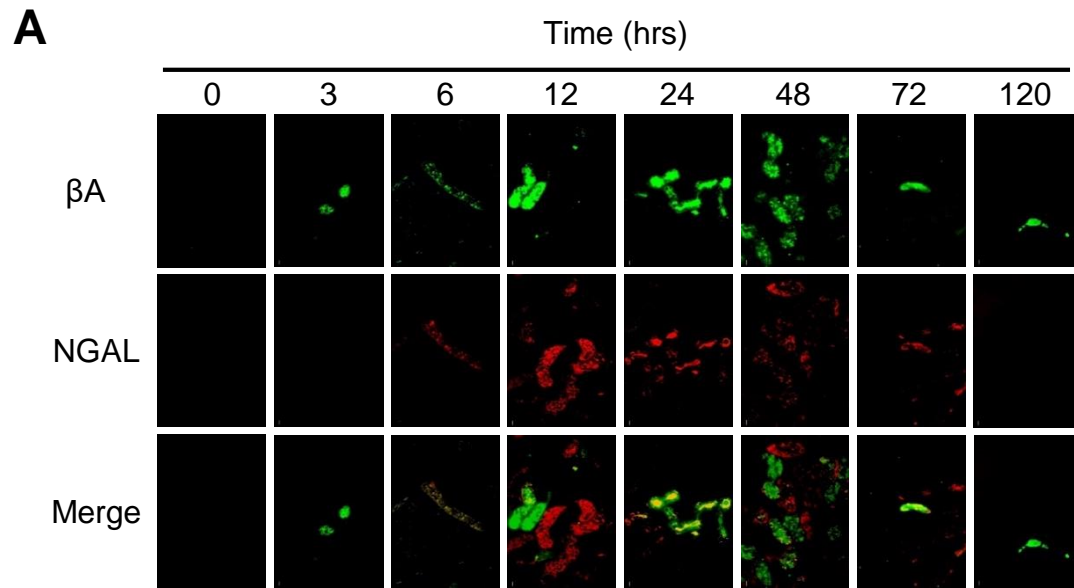


Figure 3

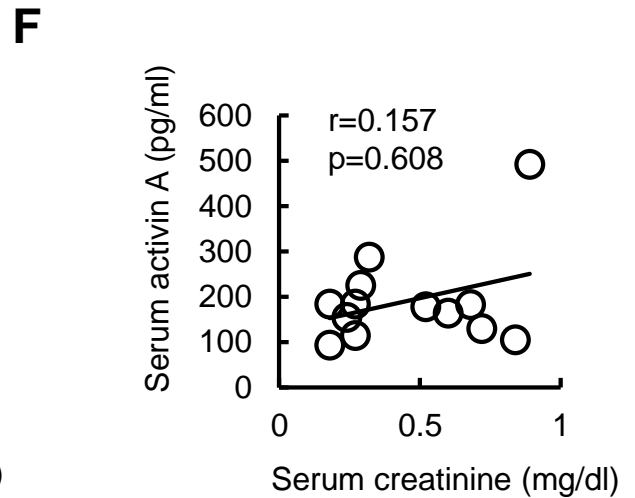
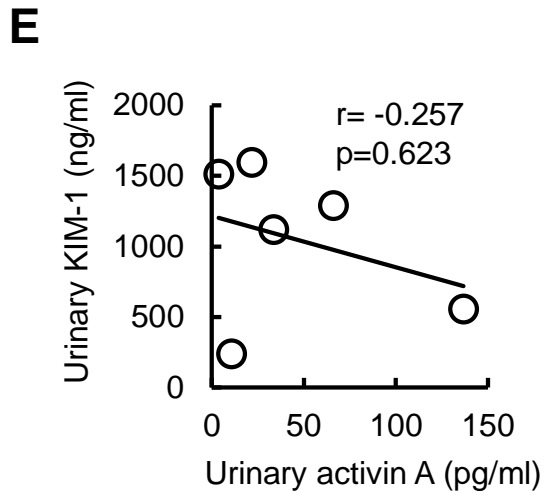
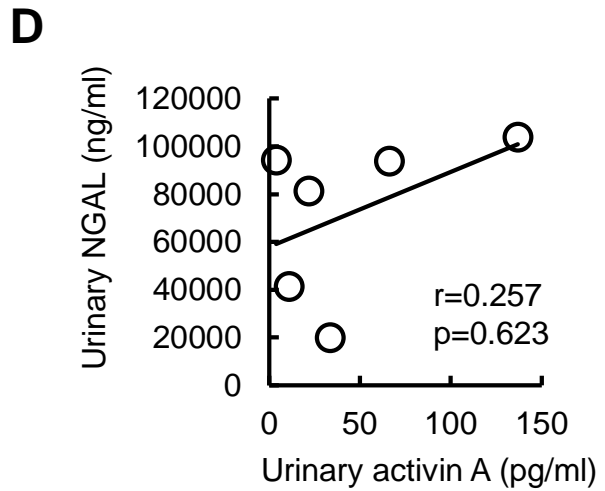
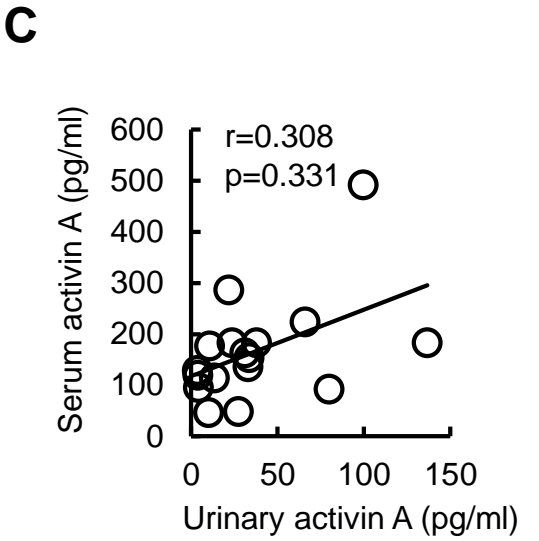
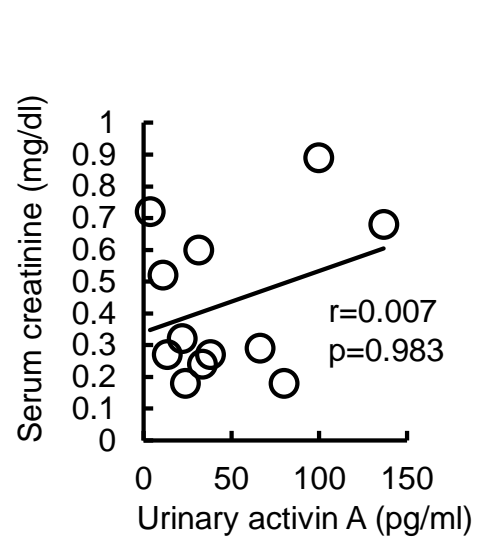
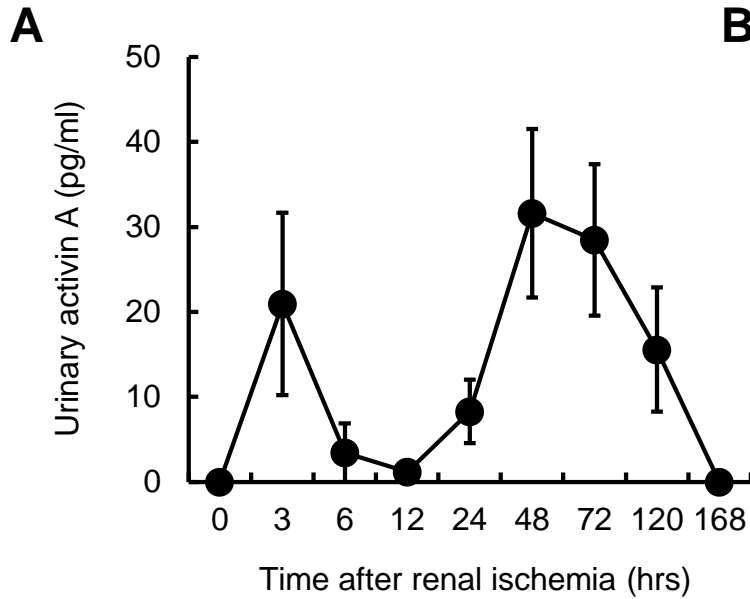
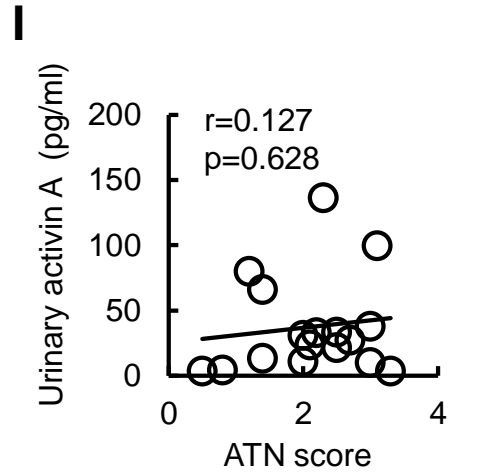
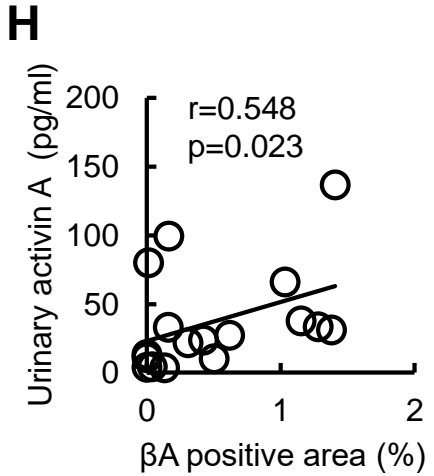
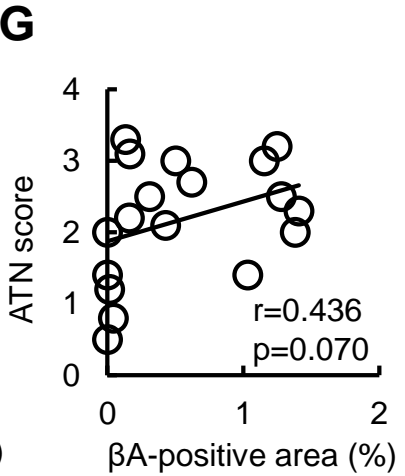
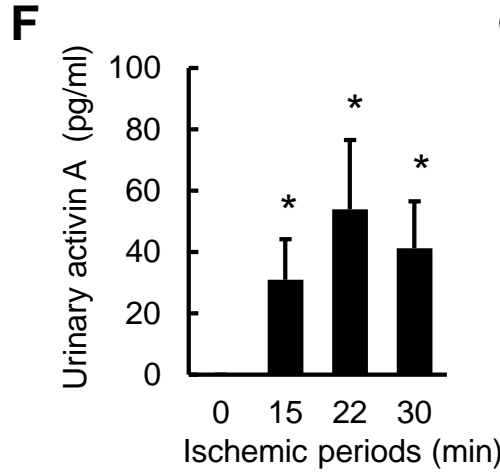
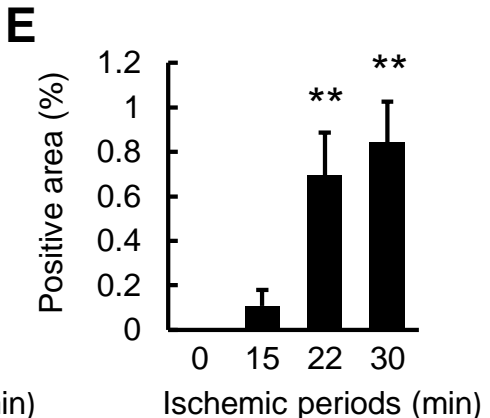
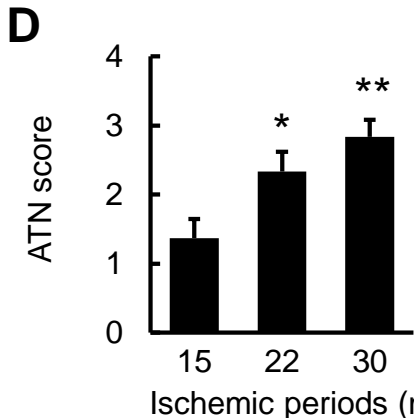
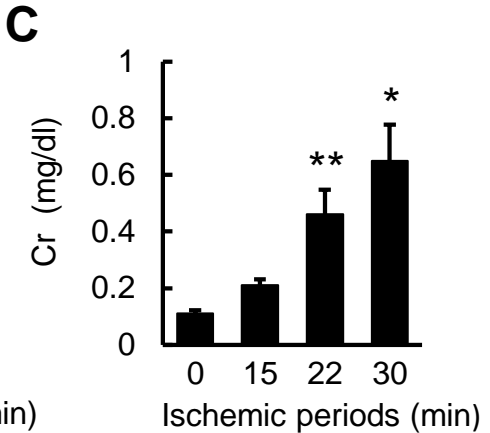
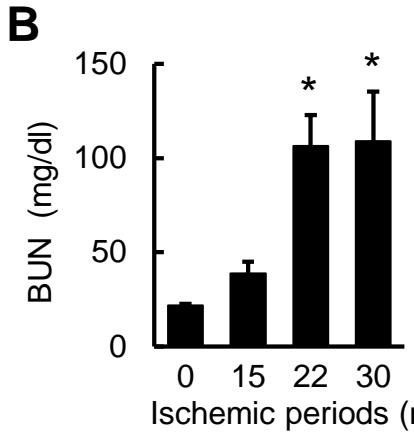
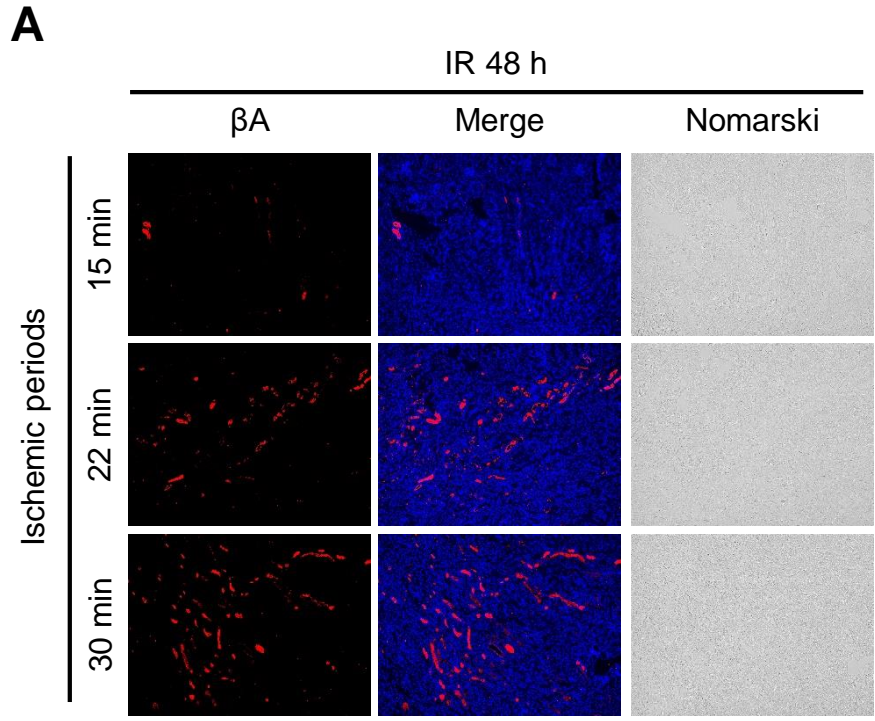


Figure 4



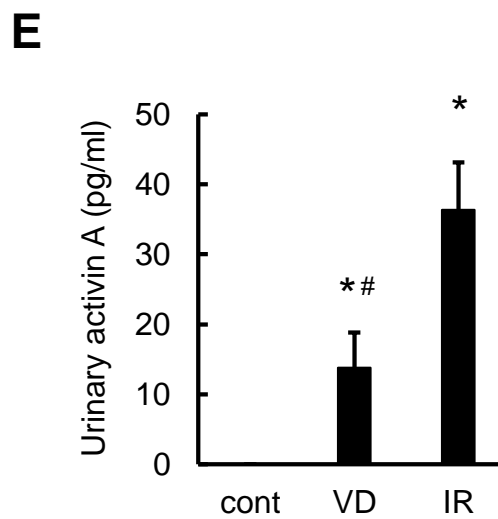
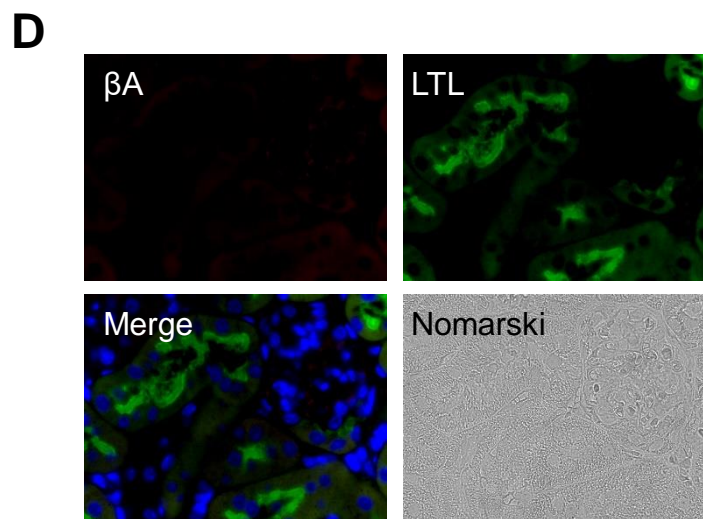
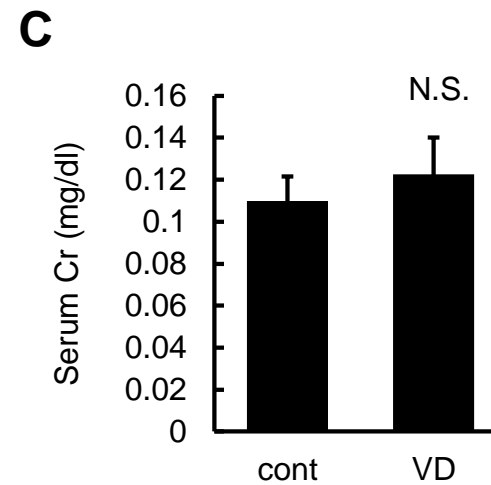
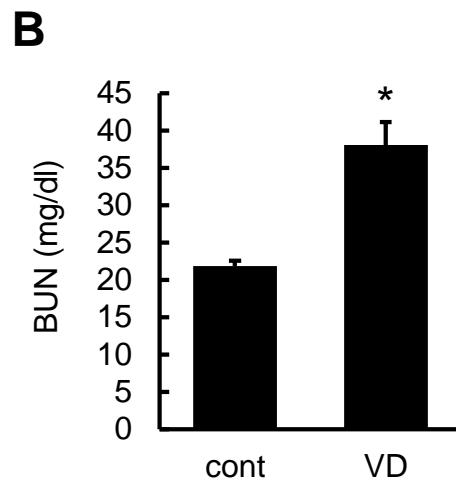
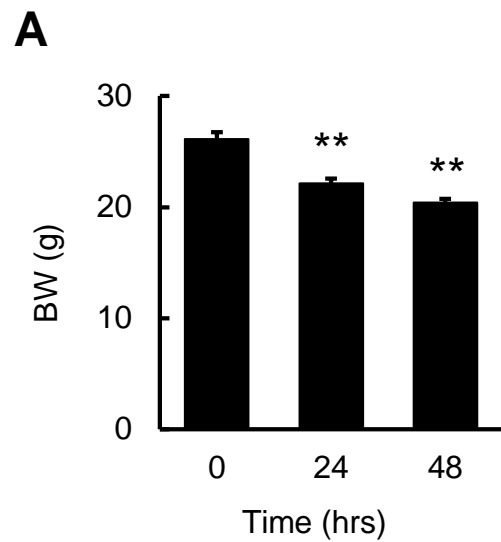


Table 1 Baseline Characteristics of AKI Patients

	Total	Renal AKI	Pre-renal AKI	P-value (renal vs. pre-renal)
Number	18	13	5	
Age (years)	63.3 ± 3.46	63.7 ± 4.46	62.2 ± 6.30	0.849
Sex (M/F)	10/8	6/7	4/1	
U-Prot/U-Cr (g/gCr)	2.25 ± 0.55	2.91 ± 0.64	0.54 ± 0.18	0.003
sCr (mg/dl)	4.86 ± 0.50	4.96 ± 0.67	4.59 ± 0.61	0.688
BUN (mg/dl)	65.3 ± 6.22	59.8 ± 7.78	79.6 ± 7.08	0.083
Hb (g/dl)	9.31 ± 0.55	8.30 ± 0.43	11.9 ± 0.92	0.012
WBC (/μl)	5,233 ± 795.8	4,892 ± 880.0	6,120 ± 1,836	0.569
Plt (× 10 ⁴)	14.0 ± 2.50	13.7 ± 3.13	14.6 ± 4.40	0.872
Na (mEq/L)	138 ± 1.24	137 ± 1.30	141 ± 2.68	0.192
K (mEq/L)	4.46 ± 0.23	4.40 ± 0.27	4.60 ± 0.46	0.729
Cl (mEq/L)	103 ± 1.27	102 ± 1.55	106 ± 1.60	0.077
NAG (IU/L)	19.3 ± 4.30	15.1 ± 2.56	26.7 ± 10.4	0.404

Data collected from patients with renal AKI (drug-induced, hypercalcemia, rhabdomyolysis, and cast nephropathy) and pre-renal AKI (dehydration, sepsis, and shock) at the initial visit are shown.