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Renal redox dysregulation in AKI: application for oxidative stress marker of AKI


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Kasuno K, Shirakawa K, Yoshida H, Mori K, Kimura H, Takahashi N, Nobukawa Y, Shigemi K, Tanabe S, Yamada N, Koshiji T, Nogaki F, Kusano H, Ono T, Uno K, Nakamura H, Yodoi J, Muso E, Iwano M. Renal redox dysregulation in AKI: application for oxidative stress marker of AKI. Am J Physiol Renal Physiol 307: F1342–F1351, 2014. First published October 1, 2014; doi:10.1152/ajprenal.00381.2013.—Oxidative stress is a major determinant of acute kidney injury (AKI); however, the effects of an AKI on renal redox system are unclear, and few existing AKI markers are suitable for evaluating oxidative stress. We measured urinary levels of the redox-regulatory protein thioredoxin 1 (TRX1) in patients with various kinds of kidney disease and in mice with renal ischemia-reperfusion injury. Urinary TRX1 levels were markedly higher in patients with AKI than in those with chronic kidney disease or in healthy subjects. In a receiver operating characteristic curve analysis to differentiate between AKI and other renal diseases, the area under the curve for urinary TRX1 was 0.94 (95% confidence interval, 0.90–0.98), and the sensitivity and specificity were 0.88 and 0.88, respectively, at the optimal cutoff value of 43.0 μg/g creatinine. Immunostaining revealed TRX1 to be diffusely distributed in the tubules of normal kidneys, but to be shifted to the brush borders or urinary lumen in injured tubules in both mice and humans with AKI. Urinary TRX1 in AKI was predominantly in the oxidized form. In cultured human proximal tubular epithelial cells, hydrogen peroxide specifically and dose dependently increased TRX1 levels in the culture supernatant, while reducing intracellular levels. These findings suggest that urinary TRX1 is an oxidative stress-specific biomarker useful for distinguishing AKI from chronic kidney disease and healthy kidneys.

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THIOREDOXIN 1 (TRX1) is a redox-regulating protein with a molecular weight of 12 kDa (8). The active site consensus sequence (-Cys-Gly-Pro-Cys-) of TRX1 contains a redox-active dithiol/disulfide that modulates the dithiol/disulfide bonds of substrate proteins, thereby regulating their function (24). Intracellular TRX1 regulates various signal transduction pathways and is secreted in response to oxidative stress. Once secreted, extracellular TRX1 has diverse actions, including anti-inflammatory and antiapoptotic effects (22). For example, TRX1 circulating in plasma exerts antichemotactic effects on neutrophils and inhibits neutrophil extravasation into inflammatory sites (19). In addition, extracellular TRX1 suppresses neovascularization via the inhibition of complement activation. Measurement of serum TRX1 is an effective means of detecting oxidative stress conditions. For example, serum TRX1 levels are increased in ischemia and oxidative stress-related disorders, including unstable angina, acute myocardial infarction, heart failure, and obstructive sleep apnea (10, 25), and are negatively correlated with left ventricular ejection fraction. On the other hand, little is known about urinary TRX1. In an earlier study, we reported that TRX1 in the kidney played a protective role against renal ischemia-reperfusion (I/R)-induced acute kidney injury (AKI) in mice (9). In the present study, we investigated the effects of AKI on the TRX1 redox system and the ability of urinary TRX1 to serve as a diagnostic marker in patients with AKI associated with oxidative stress.

MATERIALS AND METHODS

Patients. This investigation was approved by the ethics board of the University of Fukui Faculty of Medical Sciences (approval no. 2146). The subjects were 159 consecutively enrolled patients [AKI, 26; microscopic polyarteritis (MPA), 14; non-IgA mesangium proliferative glomerulonephropathy (non-IgA MsPGN), 11; focal segmental nephropathy (FSGS), 8; tubulointerstitial nephritis (TIN), 15; systemic lupus erythematosus (SLE), 11; IgA nephropathy (IgA-N), 32; membranous nephropathy (MN), 11; diabetic nephropathy (DN), 12; and miscellaneous kidney diseases including nephrosclerosis, amyloidosis, Henoch-Schonlein purpura nephritis, and acute glomerulonephritis, 19; Table 1] and 20 healthy individuals (males, 10; females, 10; mean age, 34.3 ± 9.2 yr). AKI was defined by >50% or 0.3 mg/dl increase in serum creatinine from the baseline or oliguria of <0.5 ml·kg⁻¹·h⁻¹ urine output for >6 h. Urine samples were collected for TRX1 measurements within 24 h of the initial increase in serum creatinine. The etiologies of AKI included infectious diseases other...
than sepsis in 9, dehydration in 5, sepsis in 3, non-steroid anti-inflammatory drugs in 3, nephrotic syndrome in 2, anticycnergens in 1, hypercalcemia in 1, rhabdomyolysis in 1, and congestive heart failure in 1 patient. The estimated glomerular filtration rate (eGFR) was 65.1 ± 19.1 mL/min 19.9 for AKI shown in Table 1 is based on the first time point at which the patients fulfilled the diagnostic criteria of AKI. The time-series relationship between blood and urine was investigated in 4 of the 26 AKI patients, all of whom were available to provide daily sequential samples during the AKI episode. Renal diseases other than AKI were diagnosed by renal biopsy. Exclusion criteria included peripheral vascular diseases. Another series of studies was carried out in patients who had undergone cardiopulmonary bypass (CPB; n = 10 with no postoperative AKI; n = 8 with postoperative AKI; Table 1). Preoperative measurements are shown as baseline clinical characteristics of these patients in Table 1. We obtained written informed consent from every participant. A full history was obtained from each patient before the investigation. Urine samples were centrifuged at 1,500 g for 5 min, and the supernatants was stored at −80°C until use. Serum and urinary creatinine were measured by the hospital clinical laboratory.

**Animals and cells:** Mice, weighing 25–30 g, were placed under general anesthesia using intraperitoneal pentobarbital sodium (50 mg/kg body wt). Through retroperitoneal incisions, the renal arteries were bilaterally occluded for 30 min using a clamp, after which the clamp was released to induce r/tR. The harvested kidneys were frozen in liquid nitrogen or immersed in 10% neutral-buffered formaldehyde and embedded in paraffin. Sham-operated mice were treated as above, but without occluding and releasing the arteries. For the time-series investigation, the number of mice was n = 2/3/r/tR and sham, respectively; for each indicated time point, a total of 46 mice were subjected to the experiments altogether for all 7 time points. Urine was collected using metabolic cages (Metabolica, Tokyo, Japan) and quickly frozen in liquid nitrogen until use. Human proximal tubular epithelial cells (HPTECs) were purchased as twice-passaged cells from Clonetics (San Diego, CA). The cells were grown and prepared as described previously (15). Growth-arrested confluent HPTECs were treated with 10 or 50 μM hydrogen peroxide (H$_2$O$_2$; Santoku Chemical Industries, Tokyo, Japan) or 50 μg/ml LPS (Sigma St. Louis, MO) for 1 and 3 h. The amount of TRX1 in culture supernatants and in cell lysates was measured as previously described (12).

**Measurement of TRX1, neutrophil gelatinase-associated lipocalin, and kidney injury molecule-1 using ELISAs.** Urinary and serum TRX1 concentrations were measured using an ELISA employing two murine antibodies to nonoverlapping epitopes of human TRX1 (TRX11 and TRX21) provided by Redox Biosciences (Kyoto, Japan) as per the manufacturer’s protocol (11). Briefly, samples were added to 96-microwell plates coated with TRX-21, incubated at room temperature for 2 h, and washed. Peroxidase-conjugated TRX11 antibody was then added followed by incubation at room temperature for 2 h. After washing, buffer containing horseradish peroxidase (HRP) substrate (2,2′-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid) and H$_2$O$_2$ was added and incubated at room temperature for 1 h. The absorption at 405 nm was then measured using an ELISA reader (Multiskan Bichromatic; Labsystems, Tokyo, Japan). Recombinant human TRX1 was used as a standard. Variability in urinary TRX1 concentrations was compensated for by adjustment of the concentrations based on the urine level of creatinine (mg/dl). Neutrophil gelatinase-associated lipocalin (NGAL), kidney injury molecule-1 (KIM-1), and mouse TRX1 were measured using commercially available ELISAs (BioProto Diagnostics, Grusbakken, Denmark; R&D Systems, Minneapolis, MN; and Cloud-Cloud, Houston, TX) as per the manufacturer’s protocol.

**Effect of urinary cellular components on urinary TRX1 values.** To investigate the effect of cellular components on urinary TRX1 values, the cellular components were removed by centrifugation at 1,500 rpm for 5 min. The urinary concentrations of TRX1 before and after removal of the urinary cellular components were then compared.

**Effect of urinary pH on TRX1 ELISA performance.** To investigate the influence of pH on measurements of urinary TRX1, we diazoy urine samples from 10 healthy subjects at 4°C overnight against 7 buffers of different pH, ranging from 4 to 10, prepared using the appropriate ratio of Na$_2$HPO$_4$ and NaH$_2$PO$_4$ (dialysis membrane: UC24 –32-100; Viskase, lot 801001). Urinary TRX1 was then measured to assess the effect of pH on the performance of the ELISA.

**Western blotting with urinary and serum TRX1.** Serum and urine samples were initially concentrated 10-fold using a vacuum lyophilizer (CryoCool-80; Tomy, Tokyo, Japan). These samples together were then deparaffinized with xylene, after which immunohistological staining was performed as previously described (9). Briefly, deparaffined sections were treated for 6 min at room temperature with 3% H$_2$O$_2$ to inhibit endogenous peroxidase activity and then incubated with normal rabbit serum to block nonspecific antibody binding. The sections were then incubated with anti-TRX1 monoclonal antibody (2.0 μg/ml) overnight at 4°C, or with normal mouse serum as a negative control. Signals were visualized using EnVision+ HRP mouse (for MET; Dako, K4001) plus diaminobenzidine (Dako, K3468) and a hematoxylin counterstain. In vitro, HPTECs were seeded onto BD BioCoat Culture Slides (BD Biosciences). Growth-arrested confluent HPTECs were then treated with 50 μM H$_2$O$_2$ or

Table 1. Clinical characteristics of patients

<table>
<thead>
<tr>
<th>Parameter</th>
<th>AKI</th>
<th>MPA</th>
<th>MsPGN</th>
<th>FSFGS</th>
<th>TIN</th>
<th>SLE</th>
<th>IgA-N</th>
<th>MN</th>
<th>DN</th>
<th>CPB</th>
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<tr>
<td>Age, yr</td>
<td>65.1 ± 19.1</td>
<td>68.1 ± 7.2</td>
<td>63.0 ± 15.0</td>
<td>45.0 ± 19.3</td>
<td>52.1 ± 12.2</td>
<td>32.2 ± 18.8</td>
<td>38.1 ± 16.9</td>
<td>53.9 ± 12.3</td>
<td>68.8 ± 13.4</td>
<td>67.2 ± 14.6</td>
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<tr>
<td>Male, %</td>
<td>69</td>
<td>71.4</td>
<td>18.2</td>
<td>62.5</td>
<td>42.9</td>
<td>27.2</td>
<td>66.6</td>
<td>72.7</td>
<td>91.7</td>
<td>50.1</td>
</tr>
<tr>
<td>eGFR, ml/min</td>
<td>19.9 ± 12.5</td>
<td>33.3 ± 16.1</td>
<td>92.0 ± 44.9</td>
<td>89.1 ± 35.0</td>
<td>51.7 ± 16.2</td>
<td>67.4 ± 31.5</td>
<td>71.4 ± 26.5</td>
<td>93.1 ± 32.3</td>
<td>31.4 ± 24.9</td>
<td>64.4 ± 22.6</td>
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<td>CRP, mg/dl</td>
<td>7.97 ± 6.5</td>
<td>6.7 ± 1.0</td>
<td>7.0 ± 0.6</td>
<td>4.6 ± 0.9</td>
<td>7.5 ± 1.0</td>
<td>5.8 ± 0.9</td>
<td>6.7 ± 0.7</td>
<td>5.3 ± 1.1</td>
<td>6.4 ± 0.9</td>
<td>6.6 ± 0.8</td>
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<td>Serum albumin, g/dl</td>
<td>2.7 ± 0.9</td>
<td>3.1 ± 1.0</td>
<td>4.5 ± 0.4</td>
<td>2.6 ± 0.8</td>
<td>3.7 ± 0.4</td>
<td>3.1 ± 0.8</td>
<td>4.0 ± 0.5</td>
<td>2.7 ± 0.7</td>
<td>3.3 ± 0.6</td>
<td>3.7 ± 0.7</td>
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<tr>
<td>Hemoglobin, g/dl</td>
<td>10.3 ± 1.9</td>
<td>9.5 ± 1.4</td>
<td>13.2 ± 1.2</td>
<td>12.9 ± 3.2</td>
<td>13.2 ± 0.9</td>
<td>11.4 ± 3.1</td>
<td>13.4 ± 1.8</td>
<td>13.8 ± 2.0</td>
<td>11.1 ± 2.7</td>
<td>11.5 ± 2.2</td>
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<tr>
<td>CRP, mg/dl</td>
<td>35.0 ± 51.7</td>
<td>16.2 ± 67.4</td>
<td>19.3 ± 52.1</td>
<td>12.2 ± 32.2</td>
<td>10.5 ± 32.8</td>
<td>5.63 ± 0.14</td>
<td>0.96 ± 0.12</td>
<td>0.72 ± 0.06</td>
<td>0.72 ± 0.06</td>
<td>0.34 ± 0.60</td>
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</table>
| Values are means ± SD. eGFR, estimated glomerular filtration rate; CRP, c-reactive peptide; AKI, acute kidney injury; MPA, microscopc polyangiitis; MsPGN, non-IgA mesangial proliferative glomerulonephritis; FSFGS, focal segmental glomerulosclerosis; TIN, tubulointerstitial nephritis; SLE, systemic lupus erythematoses; IgA-N, immunoglobulin A nephropathy; MN, membranous nephropathy; DN, diabetic nephropathy; CPB, cardiopulmonary bypass.
vehicle for 3 h, after which they were fixed with PBS containing 3% formaldehyde overnight at 4°C, permeabilized with 0.2% Triton X-100 in PBS for 15 min at room temperature, and blocked by incubation with 1% BSA in PBS for 10 min at room temperature. Thereafter, the cells were incubated first with diluted anti-TRX1 monoclonal antibody overnight at 4°C, and then with Alexa Fluor 488-conjugated anti-mouse antibody for 30 min at room temperature. 4,6-Diamidino-2-phenylindole was used as a nuclear counterstain. The cells were imaged using an Olympus BX51 fluorescence microscope.

RT-PCR to detect TRX1 mRNA. HPTECs were plated into six-well plates and treated with H2O2 or vehicle. Total RNA was then extracted using TRIzol reagent, after which CDNA was generated using reverse transcriptase and a ReverTra Ace qPCR RT Kit (Toyobo). Aliquots of the resultant cDNA were subjected to PCR performed on a StepOnePlus real-time PCR system (Applied Biosystems) using Taq DNA polymerase and Thunderbird qPCR Mix (Toyobo). The sequences of the DNA primers were as follows: for TRX1: 5'-TTGGAGCTGAGGTGATAAAC-3' (forward) and 5'-GCGATCATTTGAC TTCACACTC-3' (reverse); and for GAPDH: 5'-GCACCGTC AAGGCTGAGAAC-3' (forward) and 5'-TGTTGAGA GACCCAGTGGG-3' (reverse).

Statistical analysis. Data are expressed as the median (25th percentile, 75th percentile). Values of P < 0.05 were considered significant. The Mann-Whitney U-test was used for comparisons between two groups. The Kruskal-Wallis test was used for post hoc analysis after the Mann-Whitney test, and adjustment of the P value using the Dunn method (P < 0.002) was used to assess differences in clinical measures among more than three groups. The sensitivity, specificity, and predictive values for the diagnosis of AKI were calculated using receiver-operator characteristic curves and 2 × 2 tables. Pearson’s product-moment correlation coefficient was used to calculate r. Statistical analysis was performed using SigmaPlot version 12.0 software (Systat Software, San Jose, CA).

RESULTS

Presence of TRX1 in urine. We initially tested whether TRX1 was present at detectable levels in urine. Samples of urine and serum obtained from healthy individuals, as well as recombinant TRX1 protein, were separated on SDS gels under both nonreducing and reducing conditions and were then detected using a specific anti-TRX1 antibody. TRX1 appeared at 12 kDa in all samples under both nonreducing and reducing conditions (Fig. 1A). TRX1 thus appears to be present and detectable in the urine.

Stability of TRX1 measurements in clinical samples. Urine samples may contain various cellular components, including...
were highly consistent with the levels measured with the cells levels measured after the cellular components were removed without the cellular components. We found that urinary TRX1 measurement (21). To address that issue, we used an ELISA to erythrocytes, leukocytes, epithelial cells, and bacteria that of urinary TRX1 were stable (coefficient of variation

urinary pH varies physiologically, we next investigated the effect of pH on the performance of the ELISA used to measure urinary TRX1. At physiological pHs (pH 5–8), measurements of urinary TRX1 were stable (coefficient of variation = 0.096); only under nonphysiological conditions (pH 4, 9, or 10) did urinary TRX1 levels appear to deviate (data not shown). The temperature and freeze-thaw stability of TRX1 in urine were tested using an ELISA and Western blotting. There was no significant deterioration under the tested conditions (Fig. 1, C–E). These results indicate that a TRX1 ELISA system is suitable for use with clinical urine samples.

**Urinary TRX1 elevation in patients with AKI.** Using the ELISA system tested above, we measured serum and urinary TRX1 in patients with various renal diseases and in healthy subjects (Table 1). Our results show that urinary TRX1 levels are predominantly higher in patients with AKI [median, 124.0 μg/g of creatinine (1st quartile, 3rd quartile, 63.2, 345.6 μg/g of creatinine)] than in healthy controls [16.7 μg/g of creatinine (3.3, 27.7 μg/g of creatinine); P < 0.05] or in patients with other kidney diseases, including non-IgA MsPGN [29.5 μg/g of creatinine (21.4, 35.2 μg/g of creatinine); P < 0.05], FSGS [23.6 μg/g of creatinine (10.5, 27.8 μg/g of creatinine); P < 0.05], TIN [17.3 μg/g of creatinine (6.1, 26.6 μg/g of creatinine); P < 0.05], SLE [16.1 μg/g of creatinine (8.4, 26.2 μg/g of creatinine); P < 0.05], IgA-N [11.6 μg/g of creatinine (8.8, 28.3 μg/g of creatinine); P < 0.05], MN [10.4 μg/g of creatinine (6.9, 19.4 μg/g of creatinine); P < 0.05], and DN

![Fig. 2. Urinary TRX1 is increased in AKI. A: urinary TRX1 levels in patients with the indicated renal diseases and in normal controls. Data are expressed as the median (25th percentile, 75th percentile). AKI, acute kidney injury; MPA, microscopic polyarteritis; MsPGN, non-IgA mesangial proliferative glomerulonephritis; FSGS, focal segmental glomerulosclerosis; TIN, tubulointerstitial nephritis; SLE, systemic lupus erythematosus; IgA-N, immunoglobulin A nephropathy; MN, membranous nephropathy; DN, diabetic nephropathy. *P < 0.05. B: receiver-operating curve for identifying established AKI in the same patients. The area under the curve (AUC) was 0.94. C: urinary TRX1 levels excluding outliers with >600 μg/g of creatinine. Data are expressed as the median (25th percentile, 75th percentile). *P < 0.05. D: relationship between renal function and serum or urinary TRX1. Daily changes in serum creatinine and TRX1 in serum and urine are plotted for 4 AKI patients who were available to provide daily sequential samples during the AKI episode. Fold-increases with respect to baseline (hospital day 1) are expressed as means ± SD. E: relationship between urinary N-acetyl-β-glucosaminidase (NAG) and TRX1 levels (r = 0.63, P < 0.001).](http://ajprenal.physiology.org/)

AJP-Renal Physiol · doi:10.1152/ajprenal.00381.2013 · www.ajprenal.org
[7.1 μg/g of creatinine (5.8, 28.9 μg/g of creatinine); P < 0.05; Fig. 2A]. There were no significant differences between patients with AKI and MPA [50.6 μg/g of creatinine (27.3, 72.2 μg/g of creatinine)]. The receiver-operating characteristic curve revealed that the area under the curve (AUC) of urinary TRX1 for differentiating between AKI from other kidney diseases (MPA, non-IgA MsPGN, FSGS, TIN, SLE, IgA-N, MN, and DN) was 0.94 (95% confidence interval, 0.90–0.98). At the optimal cutoff value of 43.0 μg/g of creatinine, the sensitivity was 0.94 (95% confidence interval, 0.90–0.98), 0.96, and 0.80, respectively, at the cutoff for AKI, sensitivity, and specificity were 0.88 for the diagnostic accuracy of AKI (Fig. 2B). This cutoff serves as a diagnostic marker for AKI that minimizes the number of false positives and false negatives. Because the results could have been influenced by the presence of extreme outliers, we performed an additional analysis with the outliers excluded. When a urinary TRX1 outlier >600 μg/g of creatinine was excluded, the AUC, sensitivity, and specificity were 0.94 (95% confidence interval, 0.89–0.98), 0.96, and 0.80, respectively, at the cutoff value of 33.6 μg/g of creatinine (Fig. 2C).

Early elevation of urinary TRX1 in patients with AKI. We then investigated the time-series relationship between TRX1 levels in urine and blood. In patients with AKI, urinary TRX1 levels peaked on hospital day 2, whereas serum levels peaked on hospital day 4 (Fig. 2D). To quantitatively assess the relationship between urinary TRX1 and tubular injury, we compared the urinary TRX1 and N-acetyl-β-glucosaminidase (NAG) levels in these patients and found that there was a significant positive correlation between them (Fig. 2E). To investigate how conditions that cause oxidative stress but do not lead to AKI (e.g., hyperglycemia) may affect urinary TRX1 levels, the correlation between blood glucose and urinary TRX1 levels was tested in patients with DN. The results confirmed that blood glucose does not correlate with urinary TRX1 levels and that patients with acute hyperglycemia do not show a corresponding elevation of urinary TRX1 levels (r = 0.10, P = 0.75, data not shown).

Early elevation of urinary TRX1 after CPB. To investigate the ability of urinary TRX1 to serve as a marker for early detection of AKI, we compared levels of TRX1 with serum creatinine and the rapid AKI marker neutrophil gelatinase-associated lipocalin (NGAL) in urine samples from patients undergoing CPB (18). We found that urinary TRX1 increased...
much faster (6 h) than serum creatinine, which peaked 36 h after the onset of CPB (Fig. 3A). By contrast, both TRX1 and NGAL peaked 6 h after the onset of CPB and followed entirely the same course (Fig. 3, B and C). Thus urinary TRX1 levels correlated with those of NGAL, especially during the first 6 h after the onset of CPB (from 0 h to the peak for both TRX1 and NGAL, \( n = 126, r = 0.775 \), Fig. 3D). Interestingly, the CPB-related increment in TRX1 was greater (1,004 ± 588-fold at hour 6) than the increment in NGAL (436 ± 287-fold at hour 6; Fig. 3, B and C).

Oxidative stress-dependent excretion of TRX1. Oxidative stress is an important etiology of AKI (7). To confirm the association between TRX1 and oxidative stress, we stimulated cultured HPTECs with \( \text{H}_2\text{O}_2 \). Upon addition of \( \text{H}_2\text{O}_2 \) to the cultures, TRX1 levels in the supernatants increased dose dependently, whereas levels of NGAL and KIM-1 remained unchanged (Fig. 4A). Moreover, the elevation in TRX1 was selectively induced by \( \text{H}_2\text{O}_2 \), as lipopolysaccharide had no effect (Fig. 4B). Conversely, intracellular TRX1 levels were dose dependently reduced upon addition of \( \text{H}_2\text{O}_2 \) to the cultures, and again lipopolysaccharide had no effect (Fig. 4C). This result was confirmed by immunohistochemical analysis, which showed that \( \text{H}_2\text{O}_2 \) reduced intracellular staining for TRX1 (Fig. 4D). To assess the involvement of transcriptional upregulation, we also determined the effect of \( \text{H}_2\text{O}_2 \) on TRX1 mRNA levels. The results showed no upregulation of TRX1 mRNA after 3 h of exposure, the time at which supernatant levels of TRX1 peaked. Only after exposing the cells to \( \text{H}_2\text{O}_2 \) for 24 h were TRX1 mRNA levels increased (Fig. 4E).

rI/R increases urinary TRX1 in mice. We next investigated the origin of urinary TRX1 in AKI using a mouse model in which rI/R was used to induce AKI. Western blot analysis of serum samples showed no remarkable changes in TRX1 levels after rI/R. By contrast, TRX1 levels were significantly lower in kidneys subjected to rI/R than in sham-operated kidneys, and there was a corresponding increase in urinary TRX1 after rI/R (Fig. 5, A and B). To confirm that urinary TRX1 is not derived from a preceding increase in serum TRX1 and glomerular filtration, we carried out a time-series and observed the relationship between TRX1 levels in blood and urine at various intervals in mice with rI/R. The development of AKI was confirmed by a 19.4-fold increase in serum creatinine from baseline after 24 h postreperfusion. In these mice, serum TRX1 levels remained stable throughout the observation period, despite a marked increase in urinary TRX1 observed after 12 h of reperfusion (Fig. 5C). Immunohistochemical analysis showed that before rI/R, TRX1 was diffusely distributed throughout the renal tubular epithelium with localization adjacent to the basement membrane (Fig. 5D, a and b). After rI/R, TRX1 positivity had shifted to the apical side of the tubular epithelial cells and into the urinary lumen of the tubuli; the basal side of the

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**Fig. 4.** Oxidative stress-dependent excretion of TRX1 in experiments with human proximal tubular cells. A: hydrogen peroxide (\( \text{H}_2\text{O}_2 \)) specific excretion of TRX1. Cells were stimulated with \( \text{H}_2\text{O}_2 \) at the indicated concentrations for 1 or 3 h, after which supernatant levels of TRX1, NGAL, and KIM-1 were measured by ELISA. Three independent experiments were performed in triplicate. B: cells were stimulated for 3 h with lipopolysaccharide (LPS) or \( \text{H}_2\text{O}_2 \) at the indicated concentrations, after which supernatant levels of TRX1 were measured by ELISA. Three independent experiments were performed in triplicate. C: cells were stimulated for 3 h with LPS or \( \text{H}_2\text{O}_2 \) at the indicated concentrations, after which intracellular TRX1 levels were measured by ELISA. Three independent experiments were performed in triplicate. D: immunohistochemical staining of intracellular TRX1 (green). Cells were stimulated for 3 h with 50 \( \mu \text{M} \) \( \text{H}_2\text{O}_2 \) or vehicle and visualized using fluorescence microscopy. Scale bar = 50 \( \mu \text{m} \). E: late increase in TRX1 mRNA induced by oxidative stress. Human proximal tubular epithelial cells were exposed to \( \text{H}_2\text{O}_2 \) for the indicated times. Isolated total RNA was reverse-transcribed and real-time quantitative PCR was performed. Real-time PCR data were analyzed using standard curves and normalized to 18S ribosomal RNA. Values are means ± SD. *Significant increases or decreases against vehicle control.
tubular epithelium was negative for TRX1. Notably, in some tubuli the epithelial cells were completely negative for TRX1; only the urinary lumen was positive (Fig. 5D, c and d). The vascular lumen, glomerular capillary lumen, and urinary space in Bowmen’s capsule were negative (Fig. 5E, e and f). To investigate the redox status of urinary TRX1, urine samples were analyzed by redox Western blotting. The increased TRX1 in the urine from patients with AKI was predominantly in dimer form, corresponding to oxidized TRX1 (Fig. 5, F and G).

**DISCUSSION**

The studies summarized above demonstrate that the redox-regulatory protein TRX1 in the urine primarily originates from tubular cells in association with oxidative stress and that urinary TRX1 levels can usefully serve as an oxidative stress-specific marker of AKI. Using Western blotting and an ELISA, we confirmed that TRX1 is present in the urine. It was previously reported that cellular components, including blood cells and epithelial cells, contain intracellular TRX1. We therefore examined whether the presence of cellular components influenced the data. The volume of cellular components in the urine is negligible, which makes the strong correlation a reasonable outcome. Furthermore, measurements of urinary TRX1 are not affected by pH, incubation, or freeze-thaw cycles. This suggests that a conventional ELISA could be used to measure urinary TRX1 levels in clinical urine samples.

The patients with AKI were older than those with other diseases, which likely reflects the fact that kidney injury is especially common among elderly ICU patients, 6% of whom develop AKI (median age: 67) (6). In addition, we observed no correlation between age and urinary TRX1 levels (data not shown), suggesting that age did not affect urinary TRX1. Compared with other diseases, patients with AKI exhibited lower eGFR and serum albumin levels and higher levels of C-reactive protein. These features correspond to the epidemiology of AKI (4), but none correlated with the urinary TRX1 levels, suggesting none affected urinary TRX1 levels. Urinary TRX1 levels were specifically elevated in established AKI, which is different from all other renal diseases (13, 23) except MPA. It was recently reported that NGAL and KIM-1 are also increased in patients with MPA (16). Given the similarity of the clinical significances of these urinary biomarkers, increased urinary TRX1 in patients with MPA seems reasonable.

The AUC of urinary TRX1 for diagnosis of established AKI was 0.94, which indicates TRX1 is an excellent AKI marker, comparable to other established markers (2). For example, urinary KIM-1 and IL-18 are highly effective for distinguishing between true acute tubular necrosis and other types of renal injury (including CKD) or controls. The AUCs of KIM-1 and IL-18 for the diagnosis of AKI were 0.90 and 0.95, respectively. The levels of the tubular injury marker NAG positively correlated with urinary TRX1 levels (Fig. 2E). Conditions that caused oxidative stress but did not lead to AKI did not increase urinary TRX1 levels in either DN or CPB (Fig. 3B). All of these results confirmed that urinary TRX1 increased according to the severity of tubular injury and that conditions that cause oxidative stress but do not lead to AKI do not increase urinary TRX1 levels. In addition, urinary TRX1 peaked before the increase in serum TRX1, which increased and decreased in parallel with serum creatinine in patients with AKI (Fig. 2D), and showed excellent quantitative performance against tubular injury, increasing as early as the earliest AKI marker, NGAL (Fig. 3, C and D). For example, TRX1 levels began to increase within 2 hours and peaked within 6 hours after initiation of CPB (Fig. 3B). This rapid response occurred simultaneously with the increases in urinary NGAL (Fig. 3C). In fact, urinary TRX1 peaked at the same time and correlated with NGAL during CPB. Taken together, these results suggest that urinary TRX1...
increases much earlier than serum creatinine and is a useful marker for early and quantitative detection of AKI.

The peak urinary TRX1 in AKI patients and mice preceded the peak in serum, which suggests that urinary TRX1 does not increase due to elevation of serum TRX1 and that urinary TRX1 does not derive from an excess of serum TRX1 filtered by the glomeruli (Figs. 2D, 5C). Supporting this hypothesis is our observation that in human tubular epithelial cells, H2O2 increased extracellular and decreased intracellular TRX1 levels (Fig. 4, A–D). The effects of H2O2 were specific and dose...
dependent and were detectable within as little as 3 h. By contrast, H\textsubscript{2}O\textsubscript{2} had no effect on TRX1 mRNA in that time frame (Fig. 4E). These in vitro observations of TRX1 mRNA expression are consistent with our earlier study in mice, which showed that rI/R had no effect on TRX1 mRNA expression after 3 h (9). These findings suggest that tubular epithelial cells secrete intracellular TRX1 in response to oxidative stress and that this secretion is not accompanied by upregulated transcription of TRX1 mRNA. Furthermore, we observed in mice that rI/R decreases TRX1 levels in the renal parenchyma, which is consistent with the reduction in intracellular TRX1 levels induced by H\textsubscript{2}O\textsubscript{2} in human tubular epithelial cells. That TRX1 was increased in the urine but not the serum of mice subjected to rI/R (Fig. 5, A–C) was also consistent with the TRX1 levels in urine and serum samples from patients with AKI (Fig. 2D). Immunohistochemical analysis showed that rI/R induced a shift in TRX1 staining from diffuse, adjacent to the tubular basement membrane, to the apical side, away from the tubular basement membrane, or into the urinary lumen. Similar changes in staining were observed in tissue from AKI patients. However, the most critical finding was the lack of staining in the blood vessels and intraluminal spaces of Bowman’s capsule in both mice and humans with AKI (Fig. 5E). These findings support the hypothesis that urinary TRX1 does not derive from serum TRX1 filtered by glomeruli, but from excretion from tubular epithelial cells.

The mechanism underlying TRX1 release remains unknown; however, TRX1 is secreted by cells in response to oxidative stresses such as viral infection, ultraviolet irradiation, and H\textsubscript{2}O\textsubscript{2}, despite its lack of a typical secretory signal sequence (12). Previous studies demonstrated that the level of TRX1 release is enhanced upon addition of H\textsubscript{2}O\textsubscript{2} but suppressed upon addition of N-acetylcysteine, which suggests oxidative stress-induced release of TRX1 is dependent upon a redox-sensitive event. After oxidation, an intermolecular disulfide bond is formed between C62 and C69 of TRX1, resulting in dimer or multimer formation (20). The reduction of oxidized TRX1 is mediated by NADPH and TRX1 reductase intracellularly; however, it is not yet known whether the recycling of oxidized/reduced TRX1 is possible under extracellular conditions, such as in urine. In the present study, we also confirmed that H\textsubscript{2}O\textsubscript{2} specifically stimulates the release of TRX1 from human proximal tubular cells, but has no effect on NGAL or KIM-1. Furthermore, we observed that the increased urinary TRX1 seen with AKI is predominantly in the oxidized form (Fig. 5, F and G). Conversely, overexpression of intracellular TRX1 was shown to inhibit rI/R-induced AKI in the mouse kidney (9). Over the years, a variety of urinary biomarkers have been proposed, but few appear to be directly involved in oxidative stress or redox regulation (1). Our present findings suggest TRX1 released into the urine has a closer relationship with the cellular redox response to oxidative stress than do other urinary biomarkers. This clearer causal relationship between redox dysregulation and AKI represents an advantage of using TRX1 over other biomarkers. By using biomarkers with a clearer pathophysiological relationship, such as TRX1, one obtains a better understanding of AKIs, which can have diverse combinations of etiologies.

One limitation of this study is the small number of patients, which precluded subgroup analysis between the etiologies of AKI. AKI is caused by a number of disorders, including septic shock, major surgery, cardiogenic shock, hypovolemia, and drug-related injury (26). Neither the pathogenesis nor the process underlying any form of AKI is completely understood. It has been proposed that oxidative stress is one of the processes associated with AKI (6, 7) related to septic shock (3), major surgery (5), injury to the heart or other organs (14), and contrast medium (17). Urinary TRX1 was elevated in all forms of AKI in our present study. Because the interplay and sometimes overlap of multiple processes may contribute to the development of AKI in each etiology, it may be difficult to show the specificity of the relationship between urinary TRX1 and oxidative stress in clinical samples. Further study of a larger population of patients may provide additional information about the advantages and disadvantages of TRX1 with respect to other biomarkers, as well as the degree to which oxidative stress contributes to each AKI etiology. Consideration of AKI from various viewpoints, along with pathophysiologically specific biomarkers such as TRX1, could potentially enable etiological diagnosis of AKI and facilitate the development of more targeted therapies.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


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