Downregulation of renal type IIa sodium-dependent phosphate cotransporter during lipopolysaccharide-induced acute inflammation

Shoko Ikeda,1* Hironori Yamamoto,3,4* Masashi Masuda,1 Yuichiro Takei,1 Otoki Nakahashi,1 Mina Kozai,1 Sarasa Tanaka,1 Mari Nakao,1 Yutaka Taketani,1 Hiroko Segawa,2 Masayuki Iwano,4 Ken-ichi Miyamoto,2 and Eiji Takeda1

1Department of Clinical Nutrition, Institute of Health Biosciences, University of Tokushima, Karamoto-cho, Tokushima, Japan; 2Department of Molecular Nutrition, Institute of Health Biosciences, University of Tokushima, Kuramato-cho, Tokushima, Japan; 3Department of Health and Nutrition, Faculty of Human Life, Jin-ai University, Ohde-cho, Echizen City, Fukui, Japan; and 4Division of Nephrology, Department of General Medicine, Faculty of Medical Sciences, University of Fukui, Fukui, Japan

Submitted 21 August 2013; accepted in final form 29 January 2014

Electrolyte disorders in critically ill patients during a stay in the intensive care unit (ICU) are a significant concern. Hypocalcemia and hyperparathyroidism are common findings in critically ill patients. These disorder alterations are related to the severity of disease and poor prognoses (8, 24, 49). Abnormal serum inorganic phosphate (Pi) levels have also been reported in critically ill patients such as sepsis and are associated with illness severity or higher mortality (3, 41).

Pi plays a critical role in mineral metabolism and diverse cellular functions involving intermediary metabolism and energy transfer mechanisms. Serum Pi levels are normally maintained within a narrow range through a complex interplay among intestinal absorption, exchange with intracellular and bone storage pools, and renal tubular reabsorption. Renal proximal tubular reabsorption is thought to be the primary mechanism for regulating Pi metabolism and mainly mediated by type II sodium-dependent phosphate (NaPi2a) cotransporters (NaPi2a and NaPi2c) in the brush border membrane (BBM) (6, 26, 42). The importance of NaPi2a and NaPi2c has been demonstrated in gene-deficient mice (5, 36, 38). Type III sodium-dependent phosphate cotransporters (PiT1 and PiT2) are known to be Pi housekeeping transporters. However, it has been reported that PiT2 is a novel mediator of Pi reabsorption in the BBM (6, 44). Intact parathyroid hormone (iPTH) and intact fibroblast growth factor 23 (iFGF23) have been known as Pi-regulating hormones, and both iPTH and iFGF23 downregulate the BBM expression of NaPi2a and NaPi2c and reduce Pi reabsorption, whereas these factors have opposing effects on renal vitamin D metabolism (6, 10, 12). We have previously reported that renal BBM NaPi2a and NaPi2c expression is posttranslationally downregulated by iPTH, iFGF23, and a high Pi diet (16, 28, 34, 39), and 1,25-dihydroxyvitamin D3 [1,25(OH)2D3], thyroid hormone, and all-trans retinoic acid transcriptionally upregulate NaPi2a gene expression in renal proximal tubular cells (15, 25, 43, 46). However, the regulation of Pi homeostasis during inflammation, such as sepsis, has not been examined.

In vivo studies have reported that the injection of inflammatory cytokines in mice induced hypophosphatemia (3), and Escherichia coli ETx induced phosphaturia associated with hypocalciuria and plasma iPTH elevation in rats (27). However, the regulatory mechanisms of these alterations, including other Pi regulators like Npt2a, Npt2c, and iFGF23, are still unclear. In this study, we investigated the regulation of Pi metabolism and its mechanism in the context of acute systemic inflammation, using animal models treated with lipopolysaccharide (LPS).

Materials and Methods

Animal experiments. Male C57BL/6J mice aged 7–9 wk and male Wistar rats aged 10 wk with parathyroidectomy (PTX) or sham surgery and male C3H/HeN and C3H/HeJ mice aged 7 wk were purchased from Japan SLC (Hamamatsu, Japan). All animals were maintained with 12:12-h light-dark cycles with free access to water and normal diet under pathogen-free conditions. PTX or sham oper-
Downregulation of Npt2a by LPS

Treatment. LPS (lipopolysaccharides from Escherichia coli 055: B5; Sigma, St Louis, MO) was diluted in autoclaved saline. Mice were anesthetized with diethyl ether and injected intraperitoneally with LPS or saline at 0–20 mg/kg body weight (BW). Rats were injected intravenously with LPS or saline at 20 mg/kg BW without anesthesia. All animals were killed at each time point, and blood, urine, and organ samples were collected for analysis. For the time-course study, samples were analyzed 0.5, 1.5, 3, 6, and 9 h after LPS infusion. TNF-α (recombinant human TNF-α, PHC3016; Invitrogen, Carlsbad, CA) was diluted in autoclaved saline. Mice were anesthetized with diethyl ether and injected intraperitoneally TNF-α or saline at 10 μg/mouse. Three hours after injection, all mice were killed, and blood, urine, and organ samples were collected for analysis.

Blood and urine parameters. Plasma and urine concentrations of Pi and calcium (Ca) were determined using the Phospha-C test (Wako, Osaka, Japan) and the calcium-E test (Wako). Urinary creatinine (Cre) and calcium (Ca) were determined using the Phospha-C test (Wako, Osaka, Japan). Serum blood urea nitrogen (BUN) was measured by the Jaffe method (the creatinine-Wako). Urinary calcium (Ca) and creatinine (Cre) were determined using the Phospha-C test (Wako, Osaka, Japan), and the calcium-E test (Wako). Urinary Pi was determined using the Jaffe method (the Pi-Wako). Urinary Pi excretion was calculated as urinary Pi concentration divided by urinary Cre concentration (Urine Pi/Cre).

RESULTS

Pharmacological effect of LPS. First, we injected LPS (20 mg/kg BW) or saline in mice and analyzed after 3 h. Plasma Pi, levels and urinary Pi excretion significantly increased by LPS injection when compared with saline-injected control group (Fig. 1, A and B). Although plasma Ca levels did not substantially change, urinary Ca excretion was markedly decreased in LPS-treated mice compared with control mice (Fig. 1, C and D). In addition, plasma iPTH and iFGF23 values were significantly increased by LPS injection (Fig. 1, E and F). We also confirmed a significant induction of serum TNF-α levels following LPS stimulation (Fig. 1G). Moreover, serum BUN and Cre levels in LPS group were higher than control group (Fig. 1, H and I).

Western blot analysis. Brush border membrane vesicles (BBMVs) were prepared from mouse or rat kidney by the Ca2+ precipitation method, as described previously (43). BBMVs protein samples were heated at 95°C for 5 min in sample buffer in the presence of 5% (vol/vol) 2-mercaptoethanol and subjected to SDS/PAGE. The separation was performed on rats before 2 wk of LPS treatment. The gel was stained with Coomassie blue and subjected to immunostaining, serial sections (5-μm thick) were incubated with affinity-purified anti-Npt2a antibodies (1:2,500), anti-Npt2c antibodies (1:2,500), and anti-Ca2+/H+ ATPase antibody (1:5,000) for 1 h at room temperature. Immunoreactivity was detected by treatment with ImmPACT DAB peroxidase substrate (Vector Laboratories, Burlingame, CA).

Immunohistochemical analysis. Immunostaining of mouse kidney sections was performed as described previously (37). For immunostaining, serial sections (5-μm thick) were incubated with affinity-purified anti-Npt2a antibodies (1:1,000) overnight at 4°C. Sections were then treated with Envision ( Dako) for 1 h at room temperature. Immunoreactivity was detected by treatment with ImmPACT DAB peroxidase substrate (Vector Laboratories, Burlingame, CA).

Statistical analysis. Results were presented as the means ± SE. Statistical significance was determined by Student’s unpaired t-test or one-way ANOVA followed by the Tukey-Kramer post hoc test. Time-course data were analyzed by two-way ANOVA followed by Tukey-Kramer test. A P < 0.05 was considered statistically significant.

RESULTS

Plasma and urinary Pi levels and its regulating hormone are dramatically altered by LPS. First, we injected LPS (20 mg/kg BW) or saline in mice and analyzed after 3 h. Plasma Pi, levels and urinary Pi, excretion significantly increased by LPS injection when compared with saline-injected control group (Fig. 1, A and B). Although plasma Ca levels did not substantially change, urinary Ca excretion was markedly decreased in LPS-treated mice compared with control mice (Fig. 1, C and D). In addition, plasma iPTH and iFGF23 values were significantly increased by LPS injection (Fig. 1, E and F). We also confirmed a significant induction of serum TNF-α levels following LPS stimulation (Fig. 1G). Moreover, serum BUN and Cre levels in LPS group were higher than control group. Serum BUN levels in control and LPS groups were 19.3 ± 0.4 and 32.0 ± 1.9 mg/dl, respectively (P < 0.05). Serum Cre levels in
Table 1. Biochemical parameters in plasma and urine after injection of different dose levels of LPS

<table>
<thead>
<tr>
<th>LPS, mg/kg BW</th>
<th>Plasma Pi, mg/dl</th>
<th>Plasma Ca, mg/dl</th>
<th>Urine Pi/Cr</th>
<th>Urine Ca/Cr</th>
<th>Plasma iPTH, pg/ml</th>
<th>Plasma iFGF23, pg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>7.2 ± 0.4</td>
<td>8.1 ± 0.1</td>
<td>0.9 ± 0.2</td>
<td>0.18 ± 0.03</td>
<td>27.4 ± 12.3</td>
<td>82.6 ± 4.4</td>
</tr>
<tr>
<td>2</td>
<td>9.1 ± 0.9</td>
<td>7.9 ± 0.1</td>
<td>1.9 ± 0.3</td>
<td>0.09 ± 0.02</td>
<td>109.3 ± 16.1*</td>
<td>161.7 ± 12.5*</td>
</tr>
<tr>
<td>5</td>
<td>9.4 ± 0.6</td>
<td>8.2 ± 0.1</td>
<td>4.1 ± 0.9*</td>
<td>0.09 ± 0.01</td>
<td>97.0 ± 12.1*</td>
<td>160.8 ± 8.4*</td>
</tr>
<tr>
<td>10</td>
<td>9.7 ± 0.9</td>
<td>8.1 ± 0.1</td>
<td>4.5 ± 1.4*</td>
<td>0.11 ± 0.01*</td>
<td>99.6 ± 21.0*</td>
<td>172.6 ± 7.5*</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SE; n = 5–6. Mice were intraperitoneally injected with LPS [2, 5, and 10 mg/kg body weight (BW)] or saline (control). Plasma and urine were collected 3 h after injection. LPS, lipopolysaccharide; Pi, inorganic phosphate; Cre, creatinine; iPTH, intact parathyroid hormone; iFGF23, intact fibroblast growth factor 23. *P < 0.05 vs. control.

LPS downregulated Npt2a protein levels in mouse kidneys. Next, we analyzed renal NaPi cotransporter family protein and gene expression to elucidate the contribution of these transporters to hyperphosphaturia in LPS-treated mice. Western blot analysis of BBM proteins from kidney showed a decline in immunoreactive Npt2a protein to 40% of control levels at 3 h after LPS injection, whereas the protein abundance of Npt2c and Npt1 did not materially change (Fig. 3A). Real-time PCR analysis was used to measure renal Npt2a, Npt2c, Npt1, and PiT2 mRNA levels. However, there were no significant differences in the mRNA levels of these genes when compared with the control group at 3 h after LPS (Fig. 3B). Furthermore, immunohistochemical analysis showed that Npt2a levels were

Table 2. Effects of LPS on plasma and urinary P1, levels and plasma iPTH levels in C3H/HeN and C3H/HeJ mice

<table>
<thead>
<tr>
<th>C3H/HeN</th>
<th>C3H/HeJ</th>
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<tr>
<td>Control</td>
<td>LPS</td>
</tr>
<tr>
<td>Plasma Pi, mg/dl</td>
<td>6.3 ± 0.3</td>
</tr>
<tr>
<td>Urine Pi/Cr</td>
<td>1.5 ± 0.5</td>
</tr>
<tr>
<td>Plasma iPTH, pg/ml</td>
<td>95.1 ± 2.4</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SE; n = 5–6. Mice were intraperitoneally injected with LPS (20 mg/kg BW) or saline (control). Plasma and urine were collected 3 h after treatments. *P < 0.05 vs. control.
markedly reduced in the apical membranes in LPS-treated mice compared with control mice (Fig. 3C).

**TNF-α induces significant increases in plasma iPTH levels and decrease renal Npt2a expression in mouse.** TNF-α is one of the major LPS-induced proinflammatory cytokines (31). To investigate the effect of TNF-α on renal Pi metabolism, we treated mice with TNF-α and analyzed plasma, urine parameters, and renal Npt2a and Npt2c expressions at 3 h after cytokine injection. As well as results of LPS experiments shown in Fig. 1 and Table 1, we observed that TNF-α significantly increased plasma iPTH levels and urinary Pi, excretion and decreased urinary Ca excretion. However, plasma Pi, Ca, and iFGF23 levels did not substantially change (Fig. 4). We also found that TNF-α downregulated renal BBM expression of Npt2a protein to 40% of control levels while Npt2c levels remained unaltered (Fig. 5).

**iPTH is an important regulator in Pi metabolism during LPS-induced inflammation.** To further elucidate the mechanism involved in LPS-induced hyperphosphaturia, we focused on the significant increase of plasma iPTH levels because iPTH is one of the main regulators of renal Pi transporters. Therefore, we investigated the role of iPTH on Pi metabolism in LPS-induced acute inflammation using PTX rats. As shown in Fig. 6, we observed that the responsiveness of all parameters to LPS in sham-operated rats was similar to that of mice in our previous experiments. On the other hand, in the PTX group, surprisingly, plasma and urine levels of Pi and Ca did not change. However, serum TNF-α levels were remarkably increased in both LPS groups (sham, 357.9 ± 81.8 pg/ml; PTX, 1,489.9 ± 260.7 pg/ml), and it was not detectable in both control groups. Interestingly, plasma iFGF23 levels significantly increased in LPS-treated PTX rats compared with controls (Fig. 6F). Moreover, Western blot analysis of kidney BBM proteins revealed that the downregulation of renal Npt2a gene expression by LPS was abolished in PTX rats (Fig. 7).

**DISCUSSION**

The present study demonstrated altered Pi metabolism and regulation of renal NaPi cotransporter protein expression during LPS-induced acute inflammation. After LPS injection, plasma Pi increased relative to controls over time. An increase in plasma Pi levels was also observed in the cecal ligation and

![Fig. 3. Effects of LPS on the expression of NaPi cotransporters in mouse kidney.](http://ajprenal.physiology.org/)

![Fig. 4. TNF-α induces significant increase in plasma iPTH levels.](http://ajprenal.physiology.org/)

![Fig. 5. TNF-α downregulates expression of Npt2a in mouse kidney.](http://ajprenal.physiology.org/)
Fig. 6. Plasma and urine levels of Pi, and Ca did not change in parathyroidectomized (PTX) rats. Rats with PTX or sham surgery were intravenously injected with LPS (20 mg/kg BW) or saline (control). Plasma and urine were collected 3 h after injection. A: plasma P, B: urine P/Cr. C: plasma Ca. D: urine Ca/Cr. E: plasma iPTH. F: plasma iFGF23. Values are expressed as means ± SE. *P < 0.05 vs. control (n = 4–6).

puncture model rat (33). As reported previously for rats (27), we confirmed that plasma iPTH levels and urinary Pi, excretion significantly increased following LPS treatment in mice. In addition, we found that plasma iFGF23 levels were transiently induced 3 h after LPS injection despite the persistent elevation of P, and iPTH plasma levels. iPTH and iFGF23 are well known for their roles as phosphaturic hormones in kidneys, and their production is in response to elevated serum Pi levels (11, 29). Therefore, the elevation of iPTH and iFGF23 in plasma is strongly associated with the increase of urinary Pi, excretion related to the reduction of Npt2a and Npt2c proteins in BBM. A complicated mechanism for the iPTH-dependent endocytosis of Npt2a and Npt2c has been reported that iPTH could reduce the Npt2a protein within 2 h, whereas iFGF23 required ~9 h (17, 39, 40). Indeed, LPS-treated mice showed a decrease of renal Npt2a protein levels that was not reflected in mRNA levels. In general, following stimulation of the PTH receptor, a decrease in apical Npt2a abundance occurs within minutes without changes in mRNA levels. In contrast, Npt2c requires several hours for iPTH-induced reduction (6, 17, 39). Accordingly, these reports will support the case of unchanging renal Npt2c expression at 3 h after LPS injection.

LPS treatment is also known as inducer of acute kidney injury (AKI). It has been reported that LPS administration induced a gradual increase in serum BUN and Cre concentrations that peaked at 18 h; BUN and Cre concentrations in control and LPS groups were 26 ± 1 vs. 90 ± 8 mg/dl and 0.20 ± 0.03 vs. 0.51 ± 0.05 mg/dl, respectively. In addition, renal histology revealed mild morphological damage at 18 h (45). In this study, the increases of serum BUN and Cre levels at 3 h after LPS treatment were not severe: BUN and Cre levels in control and LPS groups were 19.3 ± 0.4 vs. 32.0 ± 1.9 mg/dl and 0.08 ± 0.004 vs. 0.10 ± 0.007 mg/dl, respectively. Therefore, we consider that effects of AKI on Npt2a down-regulation at 3 h after LPS injection might be relatively small. LPS-induced hyperphosphaturia in the acute phase would be mediated by iPTH-dependent Npt2a protein reduction in kidney BBM. Previous reports using other animal models of AKI induced by ischemia-reperfusion or unilateral ureteral obstruction showed the reduction of kidney function and the down-regulation of renal Npt2a expression at 24 h after injuries (20, 23). In the late phase (more than 18 h), advanced AKI possibly is more contributive to Npt2a protein suppression.

1,25(OH)2D3 is also central to P, homeostasis, and its production is regulated by both iPTH and iFGF23 (10). In addition, 1,25(OH)2D3 is known to regulate immunity (2). However, the effect of LPS on 1,25(OH)2D3 metabolism in vivo remains unknown. In this study, plasma iPTH and iFGF23 levels were significantly increased by LPS while plasma 1,25(OH)2D levels remained unchanged until 9 h after of LPS injection. Moreover, we also indicated that LPS significantly decreased urinary Ca excretion, in agreement with a previous report (27). iPTH regulates renal NaPi cotransporters in addition to renal Ca2⁺ transport proteins, including the epithelial Na⁺/Ca2⁺ channels, transient receptor potential, vanilloid, member 5 (TRPV5), calbindins, and the Na⁺/Ca2⁺-exchanger (NCX1) (21). In fact, we confirmed an increase of TRPV5 mRNA levels by LPS (data not shown).

Importantly, a significant increase in plasma iPTH levels and a decrease in Npt2a protein expression in kidney BBM were also shown in TNF-α-treated mice. These results suggest that TNF-α is an important mediator for plasma iPTH elevation and renal Npt2a downregulation during LPS-induced acute inflammation. Although TLR4 and TNF receptors (TNFR1 and TNFR2) are expressed in the kidney (9, 32), the direct effects of LPS and TNF-α on the expression of Npt2a, Npt2c, and other NaPi cotransporters in renal proximal tubular cells have not been conclusively demonstrated. To better understand the regulation of renal Npt2a gene expression by LPS and/or TNF-α, we performed LPS injections in PTX rats. Surprisingly, PTX rats showed no response of P, metabolism and renal Npt2a gene expression to LPS. These results suggested that the role of iPTH is critical for altering P, levels in plasma, urine, and Npt2a downregulation during LPS-induced acute inflam-
mation. Furthermore, it has been reported that LPS and TNF-α are involved in inflammatory bone loss (1) and that stimulation of endogenous iPTH levels in healthy men caused an acute increase of the bone resorption marker (48). Based on these findings, we generated the hypothesis that the direct or indirect elevation of iPTH by LPS and/or TNF-α might affect kidney and bone, causing renal Npt2a downregulation and bone resorption, resulting in hyperphosphatemia and/or hyperphosphaturia. Interestingly, one past report suggested that parathyroid hormone related protein (PTHrP) contributed to LPS-induced mortality (13). Considering that iPTH and PTHrP act via the same receptor (PTH/PTHrP receptor) (14), iPTH may also contribute to mortality and the appropriate control of iPTH may improve the mortality associated with sepsis.

The production of iPTH is mainly controlled by P₃, Ca²⁺ and its sensing receptor (CaSR), and 1,25(OH)₂D₃ and its receptor (VDR) (4, 19). Based on the results of time course study, LPS increased plasma P₃ levels at 1.5 h without elevation of plasma iPTH levels. On the one hand, blood ionized Ca levels in the LPS group were significantly lower than in the control group at 3 h but not 0.5 or 1.5 h, and plasma 1,25(OH)₂D₃ levels remained unchanged until 9 h after LPS treatment. These data suggest that LPS increases plasma iPTH levels by mediating the alteration of both P₃, and ionized Ca concentrations in blood. In addition, it has been shown that LPS and inflammatory cytokines dramatically alter the compartmental fluxes of Ca²⁺ (35) and vitamin D metabolism in nonrenal cells such as endothelial and immune cells (30, 47). The direct actions of LPS and TNF-α on iPTH production mediated by TLR4 and TNFRs in the parathyroid gland should also be considered. Furthermore, we encountered another interesting finding in that plasma iFGF23 levels significantly increased relative to controls in both sham and PTX rats. It has recently been demonstrated that iPTH and 1,25(OH)₂D₃ increase serum iFGF23 and FGF23 mRNA levels in human and animals. Moreover, in vitro studies have demonstrated that iPTH directly regulates FGF23 expression (7, 18, 22). Thus iPTH is known to be a positive regulator of iFGF23; however, our study suggested that iPTH is not necessary for the increase of plasma iFGF23 in LPS-induced acute inflammation.

Hyperphosphatemia has been reported in critically ill patients and TNF-α-treated mice (3, 41). However, urinary P₃ excretion has not been assessed in previous studies of patients and animals with inflammation. Therefore, the alteration of P₃ metabolism during systemic inflammation in critically ill patients is still unclear. We have shown that LPS and TNF-α induces the elevation of plasma iPTH levels and the downregulation of renal Npt2a gene expression, resulting increased urinary P₃ excretion in mice. Furthermore, we clarified the important role of iPTH on renal P₃ homeostasis during LPS-induced acute inflammation using PTX rats, and our data strongly suggest that the downregulation of renal Npt2a expression through the induction of plasma iPTH levels mainly contributes to a developmental abnormality of P₃ homeostasis in acute inflammation status.

ACKNOWLEDGMENTS

We thank K. Nakahara, N. Okamoto, M. Sogo, R. Mukohara, M. Tajiri, and N. Yokoyama (Department of Clinical Nutrition, Institute of Health Biosciences, University of Tokushima Graduate School, Tokushima, Japan) for technical assistance and Dr. T. Michigami and Dr. M. Yamazaki (Department of Bone and Mineral Research, Osaka Medical Center and Research Institute for Maternal and Child Health, Osaka, Japan) for helpful discussions and comments.

Present address of M. Masuda: Dept. of Medicine, Div. of Renal Diseases and Hypertension, Univ. of Colorado Denver, Denver, CO 80045.

Present address of Y. Takei: Dept. of Calcified Tissue Biology, Hiroshima Univ. Institute of Biomedical and Health Sciences, Hiroshima, 734-8553, Japan.

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