Type 2a sodium-phosphate co-transporter serves as a histological predictor of renal dysfunction and tubular apical damage in the kidneys of septic mice

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Abstract
Acute renal failure (ARF) occurs in septic patients and is histologically characterized by tubular apical damages, including brush border breakdown. Nevertheless, little information is available to identify the apical injury at a molecular level. Type 2a Na-phosphate (Pi) co-transporter (NaPiT2a) is constitutively expressed by brush borders of proximal tubules under a healthy condition. Therefore, we investigated if NaPiT2a could be used as a negative marker to predict the renal dysfunction, using an animal model of septic ARF. After the treatment of lipopolysaccharide (LPS), mice manifested the tubular apical injury and renal dysfunction, as evidenced by the increase in blood urea nitrogen (BUN) levels. Immunohistochemical examination revealed that the expression of NaPiT2a by renal proximal tubules became faint, being reciprocal to the development of tubular hypoxia during sepsis. Inversely, the loss in apical NaPiT2a was restored in a regenerating stage, associated with the recovery from renal hypoxia. Overall, there was a negative correlation between the NaPiT2a expression and BUN levels or tubular injury scores in septic mice. Our data indicate that the loss of NaPiT2a is a reliable marker for predicting the progression of septic ARF, while local hypoxia might be involved in the decrease of NaPiT2a expression.

Septic acute renal failure (ARF) is now an emerging disease in the intensive care unit (ICU), which occurs due to antibiotics-induced bacterial breakdown or steroid-induced immune suppression. Indeed, ARF is a serious condition that affects as many as 20% of ICU patients. In the United State, approximately 700,000 patients develop sepsis each year with 200,000 fatalities, and when sepsis is associated with ARF, the mortality rate approaches 70%, as compared with 45% mortality among patients with ARF alone (19, 20). Since ARF constitutes a particularly serious problem, it is important to detect patho-physiological events as soon as possible in the early stage of endotoxemia.

The pathogenesis of septic ARF is not fully understood, but it involves systemic inflammation and hyper-coagulation with glomelular thrombosis, resulting in a possible decrease in glomerular blood flow. Under such a hypoxic condition, the loss of tubular brush border membrane (BBM) becomes evident (6, 9, 22), and systemic electrolytic abnormality due to BBM injury may further accelerate a decline in glomerular filtration rate (GFR), via a tubulo-glomerular feedback (5, 25). Throughout the pathological circuit, renal tubular hypoxia becomes more extensive, leading to the acceleration of ARF. Thus, it is important to delineate BBM injury at a molecular level. Nevertheless, it is still unclear which molecular marker(s) is available for the identification of apical damages.

It is known that type 2a sodium-phosphate co-transporter (NaPiT2a) is one of the most abundant
transporters in mammalian kidneys and is localized at the apical sides (i.e., BBM) of the proximal tubular epithelium (14, 29). This co-transporter is electrogenic and operates with a 3:1 Na$^+$:HPO$_4^{2-}$ stoichiometry (i.e., one net positive charge per transport cycle) (29). More of importance, mice homozygous for the disrupted NaPiT2a gene (Npt2$^{-/-}$) exhibit an increase in urinary Pi excretion and more than an 80% loss in BBM Pi re-absorption, which is followed by the onset of severe hypo-phosphatemia (4). Thus, the constitutive expression of NaPiT2a at the apical side of the proximal tubules is critical to control the phosphate homeostasis in vivo.

The fact that NaPiT2a is constitutively expressed by normal proximal tubules prompted us to hypothesize that chronological analysis of apical NaPiT2a may be helpful to predict the ARF-related pathological conditions. To test our hypothesis, we used septic mice, as apical damage of tubular cells is known to be a typical feature of septic ARF (1, 19, 20). Using the histological and Western blot techniques in septic mice, this study shows that the loss and reverse of apical NaPiT2a expression may represent the progression and recovery of renal dysfunction and hypoxia, respectively.

MATERIALS AND METHODS

Septic mouse model. We attempted to induce septic illness in 8-week-old female C57Bl/6J mice (SLC, Hamamatsu, Japan), based on a recent report (31). The mice were treated with lipopolysaccharide (LPS: 0111:B4, Lot L-2630) (Sigma, St. Louis, MO, USA) at a dose of 6 mg/kg (i.p.). To determine the natural course of ARF, 20 mice were autopsied at 0, 3, 12, 24 and 36 h after the injection of LPS (n = 4/group). Additional mice were killed at 72 h post-LPS challenge for a recovery test of NaPiT2a. At the scheduled autopsy, mice were anesthetized with ketamine chloride (80 mg/kg, s.c.) and xyladine sulfate (8 mg/kg, s.c.), and blood was collected from the submaxillary artery. Some septic mice were treated with pimonidazole (Hypoxyprobe-1™; Chemicon, Temecula, CA, USA) (60 mg/kg, i.p.) 1 h before the autopsy to determine the degree of renal hypoxia, as described (13).

Blood chemistry. The plasma samples were obtained from heparinized blood and were frozen at −80°C until use. To assess the renal function after the LPS treatment, blood urea nitrogen (BUN) levels were determined using a kit (urea nitrogen-B test; Wako, Osaka, Japan) (12, 13).

Histopathology and immunohistochemistry. At the scheduled necropsy, renal tissues were removed, fixed in 10% neutral buffered formalin (pH 7.4) and embedded in paraffin. Each section was cut into 4 μm slices, stained with periodic acid-Schiff (PAS) reagent (Wako). To detect the apical changes during sepsis, the renal tubular co-transporter was identified in renal sections, using an anti-NaPiT2a rabbit IgG (Dako, Glostrup, Denmark), followed by the second reaction with biotin-labeled anti-rabbit IgG (Vector, Burlingame, CA, USA). An avidin-biotin coupling reaction was performed on the renal sections, using an ABC Elite kit (Vector). Likewise, pimonidazole was detected in the kidney, using an anti-pimonidazole mouse IgG (Chemicon) (13). All antigens were visualized as brown, with 3,3′-diaminobenzidine (Nacalai, Kyoto, Japan) (12, 13).

Immunofluorescence staining. The distribution of NaPiT2a and Hypoxyprobe-1 were examined in the same sections, using double fluorescence techniques. The renal sections were incubated with anti-NaPiT2a rabbit IgG and anti-pimonidazole mouse IgG (Chemicon) (13) at 4°C overnight. The tissue sections were then washed with saline, followed by incubation with Alexa488-conjugated anti-rabbit IgG and Alexa546-conjugated anti-mouse IgG (Invitrogen, Carlsbad, CA, USA). In this method, NaPiT2a and pimonidazole were visualized as green and red signals, respectively, under a confocal microscope (LSM5 Pascal, Zeiss, Germany) (13).

Morphometric scores. The tubular injury (such as tubular dilatation and epithelial desquamation) was evaluated in the PAS-stained sections and graded as follows: 0, intact; 1, < 10%; 2, 10–25%; 3, 25–50%; 4, 50–75%; 5, > 75% of the tubules were affected, as reported (12). The NaPiT2a-positive cells were counted in more than 1000 tubular cells in the kidneys, and its percentage was defined as the tubular NaPiT2a staining score.

Western blot analysis. Renal tissues were homogenized in a lysis buffer [50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 25 mM glycerophosphate, 50 mM NaF, 1 mM Na$_3$VO$_4$, 1% Triton X-100, 10% glycerol, 1 mM phenylmethanesulfonyl fluoride, 2 μg/mL antipain, pepstatin A, leupeptin and 1% aprolin, pH 7.4], incubated with anti-NaPiT2a IgG (the same as in the immunohistochemistry) at 4°C overnight and then precipitated with Protein-G (Amersham Pharmacia, Little Chalfont, UK), as reported previously (13). These samples were subjected to SDS-
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PAGE and transferred to PVDF membranes. The anti-NaPiT2a IgG was applied to PVDF membranes, followed by a second reaction with peroxidase-labeled goat anti-rabbit IgG (Dako). The positive signals were visualized on PVDF membranes using a commercial kit (ECL system, Amersham Pharmacia) (12, 13).

Statistical analysis. A Student’s t-test, ANOVA analysis, or Mann Whitney U-test was used to compare the group means and a value of $P < 0.05$ was considered to be significant.

RESULTS
Progression of renal damage and dysfunction in LPS-treated mice
We first determined whether the single injection of LPS (6 mg/kg, i.p.) successfully induced the renal damages in mice. Indeed, BUN levels rapidly increased within 12 h, reaching a peak value at 36 h post-LPS treatment (Fig. 1A). In the present protocol, more than 60% of mice died within 2 days due to severe multiple organ failure, including ARF and acute respiratory dysfunction (not shown). Thus, we focused on the initial morphological event (< 36 h) after the LPS treatment. The BBM of renal tubules was identified as PAS-stained microvilli in the renal section (Fig. 1B, inset), while there was the gradual loss in the PAS-stained villi between 12 and 36 h post-LPS injection (Fig. 1B, inset), along with the increase in BUN levels. Hypoxia is responsible for the onset of BBM injury (22), but little information is available about the role of renal hypoxia during a natural course of septic ARF. Exogenously administered pimonidazole (Mr = 290.7) is converted to active intermediate specifically under the ischemic conditions (i.e., $O_2$ tension < 10 mmHg) (28). This active intermediate (i.e., hydroxylamine) is covalently bound to thiol-containing proteins in hypoxic cells (28). Thus, we evaluated the renal hypoxia, based on the immunohistochemical detection of pimonidazole (13). In the healthy kidneys, no significant signal of pimonidazole was seen in the renal cortex, while pimonidazole-positive areas became extensive with strong staining between 12 and 36 h post-LPS injection (Fig. 1C). Indeed, pimonidazole-positive signal was detected mainly in cytoplasm (and partially in nucleus) of the renal tubular epithelium, being agreement with previous findings (13, 17), thereby indicating the possible involvement of renal hypoxia in the onset of endotoxin-induced ARF.

![Fig. 1](image-url) Changes of renal dysfunction, tubular apical injury and hypoxia in septic mice. A. Changes of BUN levels before and after the LPS treatment (6 mg/kg, i.p.). B. Alteration of tubular injury score and representative microphotographs of PAS-stained renal sections (x80). Inset: expanded features of BBM. C. Alteration of renal tubular hypoxia after the LPS challenge, as evidenced by immunohistochemistry of pimonidazole (x50). All data are shown as mean ± SD (n = 4 each group). Statistical analysis: *; $P < 0.05$, and **; $P < 0.01$ compared with pretreatment group. For the abbreviations, see text.
Decrease in the apical NaPiT2a of proximal tubules during progression of septic ARF in mice

Using the successful model of septic ARF, we examined if NaPiT2a expression is altered during the manifestation of renal dysfunction. Renal immunohistochemistry revealed that there was an extensive NaPiT2a-positive signal at the microvillar areas in normal kidneys (Fig. 2A). In contrast, a gradual reduction in the NaPiT2a-positive areas occurred between 12 and 36 h after LPS injection (Fig. 2A). Indeed, there was a significant decrease in the tubular NaPiT2a staining score (Pre: 56.8 ± 6.2% vs. 36 h: 17.5 ± 4.4%, \( P < 0.01 \)) (Fig. 2B). This result was reproduced by immune blot analysis: NaPiT2a was detected in the normal kidneys as an abundant band, while these NaPiT2a-positive signals became weaker at 36 h post-LPS injection (Fig. 2C).

Usefulness of NaPiT2a as a negative marker of renal dysfunction and damage

To evaluate the usefulness of apical NaPiT2a as the predictor of ARF, we determined the relationship between tubular NaPiT2a expression and renal dysfunction or damage. Multiple linear regression analysis revealed that tubular NaPiT2a staining score negatively correlated with the BUN levels, as shown in the scatter-graph (\( R^2 = 0.903 \)) (Fig. 3A). Furthermore, there was a negative correlation between the NaPiT2a-positive ratio (%) and tubular injury score in the LPS-treated mice (Fig. 3B). These findings supported our hypothesis that the decline of NaPiT2a expression reflects well the degree of renal dysfunction and apical damage during septic ARF.

Differential expressions of NaPiT2a and Hypoxi-probe-1 in the renal tubules of septic mice

Considering that hypoxia is able to cause BBM injury (20, 22), we hypothesized that tubular hypoxia may elicit the loss in NaPiT2a at the tubular apical surfaces. Double immunofluorescence revealed that NaPiT2a expression was extensive at the microvillar areas of proximal tubules, while no significant signal of the hypoxic marker was seen in the NaPiT2a-rich tubules (Fig. 4A). Inversely, the NaPiT2a-positive signal became faint in the pimonidazole-positive (i.e., hypoxic) tubules, as observed at 36 h after the LPS challenge (Fig. 4B), hence indicating the possible involvement of hypoxic stress in the decrease of apical NaPiT2a, as suggested elsewhere (6, 22).

Lastly, we examined the reversibility of NaPiT2a, focusing on the regenerating stage of ARF (i.e., 72 h post-LPS challenge). As a result, the loss in the apical NaPiT2a-positive signal was restored, accompa-
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...nied by a decrease in pimonidazole-positive signals (Fig. 4C). At this time-point, the BUN levels almost returned near the normal range (20–30 mg/dL). These data indicate that the detection of changes in apical NaPiT2a is helpful in determining the diagnosis of renal dysfunction, although it is still difficult to determine if tubular hypoxia is a cause or result of ARF.

![Graph A](image1.png)  
**Fig. 3** Scatter-graphs showing correlation between renal apical transporter expression and functional or morphological damages in septic mice. **A.** Relationship between the tubular NaPiT2a expression (%) and BUN levels. **B.** Link of NaPiT2a expression to tubular injury score, as evaluated in the PAS-stained renal sections. Multiple linear regression analyses were undertaken in these scatter-graphs for evaluation of the relationship between renal histological and biochemical parameters (n = 16). Symbols are: (■) Pretreatment; (●) 12 h; (▲) 24 h; and (◆) 36 h post-LPS challenge.

![Graph B](image2.png)

**Fig. 4** Differential localization of NaPiT2a and pimonidazole in septic kidneys. **A.** Localization of NaPiT2a and pimonidazole in normal mice. The NaPiT2a expression is evident in a form of microvillar clusters along the non-hypoxic areas. **B.** The loss in NaPiT2a expression in reciprocal to expansion of hypoxic areas, as noted in an ARF-progressing phase (i.e., 36 h after LPS challenge). **C.** Restoration of the apical NaPiT2a expression in a regenerating phase (i.e., 72 h post-LPS). Fluorescence image: NaPiT2a (green); and pimonidazole (red).
DISCUSSION

So far, electron microscopic studies have revealed that the loss of apical microvilli is a histological hallmark of ARF (1, 20). In our ARF model, PAS-stained BBM areas disappeared in conjunction with the increase in BUN levels, suggesting a cause-and-result relationship between apical injury and renal dysfunction, as reported (9, 19). There are several types of ion transporters on tubular apical surface for regulating electrolyte balance (7, 8, 18, 26), but no information is available to determine if apical molecules predict a prognosis of ARF. Using the mouse model for human ARF, we found that apical NaPiT2a expression became faint during the progression of renal dysfunction and hypoxia (i.e., from 12 to 36 h post-LPS challenge), while the reverse occurred in the regenerating period (i.e., 72 h post-LPS challenge). Based on these data, we conclude that the dynamism of renal NaPiT2a expression sheds light on identifying the pathological status during septic ARF.

It is critical to discuss the possible events, required for the loss in apical NaPiT2a during septic ARF. The decrease in PAS-stained microvilli indicates two possibilities: One is the desquamation of the BBM itself, caused by hypoxic stress (6, 22). Another is the down-regulation of functional molecules, including NaPiT2a, irrespective of the underlying cause of ARF. Indeed, tubular NaPiT2a expression levels were mildly decreased in our ARF model, even if BBM was still intact at the earlier phase of septic ARF (not shown), suggesting that NaPiT2a-staining, rather than PAS-staining, might be useful for early detection of ARF-related pathological events during sepsis. With regard to this, several lines of evidence demonstrated that fibroblast growth factor-23 (FGF-23) down-regulates NaPiT2a production both in vitro (3) and in vivo (10, 23). For example, FGF-23-overexpressed mice manifested the decreased levels of apical NaPiT2a (10). Since plasma FGF-23 levels are known to be elevated during renal diseases (33), circulating FGF-23 may elicit the decreased localization of NaPiT2a. In our model, tubular hypoxia may be primarily responsible for the BBM injury, as reported (6, 22), but we cannot exclude that BBM injuries also enhance the tubular hypoxia via the possible decline of GFR (e.g., tubulo-glomerular negative feedback system), as suggested (5, 20). Although further studies are required to address this notion, it is possible that not only the hypoxia-induced desquamation of BBM itself but also the down-regulation of NaPiT2a production would lead to the decreased expression of NaPiT2a during septic ARF.

We next determined whether the loss in apical NaPiT2a expression is reversed during sepsis, although the initial focus was on the early stage of ARF. The follow-up study revealed that the increase in the renal NaPiT2a score occurred at 72 h post-LPS challenge. This was accompanied by an improvement in tubular hypoxia, indicating a reversibility of reduced NaPiT2a expression. Renal hypoxia causes microvillar damage in proximal tubules at the early stage, as reported (9, 22), and vice versa in the advanced stage via a tubulo-glomerular feedback (5, 25). Although growth factors and extracellular matrix have been implicated in a process of renal tubular morphogenesis (11, 32), it is still unclear which of molecules is critically involved in BBM repair (including re-induction of apical cotransporters). Thus, our ARF model is useful for elucidating the molecular mechanism whereby the loss in NaPiT2a could be restored during a recovery from renal hypoxia.

As hypoxia is causative for the renal tubular BBM desquamation (6, 22), it is important to note the up-stream events leading to hypoxia and apical injuries. In our ARF model, glomerular P-selectin expression and fibrin deposition became evident at an earlier phase (<12 h), followed by the onset of tubular hypoxia and apoptosis (manuscript in preparation). Overall, we predicts the possible sequence of events as follows: (a) glomerular tuft thrombosis occurs, associated with the systemic inflammatory response syndrome, as reported (30); (b) renal blood flow and GFR decrease in response to tuft thrombosis; (c) under such a hypoxic condition, desquamation of BBM and/or down-regulation of NaPiT2a (e.g., by FGF-23) are enhanced; and (d) the loss in BBM leads to a further decrease in GFR via a tubulo-glomerular negative-feedback (5, 25). Considering that glomerular thrombosis triggers the onsets of tubular BBM injury, hypoxia and renal dysfunction (20, 30), how to inhibit the production or function of thrombosis-priming factors (such as tumor necrosis factor-α and interleukin-6) would be critical for maintenance of apical surface structure as well as for prevention of renal tubular hypoxia, especially at the earlier phase of septic ARF.

By the way, it is interesting to note that hypophosphatemia occurs in more than 80% of septic patients (2, 16, 24). Indeed, hypophosphatemia, as defined by a serum level <1.0 mg/dL causes severe respiratory failure, myocardial depression and seizures (24). Thus, molecular mechanisms whereby
hypo-phosphatemia occurs in septic patients should be discussed. The onset of hypo-phosphatemia is evident in NaPiT2a-knockout mice (4). Furthermore, hypo-phosphatemia was also identified in several strains of mutant mice when microvillar NaPiT2a expression was suppressed (15, 21, 23). This indicated the pivotal role of apical NaPiT2a for re-absorption of urinary Pi. In other words, our finding that the decrease in apical NaPiT2a is associated with the progression of ARF might explain possible hypo-phosphatemia, as noted in patients with septic ARF (2, 16, 24). Although further studies are required to confirm the reciprocal balance between decreased NaPiT2a and hypo-phosphatemia, it is likely that maintenance of Pi-absorbing transporters on tubular apical surfaces is important as a strategy to minimize the onset of hypo-phosphatemia, a common event during septic ARF.

In this study, we for the first time demonstrated the usefulness of NaPiT2a as the predictor of renal dysfunction and apical damage in septic ARF. Since NaPiT2a is critical for re-absorption of urinary Pi (7, 23, 29), this marker may be helpful to predict not only renal damage but also phosphate metabolism. Given that plasma Pi levels are known to markedly decrease in septic patients (2, 16, 24), the loss in renal tubular NaPiT2a may provide a rationale to explain why hypo-phosphatemia occurs in septic humans (2, 24) and animals (27). Although further study is required to extend the value of NaPiT2a as a predictor in other kidney diseases, the present findings may provide new diagnostic approaches to understanding pathological status during sepsis.

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REFERENCES