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, Karamoto-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

Am J Physiol Renal Physiol 2014; doi:10.1152/ajprenal.00474.2013.—The type IIa sodium-dependent phosphate cotransporter (Npt2a) plays a critical role in reabsorption of inorganic phosphate (Pi) by renal proximal tubular cells. Pi abnormalities during early stages of sepsis have been reported, but the mechanisms regulating Pi homeostasis during acute inflammation are poorly understood. We examined the regulation of Pi metabolism and 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 hypercalciumia and plasma iPTH elevation in rats (27). However, the regulatory mechanisms of these alterations, including other P, 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-
atation was performed on rats before 2 wk of LPS treatment. The breeding and handling of all animals in these experiments were approved by the Animal Experimentation Committee of the University of Tokushima.

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 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) was measured with a radioimmunoassay (RIA) kit (TFB, Tokyo, Japan). All animals were killed at each time point, and blood, urine, and organ samples were collected for analysis.

Western blot analysis. Brush border membrane vesicles (BBMVs) were prepared from mouse or rat kidney by the Ca²⁺ precipitation method, as described previously (46). BBMVs protein samples were heat treated at 95°C for 5 min in sample buffer in the presence of 5% (vol/vol) 2-mercaptoethanol and subjected to SDS/PAGE. The separated proteins were transferred by electrophoresis on to PVDF membranes (Immobilon-P; Millipore). The membranes were treated with the secondary antibody, and signals were detected using the ECL plus system (GE Healthcare).

Real-time RT-PCR analysis. Real-time PCR analysis was performed by using the Light Cycler (Roche Diagnostics, Mannheim, Germany). Extraction of total RNA, cDNA synthesis, and real-time PCR were performed as previously described (46). Amplified products were then analyzed using a melting curve, which confirmed the presence of a single PCR product in all reactions (apart from the negative control). The PCR products were quantified by fit-point analysis, and results were normalized to those of β-actin. The primer sequences were as follows: mouse Npt2a (NM_011392: 5'-AGAGCCCCCTCACAAGACTCATC3' and 5'-TACCCCTGGAACATAGTGAAGC3'), mouse Npt2c (NM_080854: 5'-TGAAGAACCTGCCAACTTAATG3' and 5'-AGCACGCTGAGGTAGTCC3'), mouse P1t1 (NM_009198: 5'-CTCTCTCGGACACATCCATG3' and 5'-AACCAGCAAAGGAGAAATC3'), mouse Pit2 (NM_011394: 5'-TTCCTGCAAGTCCACTGC3' and 5'-ACAGGGACGCTAGTTGAA3'), and mouse β-actin (NM_007393: 5'-CTGACCCTGAATGGCCATTGA3' and 5'-CGGTTGTTGAAGGCTTACAAA3').

Immunohistochemical analysis. Immunostaining analysis 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 (+) rabbit peroxidase (Dako) for 1 h at room temperature. Immunoreactivity was detected by treatment with ImmPACT DAB peroxidase substrate (Vector Laboratories, Burlingame, CA).

Statistical analysis. Results are 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 with 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
control and LPS groups were 0.08 ± 0.004 and 0.10 ± 0.007 mg/dL, respectively (P < 0.05). To evaluate the lower dose-effects of LPS, we injected different doses of LPS (0, 2, 5, and 10 mg/kg BW) in mice. As shown as Table 1, plasma P_i levels increased in every dose of LPS, and urinary P_i excretion was significantly elevated in 5 and 10 mg/kg LPS groups. In addition, we observed that LPS significantly decreased urinary Ca excretion and increased plasma iPTH and iFGF23 levels in each dose group. To elucidate the role of TLR-like receptor 4 (TLR4), which is known to be the LPS receptor, we examined the effect of LPS on C3H/HeJ mice, which lack functional TLR4 (31). We found that the plasma P_i and iPTH elevation and increase of urinary P_i excretion by LPS were lost in C3H/HeJ mice (Table 2). A time-course study revealed that plasma levels of P_i and iPTH significantly increased at 3, 6, and 9 h after LPS treatment (Fig. 2, A and B). Interestingly, LPS transiently increased plasma iFGF23 levels at 3 h after injection (Fig. 2C). In contrast, LPS had little effect on plasma 1,25(OH)2D values until 9 h after injection (Fig. 2D). Furthermore, we checked blood ionized Ca levels at early time point (0.5, 1.5, and 3 h), which resulted in ionized Ca levels in LPS group were 1.16 ± 0.01 mmol/L, significantly lower than in control group (1.21 ± 0.04 mmol/L; P < 0.05) at 3 h; there were no difference between control and LPS groups at 0.5 h (1.17 ± 0.01 and 1.19 ± 0.01 mmol/L, respectively) and 1.5 h (1.20 ± 0.01 and 1.23 ± 0.02 mmol/L, respectively).

LPS downregulate Npt2a protein levels in mouse kidneys. Next, we analyzed renal NaPi cotransporter family protein and gene expression to elucidate the contribution of these trans-porters 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 1. Biochemical parameters in plasma and urine after injection of different dose levels of LPS

<table>
<thead>
<tr>
<th></th>
<th>LPS, mg/kg BW</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Plasma P_i, mg/dL</td>
<td>7.2 ± 0.4</td>
<td>9.1 ± 0.9</td>
</tr>
<tr>
<td>Plasma Ca, mg/dL</td>
<td>8.1 ± 0.1</td>
<td>7.9 ± 0.1</td>
</tr>
<tr>
<td>Urine P/Cr</td>
<td>0.9 ± 0.2</td>
<td>1.9 ± 0.3</td>
</tr>
<tr>
<td>Urine Ca/Cr</td>
<td>0.18 ± 0.03</td>
<td>0.09 ± 0.02*</td>
</tr>
<tr>
<td>Plasma iPTH, pg/ml</td>
<td>27.4 ± 12.3</td>
<td>109.3 ± 16.1*</td>
</tr>
<tr>
<td>Plasma iFGF23, pg/ml</td>
<td>82.6 ± 4.4</td>
<td>161.7 ± 12.5*</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; P_i, inorganic phosphate; Cre, creatinine; iPTH, intact parathyroid hormone; iFGF23, intact fibroblast growth factor 23. *P < 0.05 vs. control.

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

<table>
<thead>
<tr>
<th></th>
<th>C3H/HeN</th>
<th></th>
<th>C3H/HeJ</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>LPS</td>
<td>Control</td>
<td>LPS</td>
</tr>
<tr>
<td>Plasma P_i, mg/dL</td>
<td>6.3 ± 0.3</td>
<td>9.1 ± 0.5*</td>
<td>5.7 ± 0.4</td>
<td>6.7 ± 0.4</td>
</tr>
<tr>
<td>Urine P/Cr</td>
<td>1.5 ± 0.5</td>
<td>6.7 ± 0.8*</td>
<td>0.8 ± 0.4</td>
<td>0.7 ± 0.4</td>
</tr>
<tr>
<td>Plasma iPTH, pg/ml</td>
<td>95.1 ± 2.4</td>
<td>151.9 ± 8.8*</td>
<td>86.6 ± 5.2</td>
<td>76.3 ± 4.4</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.

Fig. 2. Time course of changes in plasma biochemistry after LPS injection. Mice were intraperitoneally injected with LPS (20 mg/kg BW) or saline (control). Plasma was collected at 0.5, 1.5, 3, 6, and 9 h after injection. A: plasma P_i; B: plasma iPTH; C: plasma iFGF23; D: plasma 1,25-dihydroxyvitamin D3 [1,25(OH)2D]. Values are expressed as means ± SE. *P < 0.05 vs. control of same time point, #P < 0.05 vs. 0.5-h values in same group (n = 3–4).
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. Mice were intraperitoneally injected with LPS (20 mg/kg BW) or saline (control). Kidneys were collected at 3 h after LPS injection and brush border membrane vesicles (BBMVs) were isolated. A: Western blotting analysis. Each lane was loaded with 25 μg of BBMVs. Actin was used as an internal control. Representative blots from 3 separate experiments and densitometric analysis data are shown. Values are expressed as means ± SE. *P < 0.05 vs. control (n = 3–4). B: quantitative PCR of Npt2a, Npt2c, Npt1, and PiT2 mRNA in mouse kidney. Values are expressed as means ± SE (n = 4). C: immunohistochemical analysis of Npt2a proteins in mouse kidney (large panel: ×100; small panel: ×400). Npt2a, type IIa Na/Pi cotransporter; Npt2c, type IIc Na/Pi cotransporter; Npt1, type I Na/Pi cotransporter; PiT2, type III Na/Pi cotransporter.

**Fig. 4.** TNF-α induces significant increase in plasma iPTH levels. Mice were intraperitoneally injected with TNF-α (10 μg/mouse) or saline (control). Plasma and urine were collected 3 h after injection. A: plasma Pi, B: urine Pi, 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 = 4–5).

**Fig. 5.** TNF-α downregulates expression of Npt2a in mouse kidney. Mice were intraperitoneally injected with TNF-α (10 μg/mouse) or saline (control). Kidneys were collected 3 h after TNF-α injection. BBMVs (25 μg protein) were analyzed by Western blotting and densitometric analysis. Actin was used as an internal control. Values are expressed as means ± SE. *P < 0.05 vs. control (n = 3–4).
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 Pi 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 Cr 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 downregulation 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 downregulation 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 Pi 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)2D3 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 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 Pi metabolism and renal Npt2a gene expression to LPS. These results suggested that the role of iPTH is critical for altering Pi levels in plasma, urine, and Npt2a downregulation during LPS-induced acute inflam-
tion. 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 hyperparathyroidism. 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)₂D 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.

Hypophosphatemia 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.

GRANTS

This work was supported by Grants 16790526, 25282022 (to H. Yamamoto) and 13470013 (to E. Takeda) from the Ministry of Education, Science, Sports and Culture of Japan and the Human Nutritional Science on Stress Control 21st Century Center of Excellence Program (COE), and the Program for Promotion of Basic and Applied Researches for Innovations in Bio-oriented Industry.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES


