Indoxyl sulfate induces skeletal resistance to parathyroid hormone in cultured osteoblastic cells

T Nii-Kono[1], Y Iwasaki[2], M Uchida[3], A Fujieda[3], A Hosokawa[3], M Motojima[4], H Yamato[5], K Kurokawa[6] and M Fukagawa[1]

[1]*Division of Nephrology and Dialysis Center, Department of Internal Medicine, Kobe University School of Medicine, Kobe, Japan;* [2]*Department of Health Sciences, Oita University of Nursing and Health Sciences, Oita, Japan;* [3]*KUREHA CORPORATION Co Ltd, Tokyo, Japan;* [4]*Vanderbilt University School of Medicine, Nashville, Tennessee, USA;* [5]*Kureha Special Laboratory Co. Ltd., Fukushima, Japan and* [6]*Division of Health Policy, Research Center for Advanced Science, The University of Tokyo, Tokyo, Japan*

Skeletal resistance to parathyroid hormone (PTH) is well known to the phenomenon in chronic renal failure patient, but the detailed mechanism has not been elucidated. In the process of analyzing an animal model of renal failure with low bone turnover, we demonstrated decreased expression of PTH receptor (PTHR) accompanying renal dysfunction in this model. In the present study, we focused on the accumulation of uremic toxins (UTx) in blood, and examined whether indoxyl sulfate (IS), a UTx, is associated with PTH resistance. We established primary osteoblast cultures from mouse calvariae and cultured the cells in the presence of IS. The intracellular cyclic adenosine 3′,5′ monophosphate (cAMP) production, PTHR expression, and free radical production in the primary osteoblast culture were studied. We found that the addition of IS suppressed PTH-stimulated intracellular cAMP production and decreased PTHR expression in this culture system. Free radical production in osteoblasts increased depending on the concentration of IS added. Furthermore, expression of organic anion transporter-3 (OAT-3) that is known to mediate cellular uptake of IS was identified in the primary osteoblast culture. These results suggest that IS taken up by osteoblasts via OAT-3 present in these cells augments oxidative stress to impair osteoblast function and downregulate PTHR expression. These finding strongly suggest that IS accumulated in blood due to renal dysfunction is at least one of the factors that induce skeletal resistance to PTH.

Kidney International (2007) **71,** 738–743. doi:10.1038/sj.ki.5002097; published online 31 January 2007

Correspondence: *M Fukagawa, Division of Nephrology & Dialysis Center, Kobe University School of Medicine, Kusunoki-cho 7-5-2, Chuo-Ku, Kobe 650-0017, Japan. E-mail: fukagawa@med.kobe-u.ac.jp*

Received 4 January 2006; revised 2 November 2006; accepted 5 December 2006; published online 31 January 2007

Secondary hyerparathyroidism accompanying chronic renal failure is a state of oversecretion of parathyroid hormone (PTH). This is caused by reduced calcium absorption from the intestinal tract due to lowered activity of 1α vitamin D hydroxylase in the kidney, as a result of hyperphosphatemia due to renal dysfunction. Moreover, *in vitro* experiments using cell cultures have confirmed that phosphorus acts directly on parathyroid cells stimulating PTH secretion.[1,2] In recent years, new drugs have been developed that allow control of PTH oversecretion due to secondary hyerparathyroidism. However, a level of PTH three times the normal level is necessary to maintain normal bone turnover in patients with renal failure.[3] In addition, studies of bone biopsies from dialysis patients have reported that patients with subnormal PTH secretion have low bone turnover. These phenomena are recognized as skeletal resistance to PTH associated with renal failure.

The causes of PTH resistance have been investigated and several factors have been reported. Lowered *PTH receptor* (*PTHR*) gene expression in patients with renal failure has been proven by *in situ* hybridization of bone biopsy tissues.[4] Other studies showed that the 7–84 PTH fragment accumulates in the serum of renal failure patients and inhibits receptor binding of the native 1–84 PTH.[5,6] Osteoprotegerin is the decoy receptor of the osteoclast differentiation factor, and osteoprotegerin has been reported to accumulate in the serum of patients with renal failure.[7–9] Accumulation of osteoprotegerin in serum may inhibit the actions of PTH on the bone. Furthermore, bone morphogenetic protein-7 produced in the kidney is a strong osteoblast differentiation factor, and low serum bone morphogenetic protein-7 level has been reported in renal failure.[10] It is speculated that low bone morphogenetic protein-7 level may lead to reduced number of osteoblasts and lowered PTH response.

As a model of skeletal PTH resistance, we have previously developed an animal model of renal failure without secondary hyerparathyroidism and reported our analysis of the bone metabolism in this model.[11] Our results demonstrated that bone turnover is lowered in renal failure with no secondary PTH oversecretion, and confirmed that the

expression of the *PTHR* gene is downregulated in bone. These results indicate that skeletal PTH resistance is present even in renal failure with normal PTH secretion, as is observed in osteitis fibrosa associated with secondary hyerparathyroidism. Furthermore, we demonstrated that bone turnover is lowered dependent on the degree of renal dysfunction. From these results, we speculated that the uremic toxins (UTx) accumulated in blood as a result of lowered renal function may be associated with PTH resistance.

A diversity of UTx accumulates in the blood of patients with impaired renal function. Among them, indoxyl sulfate (IS) is produced as from the metabolism of dietary tryptophan in the body.[12] Studies so far have shown that IS accumulates in blood and promotes the progression of renal dysfunction.[13,14] IS is taken up by cells via the organic anion transporter (OAT) especially present in proximal tubular cells of the kidney,[15–19] and induces cytotoxicity.[16,17] Compared with other UTx, such as β-aminoisobutylic acid, guanidinosuccinic acid, hippuric acid, and *p*-hydroxyhippuric acid, IS at low concentrations produces higher levels of free radicals and stronger cytotoxicity than the other UTx.[17] Moreover, administration of an oral adsorbent that adsorbs indole, the precursor of IS, suppressed the progression of renal failure.[20–22] We recently reported that when rats with renal dysfunction and low bone turnover were administered an oral adsorbent, blood IS level was reduced and osteoblastic cell function was improved.[23] We also observed improvement of the suppressed *PTHR* gene expression in bone, which is implicated as a cause of PTH resistance. The results of these animal experiments strongly suggest that IS exerts some effects on osteoblastic cells or is involved in PTH resistance. We therefore focused on IS as a representative of UTx.

In the present study, we aimed to examine whether IS is associated with skeletal resistance to PTH by studying the effects of IS using cultured osteoblastic cells.

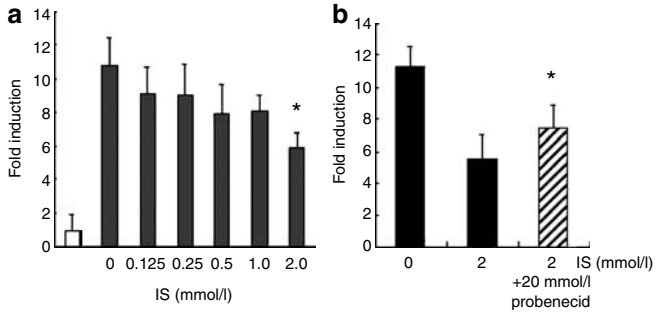

Figure 1 | Effect of IS on PTH-induced intracellular cAMP production in primary osteobalstic cell cultures. (**a**) Primary osteoblastic cell cultures pretreated for 24 h with indicated concentrations of IS were stimulated with PTH for 10 min and then intracellular cAMP production was measured. cAMP concentration was adjusted by protein concentration per well, and is expressed as fold induction compared to baseline. Open bar is negative control without addition of PTH or IS. Data are presented as mean + s.d. of eight wells. *$P<0.05$ vs 0 mmol/l IS (**b**) cAMP production upon pretreatment with 2 mmol/l of IS in the presence of probenecid, a transporter inhibitor. Shaded bar shows cAMP production in the presence of 20 mmol/l of probenecid. Data are presented as mean + s.d. of eight wells. *$P<0.05$ vs 2 mmol/l IS. IS: indoxyl sulfate

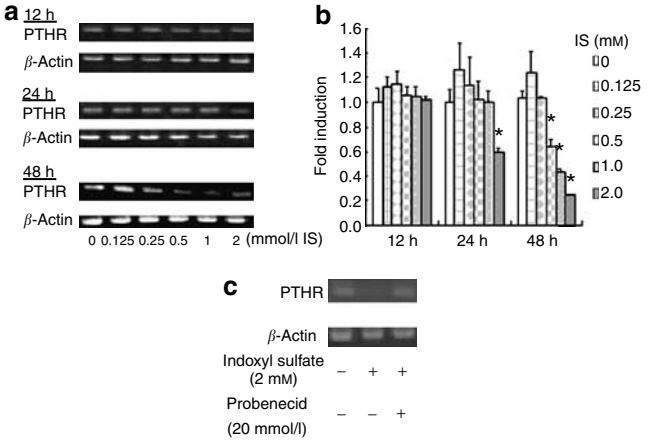

Figure 2 | Changes in expression of the PTHR gene in primary osteobalstic cell cultures by the addition of IS. All experiments were repeated three times and the representative data are shown. (**a**) Expression of PTHR gene upon treatment with various concentrations of IS for 12, 14, or 48 h. (**b**) Fold induction of PTHR gene expression compared to beta-actin. *$P<0.05$ vs 0 mmol/l of IS. (**c**) Expression of PTHR gene upon treatment with 2.0 mmol/l of IS for 48 h in the presence of probenecid, a transporter inhibitor.

RESULTS

Effect of IS on PTH action in primary cultured osteoblastic cells

To elucidate whether IS is involved in skeletal resistance to PTH, we first examined its effect on PTH signaling pathways in primary cultured osteoblastic cells derived from mouse calvariae. Eight wells were tested for each condition. As shown in Figure 1a, cyclic adenosine 3′,5′ monophosphate (cAMP) production was seven times higher when the cells were stimulated with 10^{-8} mmol/l of PTH compared to non-stimulated cells. cAMP production was diminished by pretreatment with IS in a dose-dependent manner. Pretreatment with 2 mmol/l IS significantly suppressed PTH-stimulated cAMP production compared to non-IS-treated controls (Figure 1a). IS has been reported to be taken up into cells by OAT.[15–19] To confirm that OAT is involved in IS uptake into osteoblasts, we added probenecid, a transporter inhibitor, to the cells during pretreatment with 2 mmol/l of IS. In the presence of 20 mmol/l of probenecid, the inhibition of cAMP production by IS was partially abrogated (Figure 1b). The results were almost unchanged when performed in the presence and absence of albumin (data not shown).

Next, we examined *PTHR* gene expression. As shown in Figure 2, *PTHR* mRNA level was inhibited significantly by treatment with 2 mmol/l of IS for 24 h. Furthermore, the inhibition was dose dependent at 48 h of IS treatment (Figure 2a and b), and *PTHR* gene expression was inhibited significantly by 0.5 mmol/l of IS. The results were almost unchanged when performed in the presence and absence of albumin (data not shown). Addition of 20 mmol/l of

probenecid to 2 mM of IS partially reversed the inhibition of *PTHR* gene expression as shown in Figure 2c.

Gene expression of OATs in osteoblastic cells

As past studies have reported that IS has high affinity to OAT-1 and OAT-3 in renal tubular cells,[18,19] gene expression of *OAT-1* and *OAT-3* was determined by reverse transcriptase-polymerase chain reaction (PCR) using RNA extracts. Expression of *OAT-3* was observed, whereas no expression of *OAT-1* was detected in primary cultured osteoblastic cells (Figure 3).

Uptake of IS in osteoblasts

After confirming the expression of *OAT-3* gene in cultured osteoblastic cells, we measured the amount of intracellular uptake of IS by high-performance liquid chromatography analysis. Cellular uptake of IS increased in a time-dependent manner and the uptake was significantly suppressed by the addition of a specific transporter inhibitor, probenecid (Figure 4). The results were almost unchanged when performed in the presence and absence of albumin (data not shown).

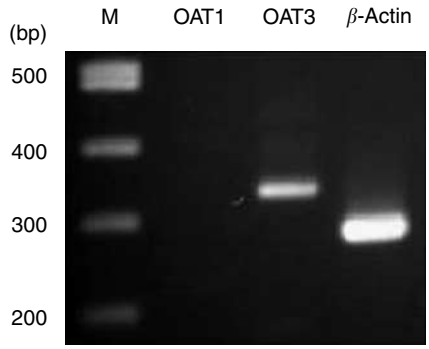

Figure 3 | Expression of the OAT gene in primary osteobalstic cell cultures. The methods were as described in Materials and Methods. The experiment was repeated three times and a representative gel is shown. OAT; organic anion transporter.

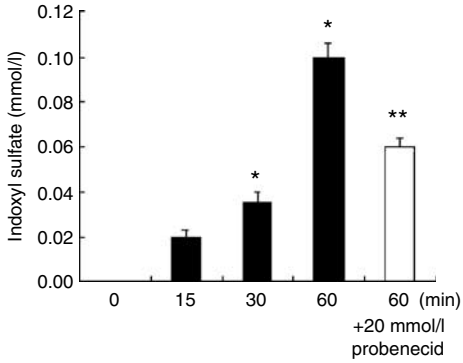

Figure 4 | Intracellular IS concentrations in primary osteobalstic cell cultures. The data are presented as mean + s.d. of eight wells. IS at 1 mmol/l was added to the cell cultures and the intracellular IS concentrations were measured with time. Open bar shows the intracellular IS concentration in cells incubated with 1 mmol/l of IS in the presence of probenecid, a transporter inhibitor, for 60 min. $^{*}P<0.05$; vs 0 min, $^{**}P<0.05$ vs 60 min with addition of IS only.

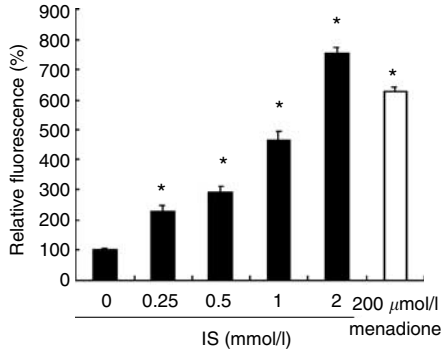

Figure 5 | Free radical production induced by the addition of IS. The data represent mean + s.d. of eight wells. Primary osteoblastic cells were seeded in 96-well plates. IS was added and free radical production was measured after 6 min. Menadione was used as positive control, because the free radicals produced by IS and menadione are both hydroxyl radicals. $^{*}P<0.05$ vs 0 min, IS: IS.

Table 1 | Free radical production induced by the addition of indoxyl sulfate

Indoxyl sulfate	Relative fluorescene (%)
1 mmol/l	300 ± 24*
1 mmol/l (+Probucol 62.5 μmol/l)	180 ± 21#
1 mmol/l (+NAC 2.5 mmol/l)	120 ± 18#
1 mmol/l	300 ± 2*
1 mmol/l (+Probenecid 10 mmol/l)	140 ± 2#
1 mmol/l(+Probenecid 20 mmol/l)	80 ± 2#
0 mmol/l	100 ± 0

NAC, *N*-acetyl-cysteine.
$^{*}P<0.01$ vs 0 mmol/l, $^{\#}P<0.05$ vs 0 mmol/l.

Assessment of intracellular oxidative stress

As shown in Figure 5, IS increased cellular oxidative stress in a dose-dependent manner. Addition of antioxidants or transporter inhibitor suppressed free radical production (Table 1). The results were almost unchanged when performed in the presence and absence of albumin (data not shown).

Effect of IS on cell viability in mouse primary osteoblastic cell culture

To determine cytotoxicity, the effect of IS on cell proliferation of mouse calvaria primary osteoblastic cell culture was studied by 3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyl-tetrazolium bromide assay. As shown in Figure 6, IS inhibited cell proliferation at the concentration range of 0.5–2 mmol/l. The results were almost unchanged when performed in the presence and absence of albumin (data not shown).

DISCUSSION

Bone metabolism in patients with renal failure is characterized by skeletal PTH insufficiency, so-called PTH resistance,

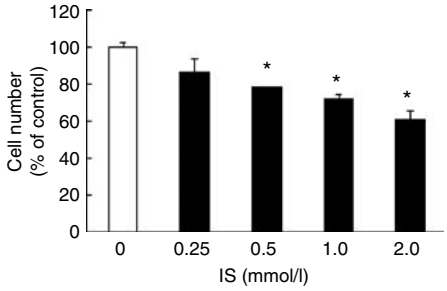

Figure 6 | Effect of IS on viability of primary mouse osteoblastic cell culture. Cell number was measured 24 h after the addition of IS at indicated concentrations, and expressed as the percentage of control cells not pretreated with IS (open bar). The data represent mean + s.d. of eight wells. *$P < 0.05$ vs 0 mM IS-treated cells, IS: indoxyl sulfate.

which presents various pathological states depending on the serum concentrations of PTH and activated vitamin D_3. Although recent development of various drugs now permits the control of PTH oversecretion in renal failure, the problem of skeletal resistance to PTH remains unsolved.

We have developed an animal model of renal failure with low bone turnover. During analysis of the bone of this model, we found decreased *PTHR* expression in the bone and confirmed the presence of skeletal resistance to PTH in low turnover bone of renal failure animals.[11] However, the factors that cause the deceased expression of *PTHR* remain unelucidated. Our finding that downregulation of *PTHR* in bone depends on the degree of renal dysfunction led us speculate that uremic substances accumulated in blood due to renal failure may be involved. Furthermore, we have recently reported that preventing accumulation of IS in blood improves bone formation and improves the inhibited *PTHR* gene expression.[23] Therefore, in the present study, we focused on IS that has been reported to accumulate in blood during renal failure, and studied whether this substance is associated with skeletal resistance to PTH.

Our results showed that culture of primary osteoblastic cells in the presence of IS suppressed the intracellular cAMP production stimulated by PTH. This suppression was dependent on the concentration of IS added; especially, a significant decrease was observed at 2 mmol/l. In addition, we also demonstrated that the addition of IS decreased *PTHR* mRNA expression in osteoblasts in a time-dependent and concentration-dependent manner. These observations suggest a possibility that PTH resistance induced by the presence of IS may be mediated by downregulation of *PTHR* expression in bone. Human study using bone biopsy samples of renal failure patients[4] and our animal model of renal failure with low bone turnover[11] both showed downregulation of PTHR expression in bone. Therefore, we speculate that downregulation of *PTHR* expression is caused by the IS accumulated in blood as a result of renal insufficiency.

It has been reported that in the proximal tubules of the kidney, IS is taken up by the tubular cells via OAT-1 and -3.[15–19] We identified the presence of *OAT-3* gene in osteoblasts and demonstrated that the intracellular concentration of IS increased in a time-dependent manner. Moreover, in the presence of a transporter inhibitor, the intracellular IS concentration decreased and the inhibition of cAMP accumulation was mitigated. These results strongly suggest that IS is taken up by osteoblasts via OAT-3 present in these cells.

The free radical species produced in cells when stimulated by IS has been identified to be hydroxyl radicals.[17] Owada *et al.* (*J Am Soc Nephrol* 2003; **14**: 664 (abstract)) conducted an *ex vivo* electron spin resonance spectroscopy study and reported that IS lowers the superoxide scavenging activity in the kidney, causes a delay in clearance of free radicals in the body, and increases the oxidative stress load. Recently, Gelasco and Raymond[24] reported that IS sulfate augments extracellular SOD-sensitive O^{2-} production and intracellular hydroxyl radical production in mesangial cells. In addition, oxidative stress induced by hydrogen peroxide[25–27] and menadione[28] has been reported to inhibit growth and mineralization of osteoblast. In the present study, free radical production in cultured osteoblasts was increased depending on the concentration of IS added, as shown in Figure 5. At the same time, IS sulfate-induced free radical production was suppressed by the addition of antioxidants and free radical scavenger, and inhibition was also obtained by the addition of probenecid, a transporter inhibitor (Table 1). These results verify that the same phenomena observed in proximal renal tubular cells occur also in osteoblasts. In other words, they suggest that IS taken up by osteoblasts via OAT-3 increases intracellular oxidative stress leading to osteoblast dysfunction.

In our previous study using a renal failure rat model with low turnover bone, we observed cytotoxicity to osteoblasts during 6 weeks.[11] When we attempted long-term culture of mouse primary osteoblastic cells to examine the effect of IS at a concentration similar to the blood level in dialysis patients, the cultures could not be maintained for more than 3 weeks. Therefore, it was not possible to examine the long-term effect of low concentration of IS. On the other hand, short-term exposure to low concentration of IS had no significant effect on osteoblastic cells. In a recent study, we demonstrated that the blood IS level increased with time in rats with renal failure.[23] From these findings, we presumed that the cytotoxic effect of IS depends on the product of concentration and time of exposure. As the objective of the present study was to examine the mechanism of action of IS on osteoblastic cells, we considered it necessary to design the experiment using a high concentration of IS for a short exposure duration.

A large variety of UTx accumulate in blood during renal failure. UTx are classified by molecular weight and mode of protein binding.[29] However, except for IS and a few other substances, the detailed effects of these toxins are largely unknown. Osteoblasts are also affected by uremic serum. Addition of serum from dialysis patients to cultured osteoblasts decreased *PTHR* expression[30] and induced

osteoblast dysfunction including impaired IL-6 secretion.[31] However, these studies did not identify the substances that induce these effects. An animal study reported that administration of two UTx, indoleacetic acid, and hippuric acid, to animals with lowered renal function accelerated the progression of renal failure. It is possible that other organic anionic UTx that depend on OAT for uptake may exert similar effects on osteoblastic cells, although the cytotoxicity of the UTx may depend on its affinity to OAT.

In summary, the UTx IS is taken by osteoblasts via OAT-3 present in these cells, where it augments free radical production inside the cells and induces osteoblast dysfunction including decreased expression of *PTHR*. As decreased *PTHR* is associated with decreased response to PTH, these observations suggest that IS accumulated in blood due to renal failure may be one of the factors inducing skeletal resistance to PTH. IS is a surrogate compound of organic anionic UTx. Many other types of UTx apart from organic anionic compounds accumulate in the patients' blood and these diverse UTx are probably also involved in the development of low turnover bone in dialysis patients. Further studies of the effects of UTx, including organic anionic compounds other than IS, individually and in combination, would elucidate the detailed mechanism of development of low turnover bone and PTH resistance.

MATERIALS AND METHODS

Primary culture of osteoblastic cells

Osteoblastic cells were isolated from calvariae of neonatal mice littermates as described previously.[32] Seven-day-old mice, delivered by timed pregnant mice (Nihon Clea, Tokyo), were killed and the calvariae aseptically harvested and dissected free of suture tissue without disturbing the periosteum. Dissected neonatal mouse calvariae were then washed in phosphate-buffered saline and digested with 1 ml trypsin/EDTA containing 10 mg collagenase (Collagenase P, Roche Applied Science, Indianapolis, IL, USA) five times for 10 min each. And cells from fractions 3 to 5 were pooled. Cells were seeded in 6-well dishes at a density of 5000 cells/cm^2 and grown to confluency (in 5–7 days) in alpha minimal essential medium containing 1 mg/ml bovine serum albumin and 100 μg/ml L-ascorbic acid phosphate and subsequently treated with the various bone resorptive factors. Then we identified the cells in this fraction as osteoblastic cells using alkaline phosphate staining (data not shown).

cAMP generation assay

To study the effect of IS on the PTH response of primary osteoblast cultures, intracellular cAMP production was measured. Osteoblastic cells (1×10^3 cells/well) were plated and allowed to grow until 80–90% confluency. After 24-h pre-incubation with various concentrations (0–2 mmol/l) of IS (Sigma, St Louis, MO, USA) in the medium, the cells were incubated with 10^{-8} mol/l PTH for 10 min. cAMP production was measured using the Tropix cAMP-screen (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol. In addition, we examined the effect of the addition of 20 mmol/l of probenecid on cAMP production. Probenecid (Sigma) was dissolved in weakly alkaline water and then added to the medium. The pH of the medium was checked after addition of probenecid to confirm that there was no change in pH before the experiment was started. The data obtained were adjusted by the cell protein concentration per well and then expressed in fold induction compared to baseline.

RNA isolation, cDNA synthesis, and PCR analysis

Total RNA was isolated using RNeasy Mini Kit (QIAGEN, Tokyo, Japan) according to the manufacturer's instruction. Total RNA (1 μg) was used as the template for cDNA synthesis in a 20 μl volume using an reverse transcriptase-PCR kit (Invitrogen, Carlsbad, CA, USA) according to the manufacture's instructions. Real-time PCR was performed on a Light Cycler (Roche, Basal, Switzerland). The PCR reactions consisted of AmpliTaq Gold PCR Master Mix (Applied Biosystems), 0.1 μM specific primers, and 50 ng of cDNA. Specific primers for mouse *PTHR* (5′-GGT GTC CAC TAC ACC GTC TTC-3′ and 5′-TGT TTC CCA TTC TTC CTG CAA C-3′), OAT1 (5′-ATG CCT ATC CAC ACC CGT GC-3′ and 5′-GGC AAA GCT AGT GGC AAA CC-3′), *OAT3* (5′-CAG TCT TCA TGG CAG GTA TAC TGG-3′ and 5′-CTG TAG CCA GCG CCA CTG AG-3′) were designed according to previous studies,[15,33–35] and *beta-actin* (5′-TCA TGA AGT GTG ACG TTG ACA TCC GT-3′ and 5′-CCT AGA AGC ATT TGC GGT GCA CGA TG-3′) was purchased from Promega (Madison, WI). The amplification reaction products (10 μl) were analyzed by agarose gel electrophoresis and visualized by ethidium bromide staining.

Cellular uptake of IS

Cellular uptake of IS was estimated by the method described previously.[13] Briefly, IS at a final concentration of 1 mmol/l was added to primary osteoblastic cell culture, and IS intake into the cells was examined up to 60 min. The cells were washed in phosphate-buffered saline and methanol was added to prepare a cell extract. The amount of intracellular IS was determined by high-performance liquid chromatography systems 10A (Shimadzu, Kyoto, Japan).

Assessment of cellular oxidative stress

Cellular oxidative stress was assessed by the method described previously.[15,17] Primary osteoblastic cells were seeded in a 96-well tissue culture plate at a density of 2×10^3 cells/well. After incubation for 3 days, the cells were washed with 100 μl D-phosphate-buffered saline (Nacalai Tesque, Kyoto, Japan) containing 5 nmol/l D-glucose and 20 μmol/l dihydro-fluorescein diacetate. IS at the indicated amount was added to the wells. Fluorescence was measured using a fluorescence plate reader (Spectromax GEMINI XS, Molecular Devices, Sunnyvale, CA) at Exλ 485 nm and Emλ 538 nm at 0 min and 60 min. Data were expressed as percent increase in fluorescence intensity compared to the control experiment. We also examined the free radical production in the presence of antioxidants, *N*-acetyl-cysteine and probucol (Sigma). The free radicals produced in cells by the addition of IS are known to be hydroxyl radicals.[17,24] Therefore, we used menadione that also produces hydroxyl radicals[36] as positive control.

Cytotoxicity assays

Cell viability was assessed using the 3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyl-tetrazolium bromide (DOJIN chemicals, Kumamoto, Japan) assay as described previously.[16] Primary osteoblastic cells were incubated in a medium with or without IS at 37°C for 48 h. After the cells were lysed with isopropanol/hydrochloric acid solution, the optical density was measured at 570 nm using the optical density at 630 nm as reference (Spectra Fluor; GmbH, TECAN, Austria).

Statistical analysis

All data are expressed as mean ± s.d. Means of groups were compared by analysis of variance.

ACKNOWLEDGMENTS

This work was supported in part by a grant-in aid from Japanese Society for Promotion of Science (no. 16590787 to MF). Part of this paper was presented at American Society of Nephrology, St. Louis, 2004, appeared as an abstract (*J Am Soc Nephrol* **15**: 227A).

REFERENCES

1. Almanden Y, Canalejo A, Hernandez A *et al.* Direct effect of phosphorus on PTH secretion from whole rat parathyroid glands *in vitro*. *J Bone Miner Res* 1996; **11**: 970–976.
2. Tatsumi S, Miyamato K, Takeda E *et al.* Molecular cloning and hormonal regulation of Pit-1, a sodium-dependent phosphate cotransporter from rat parathyroid glands. *Endocrinology* 1998; **139**: 1692–1699.
3. Fukagawa M, Akizawa T, Kurokawa K. Is aplastic osteodystrophy a disease of malnutrition? *Curr Opin Nephrol Hypertens* 2000; **9**: 363–367.
4. Picton ML, Moore PR, Mawer B *et al.* Down-regulation of human osteoblast PTH/PTHrP receptor in end-stage renal failure. *Kidney Int* 2000; **58**: 1440–1449.
5. Slatopolsky E, Finch J, Clay P *et al.* A novel mechanism for skeletal resistance in uremia. *Kidney Int* 2000; **58**: 753–761.
6. Gao P, Sheibel S, D'Amoour P *et al.* Development of a novel immunoradiometric assay exclusively for biologically active whole parathyroid hormone 1-84: implications for improvement of accurate assessment of parathyroid function. *J bone Miner Res* 2001; **16**: 605–614.
7. Kazama JJ, Shigematsu T, Yano K *et al.* Increased circulating levels of osteoclastogenesis inhibitory factor (osteoprotegerin) in patients with chronic renal failure. *Am J Kidney Dis* 2002; **39**: 525–532.
8. Gonnelli S, Montagnani A, Caffarelli C *et al.* Osteoprotegerin (OPG) and receptor activator of NF-kB ligand (RANK-L) serum levels in patients on chronic hemodialysis. *J Endocrinol Invest* 2005; **28**: 534–539.
9. Coen G, Ballanti P, Balucci A *et al.* Serum osteoprotegerin and renal osteodystrophy. *Nephrol Dial Transplant* 2002; **17**: 233–238.
10. Hruska K. New concepts in renal osteodystrophy. *Nephrol Dial Transplant* 1998; **13**: 2755–2760.
11. Iwasaki-Ishizuka Y, Yamato H, Nii-Kohno T *et al.* Down-regulation of parathyroid hormone receptor gene expression and osteoblastic dysfunction associated with skeletal resistance to parathyroid hormone in a rat model of renal failure with low turnover bone. *Nephrol Dial Transplant* 2005; **20**: 1904–1911.
12. Niwa T, Takeda N, Tatematsu A *et al.* Accumulation of indoxyl sulfate, an inhibitor of drug-binding, in uremic serum as demonstrated by internal-surface reversed-phase liquid chromatography. *Clin Chem* 1988; **34**: 2264–2267.
13. Niwa T, Nomura T, Sugano M *et al.* Inhibitory effect of oral sorbent on accumulation of albumin-bound indoxyl sulfate in serum of experimental uremic rats. *Nephron* 1991; **57**: 84–88.
14. Niwa T, Aoyama I, Takayama F *et al.* Urinary indoxyl sulfate is a clinical factor that affects the progression of renal failure. *Miner Electrolyte Metab* 1999; **25**: 118–122.
15. Motojima M, Hosokawa A, Yamato H *et al.* Uremic toxins proximal tubular injury via organic anion transpoter-1-mediated uptake. *Br J Pharmacol* 2002; **135**: 555–563.
16. Enomoto A, Takeda M, Tojo A *et al.* Role of organic anion transporters in the tubular transport of indoxyl sulfate and the induction of its nephrotoxicity. *J Am Soc Nephrol* 2002; **13**: 1711–1720.
17. Motojima M, Hosokawa A, Yamato H *et al.* Uremic toxins of organic anions up-regulate PAI-1 expression by induction of NF-kB and free radical in proximal tubular cells. *Kidney Int* 2003; **63**: 1671–1680.
18. Deguchi T, Kusuhara H, Takedate A *et al.* Characterization of uremic toxin transport by organic anion transporters in kidney. *Kidney Int* 2004; **65**: 162–174.
19. Deguchi T, Kouno Y, Terasaki T *et al.* Differential contributions of rOat1 (Slc22a6) and rOat3 (Slc22a8) to the *in vivo* renal uptake of uremic toxins in rats. *Pharm Res* 2005; **22**: 619–627.
20. Niwa T, Nomura T, Sugiyama S *et al.* The protein metabolite hypothesis, a model for the progression of renal failure: an oral sorbent lowers indoxyl sulfate levels in undialyzed uremic patients. *Kidney Int* 1997; **62**(Suppl 62): S23–S28.
21. Tsubakihara Y, Takabatake Y, Oka K *et al.* Effect of the oral adsorbent AST-120 on tryptophan metabolism in uremic patients. *Am Kidney Dis* 2003; **41**(3 Suppl 1): S38–S41.
22. Yorioka N, Ito T, Masaki T *et al.* Dose-dependent effect of an oral absorbent AST-120, in patients with early chronic renal failure. *J Int Med Res* 2002; **30**: 467–475.
23. Iwasaki Y, Yamato H, Nii-Kono T *et al.* Administration of oral absorbent (AST-120) suppressed low-turnover bone progression in ureamic rats. *Nephrol Dial Transplant* 2006; **21**: 2768–2774.
24. Gelasco AK, Raymond JR. Indoxyl sulfate induces complex redox alterations in mesangial cells. *Am J Physiol Renal Physiol* 2006; **290**: 1551–1558.
25. Bai XC, Lu D, Bai J *et al.* Oxidative stress inhibits osteoblastic differentiation of bone cells by ERK and NF-kappa B. *Biochem Biophys Res Commun* 2004; **314**: 197–207.
26. Mody N, Parhami F, Sarafian TA *et al.* Oxidative stress modulates osteoblastic differentiation of vascular and bone cells. *Free Radic Biol Med* 2001; **31**: 509–519.
27. Suzuki H, Hayakawa M, Kobayashi K *et al.* H_2O_2-derived free radicals treated fibronectin substratum reduces the bone nodule formation of rat calvarial osteoblast. *Mech Ageing Dev* 1997; **98**: 113–125.
28. Sun JS, Tsuang YH, Huang WC *et al.* Menadione-induced cytotoxicity to rat osteoblasts. *Cell Mol Life Sci* 1997; **53**: 967–976.
29. Vanholder R, Smet RD, Glorieux G *et al.* Review on uremic toxins: classification, concentration, and interindividual variability. *Kidney Int* 2003; **63**: 1934–1943.
30. Disthabanchong S, Hassan H, Charles L *et al.* Regulation of PTH 1 receptor expression by uremic ultrafiltrate in UMR-106-01 osteoblast-like cells. *Kidney Int* 2004; **65**: 897–903.
31. Steddon SJ, Mclntyre CW, Schroeder NL, *et al.* Impaired release of interleukin-6 from human osteoblastic cells in the uraemic milieu. *Nephrol Dial Transplant* 2004; **19**: 3078–3083.
32. Uchida M, Shima M, Shimoaka T *et al.* Regulation of matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) by bone resorptive factors in osteoblastic cells. *J Cell Physiol* 2000; **185**: 207–214.
33. Sweet DH, Miller DS, Pritchard JB *et al.* Impaired organic anion transport in kidney and choroid plexus of organic anion transporter 3 (Oat3 (Slc22a8)) knockout mice. *J Biol Chem* 2002; **277**: 26934–26943.
34. Nesbitt T, Fujiwara I, Thomas R *et al.* Coordinated maturational regulation of PHEX and renal phosphate transport inhibitory activity: evidence for the pathophysiological role of PHEX in X-linked hypophosphatemia. *J Bone Miner Res* 1999; **14**: 2027–2035.
35. Motojima M, Nishijima F, Ikoma M *et al.* Role for 'uremic toxin' in the progressive loss of intact nephrons in chronic renal failure. *Kidney Int* 1991; **40**: 461–469.
36. McCormic ML, Denning GM, Reszka KJ *et al.* Biological effects of menadione photochemistry: effect of menadione on biological systems may not involve classical oxidant production. *Biochem J* 2000; **350**: 797–804.