The uraemic toxin phenylacetic acid inhibits osteoblastic proliferation and differentiation: an implication for the pathogenesis of low turnover bone in chronic renal failure

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Abstract

Background. A relatively high level of serum parathyroid hormone (PTH) is required to maintain normal bone turnover in patients with chronic kidney disease (CKD). ‘Uraemic toxins’ could cause an impaired response to PTH in bone and result in low turnover bone disease. Since phenylacetic acid (PAA) has been identified as one of the uraemic toxins in patients with CKD and has an inhibiting property of monocyte function, we examined if PAA might inhibit osteoblastic functions in vitro.

Methods. Using mouse osteoblastic MC3T3-E1 cells, we performed BrdU incorporation, real-time PCR, Western blot and stainings to see the effect of PAA.

Results. PAA significantly inhibited proliferation in a dose-dependent manner ranging between 0.5 and 5 mM. PAA reduced osteocalcin mRNA level, alkaline phosphatase activity and osteoblastic mineralization. PAA pre-treatment also decreased both PTH-induced cAMP production and extracellular signal-regulated kinase (ERK) phosphorylation.

Conclusion. PAA, a newly identified uraemic toxin, affects osteoblastic functions such as proliferation, differentiation, mineralization and responsiveness to PTH, indicating that this molecule could play an important role in the pathogenesis of low turnover bone in CKD.

Keywords: chronic renal failure; low turnover bone; osteoblast; parathyroid hormone; uraemic toxin

Introduction

Not only hormonal and metabolic abnormalities such as excessive secretion of parathyroid hormone (PTH) and vitamin D deficiency but also many other unknown factors can be involved in mineral and bone disorders in chronic kidney disease (CKD-MBD). Previous bone histomorphometric studies suggested suppression of bone turnover in many of the patients with end-stage renal disease (ESRD) [1–3] and an existence of skeletal resistance against PTH [4–6]. This was a rationale for setting a target range of serum PTH in renal failure patients, and indeed, most patients with a ‘normal’ range of serum PTH represent low turnover bone or adynamic bone.

Dialysis cannot substitute all the renal functions, although its techniques and mechanics have much progressed. In ESRD patients, large numbers of molecules would be found at very high concentrations, whereas many substances are deficient in them. These molecules are called uraemic retention solutes or uraemic toxins, if they are negatively associated with biological functions. The European Uremic Toxin Work Group has successfully defined, determined and evaluated the uraemic toxins, which are classified into small molecules, midsized molecules and protein-bound solutes [7]. Phenylacetic acid (PAA) has recently been identified as one of the uraemic toxins after screening of substances to inhibit inducible nitric oxide synthase (iNOS) production in monocytes from ESRD patients [8]. The plasma PAA level has been reported to be around 3.5 mM in ESRD patients, whereas it is <5 μM in healthy subjects.

We hypothesized that a uraemic toxin might be responsible for the skeletal abnormalities or resistance to PTH by signaling disturbance and/or osteoblastic dysfunction. In this study, we first found inhibitory effects of PAA on osteoblastic cells and on PTH signaling. This observation suggests that
uraemic toxins, such as PAA, might participate in the pathogenesis of CKD-MBD.

Materials and methods

This study was performed to see an effect of PAA in osteoblastic cells. PAA, one of the derivatives of phenylalanine, was dissolved with ethanol to store at −20°C and used at <0.1% of concentration of the stock solution. In order to exclude the possibilities of just a toxic effect, we have examined the other derivatives of phenylalanine, such as homogentisic acid, phenylethylamine and phenylalanine, and found that these substances at the same concentration (1–5 mM) and the vehicle (0.1% of ethanol) did not affect osteoblastic proliferation and differentiation, indicating that an inhibitory action of PAA might be very unique. Thus, we showed results from ethanol treatment (vehicle) as a control of PAA treatment.

Materials

All routine cell culture media were obtained from GIBCO/ BRL (Grand Island, NY). PAA was purchased from Sigma Chemical Co. (St. Louis, MO). Human PTH(1–34) was generously provided by Asahi Kasei Pharma Co. (Tokyo, Japan). All other chemicals were of the highest grade commercially available.

Cell culture

Methodologies for cell culture and staining techniques were basically the same as described previously [9]. MC3T3-E1 cells, which are established as an osteoblastic cell line from normal mouse calvaria, were a generous gift from Dr H. Kodama (Ohu University, Koriyama, Japan). MC3T3-E1 cells were cultured in α-modified minimal essential medium (α-MEM) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT) and 1% penicillin/streptomycin in 5% CO2 at 37°C. The medium was changed twice weekly, and cells were subcultured into 75 cm2 culture flasks by detaching them gently with 0.5% trypsin in PBS after they reached subconfluency.

Cell proliferation

This study was started on the next day of splitting. Cells on a 96-well plate were incubated with vehicle or PAA (0.5, 1, 5 mM) for 24 h. Cell proliferation was measured using the BrdU ELISA from Roche according to the manufacturer’s instructions. For the last 2 h of the 24 h period, the cells were pulsed with BrdU. Absorbance at 450 nm was measured with a microplate reader.

Quantitative real-time PCR

Total RNA (2 μg) was reverse-transcribed with the Omniscript Reverse Transcription Kit (QIAGEN). The sense and antisense primers were designed using the Primer Express Version 2.0.0 (Applied Biosystems Inc.) based on published cDNA sequences. Primer sequences were as follows: mouse osteocalcin (OCN), 5′-TGCTTGTGAGCTATCACG-3′ and 5′-GAGGACAGGAGGATCAAGT-3′; alkaline phosphatase (ALP), 5′-CGATGACACACCTGTCT-3′ and 5′-GGACATACGCGCATACCATG-3′; PTH/PTHrP receptor (PTH1R), 5′-TGGAACAACATCGTGTGCTG-3′ and 5′-TAGGCAATGGCTTTGTGTT-3′; and housekeeping gene, 36B4 (NM007475), 5′-AAACGCGTCTCTGCAATTGTCT-3′ and 5′-CCGAGGCGACAGTGTTG-3′. PCR was performed using 1 μl of cDNA in a 25 μl reaction volume with ABI PRISM7000 (Applied Biosystems Inc.). The double-stranded DNA-specific dye, SYBR Green I, was incorporated into the PCR buffer QuantiTech SYBR PCR (QIAGEN) to allow for quantitative detection of the PCR product. The temperature profile of the reaction was 95°C for 15 min, 40 cycles of denaturation at 94°C for 15 s and annealing and extension at 60°C for 1 min. The size of the PCR product was first verified on a 1.5% agarose gel, followed by melting-curve analysis.

ALP staining

Cultured cells were rinsed in PBS, fixed in 4% formaldehyde, rinsed with PBS, and then overlaid with 1.5 ml of 0.15 mg/ml 5-bromo-4-chloro-3-indolylphosphate plus 0.3 mg/ml nitro blue tetrazolium chloride in 0.1 M Tris–HCl, pH 9.5, 0.01 N NaOH, 0.05 M MgCl2, followed by incubation at room temperature for 6 h in the dark.

Mineralization assay

Mineralization of MC3T3-E1 cells was determined in 6-well and 12-well plates using von Kossa staining and Alizarin red staining, respectively. Confluent cells on a 6-well plate were incubated with vehicle (control) or PAA (0.5, 1 and 5 mM) for 2 weeks in β-glycerophosphate supplemented to α-MEM. The cells were stained with AgNO3 by von Kossa method. At the same time, the other plates were fixed with ice-cold 70% ethanol and stained with Alizarin red (Sigma). For quantitation, cells stained with Alizarin red were destained with 10% Hexa decyclipridinium chloride in 10 mM trisodium phosphate, and then the extracted stain was transferred to a 96-well plate, and the absorbance at 562 nm was measured using a microplate reader.

cAMP measurement

Confluent cells were starved with FBS-free medium with or without PAA overnight. After aspiration of the medium, a new medium with 10−7 M of hPTH(1–34) was added onto the cells. Ten minutes later, the medium was collected and served to the measurement of cAMP. The cAMP production was measured using cAMP EIA kit (Biomedical Technologies Inc., Stoughton, MA) followed by the instruction manual.

Western blotting

In order to investigate phosphorylation levels for extracellular signal-regulated kinase (ERK1/2), we performed Western blotting. MC3T3-E1 osteoblastic cells were incubated with hPTH(1–34) for various durations after overnight serum deprivation and PAA preincubation. Cells were rinsed with ice-cold PBS and scraped on ice into lysis buffer (Cell Signaling Technology) that contained 20 mM
Tris–HCl, pH 7.5, 50 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1% Triton X-100, 2.5 mM sodium phosphate, 1 mM β-glycerophosphate and 1 mM mixed with 1 mM dithiothreitol and a cocktail of protease inhibitors (10 μg/ml each of aprotinin, leupeptin and calpain inhibitor as well as 100 μg/ml Pefabloc). The cell lysates were then sonicated for 30s. Nuclei and cell debris were removed by centrifugation (6000g for 10 min), and the resultant total cellular lysate in the supernatant was electrophoresed by 10% SDS-PAGE and transferred to nitrocellulose membrane (Bio-Rad, Hercules, CA). Immunoblot analysis was performed as described previously [10]. The blots were incubated overnight at 4°C with gentle shaking in phosphorylated ERK1/2 antibodies (Cell Signaling Technology, Beverly, MA) at 1:1000 dilution. The blots were then washed, and the signal was visualized by chemiluminescence system ECL (Amersham, Arlington Heights, IL) according to the manufacturer's protocol. After successful stripping, total ERK signal was obtained from the same membrane. NIH image software, version 1.62, was used to quantify the signal intensity.

**Statistics**

Results are expressed as a mean ± SEM. Statistical evaluations for differences between groups were carried out using one-way analysis of variance (ANOVA) followed by Fisher’s protected least significant difference (PLSD). For all statistical tests, a value of \( P < 0.05 \) was considered to be statistically significant.

**Results**

BrdU incorporation showed that PAA treatment significantly inhibited proliferation in a dose-dependent manner ranging between 0.5 and 5 mM in MC3T3-E1 cells (Figure 1). To examine if PAA affects osteoblastic differentiation, we quantified osteoblastic differentiation markers OCN and ALP mRNA expression using real-time PCR. The mRNA levels of both OCN and ALP were significantly reduced in the cells incubated with 5 mM of PAA for 48 h \((P < 0.01, \text{data not shown})\). Although 0.5 mM and 1 mM of PAA did not reach statistical significance, a dose-dependent inhibition was found. ALP staining was clearly inhibited, when cells treated with 5 mM of PAA were compared with those with vehicle treatment (data not shown). Both Alizarin red and von Kossa stainings demonstrated that osteoblastic mineralization was diminished at the presence of PAA when compared to the absence of PAA (Figure 2). These results strongly suggest an inhibitory effect of PAA on bone formation and mineralization. In addition, no effect was observed in ALP, Alizarin red and von Kossa stainings, when MC3T3-E1 cells were incubated with \( 10^{-8} \)M of hPTH(1–34), compared to those with vehicle treatment (data not shown). This might corroborate the specificity of an effect of PAA, because PTH is considered as a uraemic toxin.

Next, we investigated whether PAA could inhibit PTH action. MC3T3-E1 cells were incubated overnight with and without PAA (0.5 or 5 mM). Measurement of cAMP in the medium was performed 10 min after hPTH(1–34) treatment \((10^{-7} \text{M})\). PTH-induced cAMP production was significantly inhibited by pretreatment with PAA (Figure 3). In order to see whether PAA affects other signaling pathways, we examined ERK phosphorylation, which plays an important role in cell proliferation and is also known as mitogen-activated kinase. After pretreatment with PAA, cells were treated with hPTH(1–34), and then the whole cell lysate was collected to serve for Western blotting. ERK phosphorylation was observed 5 min after PTH treatment, whereas both basal and PTH-stimulated ERK phosphorylation levels were diminished after PAA pretreatment (Figure 4). These data indicate that PAA inhibits PTH or PTH-related peptide (PTHrP) action in MC3T3-E1 cells. To elucidate the possibility of PTH type 1 receptor (PTH1R) downregulation, we measured the mRNA level by real-time PCR. Contrary to our expectation, however, the PTH1R mRNA expression was not altered in these cells incubated with PAA (0.5, 1 and 5 mM) for 48 h (data not shown).

**Discussion**

It is well known that there are many types of mineral and bone abnormalities in patients with ESRD; osteitis fibrosa due to renal hyperparathyroidism, osteomalacia resulted from vitamin D deficiency, aluminum deposition, and mixed type. Precise observation of these clinical entities has unveiled one more skeletal abnormality, which is called ‘adynamic bone’ or ‘low turnover bone’ [1–3]. This type of abnormality is often seen in elderly, diabetes, malnutrition, inflammation,
malignancy and severely ill patients. Adynamic bone or low turnover bone is characterized by insufficient secretion of PTH and by relatively low skeletal response to PTH. In general, however, considerably a high level of serum PTH is required to maintain normal bone turnover in ESRD patients, suggesting that each uraemic patient might have functional abnormality and/or resistance to PTH in the bone. This mechanism is unclear, although a previous study suggested that decreased expression of PTH1R in osteoblasts could be partly involved [11]. We performed the present in vitro study to see if PAA, a newly identified uraemic toxin, might be involved in the pathophysiology of CKD-MBD.

In this study, we demonstrated that PAA has inhibitory effects on osteoblastic functions such as proliferation, differentiation and mineralization. Impaired PTH signaling could be associated with decreased proliferative activity as well as with suppressed ALP activity and mineralization. Our results are compatible with previous findings that serum from dialysis patients inhibits osteoblast mitogenesis [12], reduces cAMP production [13] and modifies insulin-like growth factor (IGF) systems [14]. Because the inhibitory effects of PAA can be seen at concentrations comparable to those in uraemic patients, PAA is probably related to the skeletal
Nitric oxide (NO), which is produced by different isoforms of NO synthase (NOS), has important regulatory effects on bone cell function. The endothelial NOS (eNOS) is crucial for normal osteoblastic function and for the anabolic effects of mechanical stress, estrogen and IGF-I [15–19]. NO derived from the inducible NOS (iNOS) regulates effects of proinflammatory cytokines on bone [20,21]. A recent study on mice with targeted inactivation of the neuronal NOS (nNOS) has shown that nNOS plays a role as a stimulator of bone turnover [22]. Because PAA suppressed iNOS expression in monocytes, PAA might affect NO generation in osteoblasts as well. Thus, PAA’s inhibitory effects in this study could be mediated in part by the NOS pathway.

Previous reports have demonstrated that PTH1R expression is decreased in osteoblasts in ESRD patients, compared with healthy subjects, and that the receptor expression level is much lower in subjects with low bone turnover than in subjects with high bone turnover [11]. This observation can explain the skeletal resistance to PTH in ESRD patients, especially with low turnover bone. Although exact mechanisms by which PTH1R expression is decreased are not clear, cytokines, PTH itself or other uraemic toxins, are thought to be involved in this process. Serum levels of cytokines, such as tumor necrosis factor (TNF) alpha, interleukin 1, interferon alpha and transforming growth factor (TGF) beta, are known to be elevated in ESRD patients [23], and they might be involved in PTH1R downregulation, because they have been shown to inhibit the PTH1R expression in osteoblastic cells [24–27]. It is well recognized that exposure to PTH results in homogenous desensitization and in downregulation of the PTH1R/adenilate cyclase system in osteoblasts [28–33]. Withdrawal of PTH leads to the recovery of PTH1R expression in vitro [29]. However, no change in PTH1R mRNA in liver and kidney was found in uraemic rats after parathyroidectomy [34], although others reported partial normalization [35]. This suggests an importance of uraemic environment other than PTH for PTH1R expression. In this regard, Disthabanchong et al. [13] has shown that uraemic ultrafiltrate decreases PTH1R mRNA levels in UMR106-01 cells. They have also reported that metabolic acidosis is not the cause of PTH1R downregulation in uremia [36]. Recently, Iwasaki et al. [37] have demonstrated that indoxyl sulfate, a major uraemic toxin, plays an important role in the pathogenesis of low turnover bone. They administered an oral adsorbent to renal failure rats to prevent an accumulation of indoxyl sulfate, and showed the recovery from decreased bone formation. In their study, indoxyl sulfate was found to decrease the expression of PTH1R in rat osteoblasts. In the present study, however, PAA treatment did not induce a significant reduction in PTH1R mRNA expression in MC3T3-E1 cells, suggesting that PAA might affect PTH action at a post-receptor level. The similar mechanism was also observed in vitamin D, whose signalling pathways were affected by uraemic ultrafiltrate or uraemic toxins at various cellular levels [38–40]. Thus, uraemic toxins seem to decrease the responsiveness to PTH in osteoblasts not only through the PTH1R downregulation but also through its post-receptor abnormalities, resulting in the development of low turnover bone in ESRD patients.

In conclusion, we showed that PAA, a newly identified uraemic toxin, inhibited the proliferation, differentiation and mineralization of osteoblasts as well as their responsiveness to PTH in this study. Uraemic toxins, including PAA, can lower the bone turnover rate through increased PTH, direct impairment of osteoblastic functions or alteration of the NO status. These mechanisms might, at least in part, explain the skeletal abnormalities in ESRD patients especially with low turnover bone. Thus, low turnover bone in ESRD patients should be managed by the removal of the sources of cytokine production such as inflammatory or malignant diseases, improvement in the nutritional status and elimination of uraemic toxins by sufficient dialysis/filtration or by oral adsorbent administration.

Conflict of interest statement. None declared.

References

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