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Original Articles

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Indoxyl sulphate inhibits osteoclast differentiation and function

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Abstract

Background. Patients with chronic kidney disease (CKD) develop various bone abnormalities characterized by impaired bone remodelling. Recent data suggest that accumulation of the uraemic toxin indoxyl sulphate (IS) may be one of the factors involved in bone abnormalities in CKD patients. Indeed, it was recently reported that IS induces skeletal resistance to parathyroid hormone in cultured osteoblastic cells. However, it is not yet known whether IS also affects osteoclast cells.

Methods. In the present study, we assessed the direct effect of IS at uraemic concentrations and in the presence (to reach the 3 mM concentration) or absence of added inorganic phosphate (Pi) on osteoclast (OCL) differentiation and bone-resorbing activity in two well-established cellular models of monocyte/macrophage (peripheral blood mononuclear cells and the RAW 264.7 cell line).

Results. We found that IS inhibits both OCL differentiation and bone-resorbing activity in a dose-dependent manner and that these effects were enhanced in the presence of Pi at 3mM concentration. IS induced a gradual inhibition of JNK, Akt, p38, ERK1/2 phosphorylation and AP-1 DNA-binding activity. The effects of IS on OCL differentiation and AP-1 were prevented by probenecid, a competitive inhibitor of organic anion transporters, suggesting that IS's effects occur subsequently to its intake.

Conclusion. Our findings strongly suggest that IS not only inhibits osteoblast function but also has an inhibitory effect on OCL function and thus could affect bone remodelling in CKD patients.

Keywords: adynamic bone disease; chronic kidney disease; indoxyl sulphate; osteoclast

Introduction

The uraemic toxin indoxyl sulphate (IS) is an organic anion belonging to the family of protein-bound retention solutes. It is synthesized in the liver from indole, which itself is produced by the intestinal flora from tryptophan [1]. It has been demonstrated that IS modulates the activity of many cell types and induces the production of reactive oxygen species. IS has a suppressive effect on wound repair in human umbilical vein endothelial cells [2]. The accumulation of IS in renal proximal cells promotes cytotoxic effects and thus accelerates the progression of kidney failure [3]. Shimoishi *et al.* [4] showed that IS's induction of systemic vascular oxidative stress in rats could be prevented by treatment with the oral absorbent AST-120 (Kremezin®), which absorbs indole in the intestines. In another *in vivo* study in a rat model of kidney failure with low bone turnover, Iwasaki *et al.* [5] showed that administration of AST-120 suppressed the IS-induced osteoblast cytotoxicity and the decrease in bone formation.

Recently, Fukagawa *et al.* demonstrated in a primary mouse calvariae osteoblast cell culture that addition of IS suppresses parathyroid hormone (PTH)-stimulated intracellular cAMP production, decreases PTH receptor expression and induces oxidative stress in these cells [6]. They suggested that IS accumulation in the blood of CKD patients is at least one of the factors that induces skeletal resistance to PTH implicated in the outcome of adynamic bone disease (ABD) observed in patients with end-stage renal disease [6]. ABD is characterized by quiescent osteoblasts and osteoclasts (OCLs) and markedly reduced bone turnover activity. Since 1979, uraemic toxins have been proposed to play a role in low bone turnover disease such as ABD [7]. As mentioned above, recent data suggest that accumulation of IS in the blood may be one of the uraemic factors involved in ABD via its effects on osteoblastic cells [6]. Limited data suggest that uraemic toxins may also play a role in OCL differentiation and bone resorption. We recently observed *in vitro* that the uraemic toxin, inorganic phosphate (Pi), directly inhibits both osteoclastic differentiation and bone-resorbing activity induced by receptor activator of nuclear factor kappa B ligand (RANKL) and macrophage colony stimulating factor (M-CSF) [8]. To date, the direct effects of IS on multinucleated OCL differentiation and function have not yet been evaluated. Hence, we decided in the present work to assess the effects of uraemic concentrations of IS (as defined by the EUTox work group) on OCL differentiation and bone-resorbing activity.

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Materials and methods

Chemicals

All chemicals were purchased from Sigma unless otherwise stated.

Uraemic toxin preparation

For our experiments, we referred to the list of uraemic toxins provided by EUTox and thus worked with IS at a normal concentration (ISn: 0.6 mg/L, i.e. 2 μM), a uraemic concentration (ISu: 53 mg/L, i.e. 211 μM) and the maximum concentration found in uraemic patients (ISm: 236 mg/L, i.e. 940 μM) [9]. All experiments in the presence of Pi were performed with 3 mM of NaH_2PO_4 salt, taking into account the 1.1 mM present in the alpha modified eagle's medium (αMEM) medium supplemented with 10% foetal calf serum (FCS).

Peripheral blood mononuclear cells and RAW 264.7 cell culture and differentiation

Peripheral blood mononuclear cells. Peripheral blood mononuclear cells (PBMCs) were prepared as previously described [8]. Briefly, all media and added factors were replaced twice a week; cells were cultured for 14 days to allow complete differentiation in the presence of human RANKL (25 ng/mL) and human M-CSF (30 ng/mL) (R&D Systems). In order to evaluate bone resorption activity, the cells were seeded on bovine bone slices. Cortical bone slices were obtained from bovine femora, as described in [8]. After each medium change, we pooled and stored supernatants from each experimental condition in order to quantify type I collagen C-terminal telopeptide (CTX) degradation products. In all experiments, we confirmed that there was no differentiation in the absence of RANKL.

RAW 264.7 cells. RAW 264.7 mouse monocyte/macrophage cells (ATCC) routine culture was previously described in [8]. For differentiation into OCL-like cells, RAW 264.7 cells were gently scraped and seeded in 96-well plates at a density of 10^3 cells per well in αMEM differentiation medium supplemented with 10% FCS and 30 ng/mL of recombinant murine RANKL (R&D Systems). The medium was replaced once and cells were cultured for 5 days. In all experiments, we confirmed that there was no differentiation in the absence of RANKL.

To evaluate the effects of IS on RANKL intracellular signalling, RAW 264.7 cells were seeded for 24 h at a density of 2×10^6 cells per well in αMEM. RANKL (100 ng/mL) was added for 10 min before preparation of the total cell extracts and 30 min before preparation of the nuclear extracts (50 ng/mL). IS was added 2 min before RANKL addition in order to reach the indicated concentrations.

To block the effect of IS on differentiation, we used a range of concentrations of the organic anion transporter (OAT) inhibitor probenecid (from 1 to 100 μM). Cells were treated with probenecid for the last 2 days of differentiation only because longer exposure was cytotoxic (data not shown).

Correctness of total and ionized calcium concentrations in the supernatants collected from the various experimental conditions was checked with a RXL Dimension Dade-Behring auto-analyser and a Ciba-Corning 634 Ca^{++}/pH analyser, respectively.

Tartrate-resistant acid phosphatase staining

Osteoclasts differentiated from RAW cells or PBMCs were stained for tartrate-resistant acid phosphatase (TRAP) using a commercially available kit (leukocyte acid phosphatase staining kit 387A, Sigma) according to the manufacturer's protocol. Multinucleated (more than three nuclei) TRAP-positive cells were counted as OCLs under microscopic examination.

Cell viability and specific controls

We used the water-soluble tetrazolium salt WST-1 (Roche Applied Science) to measure cell proliferation and viability (as already described in [8]). Briefly, after overnight starvation (in 0.5% FCS medium), RAW 264.7 cells seeded in 96-well plates at 5×10^3 and 1×10^3 cells per well were cultured for 2 and 5 days, respectively, under different conditions.

We checked that the effects of IS were not due to the potassium ions in the IS salt used for these experiments since KCl at 0.9 mM (the maximum concentration used with IS) inhibited neither the differentiation of PBMC progenitors into OCLs nor bone resorption after 2 weeks of culture (data not shown). Likewise, we checked that the effects of IS were not due to the presence of sulfate; we did not observe any effect of sodium sulphate (at the various concentrations used for IS) on RAW 264.7 progenitor differentiation (data not shown).

Since IS is primarily a protein-bound molecule, the free serum concentration corresponds to a small fraction of the total serum concentration. To test the role of protein-binding effects, we performed experiments with physiological concentrations (40 g/L) of human serum albumin (HSA) in murine monocyte RAW 264.7 model. Unfortunately, HSA did affect our model by itself inducing the differentiation of RAW 264.7 cells into OCL-like cells (data not shown). These phenomena prevented us from gaining a clear picture of protein-binding effects.

Bone resorption activity measurement

Morphological assessment: pit area measurement. After OCL removal, resorption lacunae (pits) were visualized by staining bone slices with haematoxylin red and a toluidine blue solution containing 1% sodium borate. The percentage of resorbed bone surface area was quantified using Countscan® (Biocom).

Quantitative assessment: measurement of type I collagen fragment release. During bone slice resorption by OCLs, the bone matrix is degraded and fragments of type I collagen are released into the supernatant. We used an enzyme-linked immunosorbent assay (ELISA) (CrossLaps®; Osteomedical) for the quantitative determination of degradation products of type I collagen C-terminal telopeptides (CTX) in PBMC culture supernatants. The level of CTX was assessed according to the manufacturer's protocol.

Immunoblot assay of kinase phosphorylation

After stimulation, cells were washed once with phosphate-buffered saline and lysed with 50 μL lysis buffer (25 nM Tris–HCl, pH 7.6, 150 nM NaCl, 10% glycerol, 1% Nonidet P-40, 2 mM Na_3VO_4 and protease inhibitors) and placed on ice for 30 min. Total cell extracts were centrifuged at 14 000 *g* (for 20 min and at 4°C) and protein-containing supernatants were collected. Equal amounts of proteins (40 μg) were resolved by sodium dodecyl sulphate–polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane and immunoblotted with specific antibodies against phospho-JNK, -Akt, -ERK1/2 and -p38 (Cell Signalling). Secondary antibodies were obtained from Santa Cruz. Equal loading was confirmed using a β-actin antibody. Immunoblots were visualized by an enhanced chemiluminescence detection kit (Amersham). Protein expression levels were quantified using a densitometer (Genegenius; Ozyme). The data were represented as the ratio of phosphorylated proteins to that of β-actin. The value under control conditions (i.e. in the absence of RANKL) was considered to be 1.

Determination of the DNA-binding activity of transcription factors using the TransAM® ELISA

NF-κB and AP-1 DNA-binding activity assays were performed using the Trans-AM® ELISA-based kits from Active Motif. Nuclear extracts were prepared using the Active Motif kit according to the manufacturer's protocol. Equal quantities of protein (10 μg) for each sample were then incubated in 96-well plates coated with an oligonucleotide containing the NF-κB or AP-1 consensus binding site. Activated transcription factors of the extracts specifically bounded to the respective immobilized oligonucleotide were detected using a primary antibody directed against p65 (NF-κB kit) or c-Jun (AP-1 kit) and a secondary antibody conjugated to horseradish peroxidase. Developing solution was then added for 10 min at room temperature and optical densities (450–655 nm) were determined with a microplate reader (Molecular Devices). Results are expressed as a percentage of the control condition (*C*t, i.e. complete medium without RANKL).

Statistical analysis

All results are expressed as mean ± SEM. Statistical significance was determined in an analysis of variance and a P-value <0.05 was considered to be significant.

Results

IS inhibits the RANKL-induced differentiation of RAW 264.7 cells into osteoclasts

As shown in Figure 1, the RANKL-induced differentiation of RAW 264.7 cells into OCLs was dose dependently and

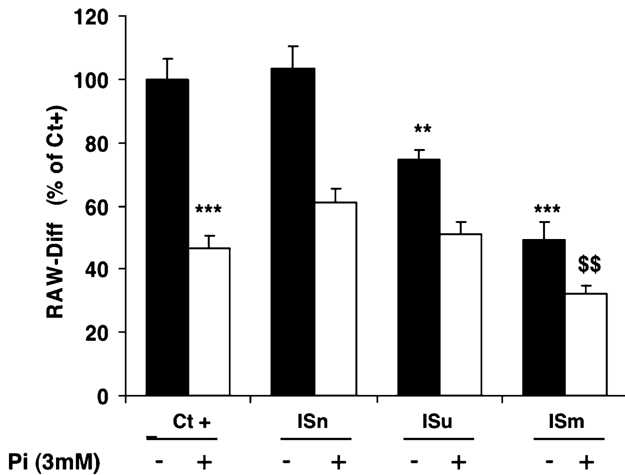

Fig. 1. IS inhibits RANKL-induced differentiation of RAW 264.7 cells into OCLs. RAW 264.7 cells were cultured for 5 days in the presence of RANKL (30 ng/mL, *C*t+) and the indicated concentrations of IS [IS at normal concentration (ISn, 0.6 mg/L), uraemic concentration (ISu, 53 mg/L) and maximum concentration (ISm, 236 mg/L)] in the presence or absence of 3 mM Pi. TRAP+ multinucleated osteoclasts (RAW-Diff OCLs) were assessed in each well. Data are represented as a percentage of *C*t+ and represent the mean ± SEM of four independent experiments. **P < 0.01, ***P < 0.001 versus *C*t+. \$\$P < 0.01 compared with cells cultured in the presence of 3 mM Pi.

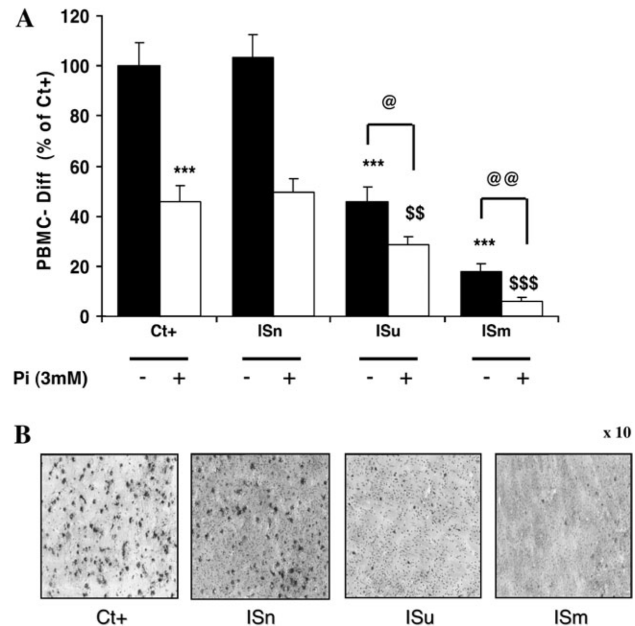

Fig. 2. IS inhibits RANKL-induced differentiation of PBMCs into OCLs. To induce differentiation, PBMCs were cultured for 14 days on bovine bone slices in the presence of RANKL (25 ng/mL) and M-CSF (30 ng/mL) (*C*t+) and the indicated concentration of IS in the presence or absence of 3 mM Pi. (**A**) The number of multinucleated TRAP+ cells was assessed in each well. (**B**) Pictures of TRAP+ OCL cells cultured on bone slices in the presence of the indicated concentrations in IS alone (ISn, ISu, ISm; see the legend to Figure 1 for details). The results are quoted as a percentage of *C*t+ and represent the mean ± SEM of four independent experiments. ***P < 0.001 versus *C*t+. \$\$P < 0.01, \$\$\$P < 0.001 compared with cells cultured in the presence of 3 mM Pi. @P < 0.01 and @@P < 0.001 IS alone compared with IS + 3 mM Pi.

significantly inhibited when IS was added to culture media at ISu and ISm concentrations, when compared with the *C*t and normal concentration of IS (ISn). When 3 mM Pi was added to ISm, we observed a marked inhibition of OCL differentiation when compared with Pi alone. In order to exclude the possibility that IS's inhibitory effect was due to a change in cell viability, we assessed the effect of IS on the viability of these cells over 2 and 5 days (using WST-1 reagent). We did not see any change in RAW 264.7 precursor cell viability in the presence of IS, regardless of the highest concentration used or the presence or absence of 3 mM Pi (data not shown).

IS reduces the RANKL-induced differentiation of PBMCs into osteoclasts

IS's effects on OCL differentiation were confirmed with PBMC cell progenitors. IS significantly and dose dependently inhibited the differentiation into active OCL cells normally induced by RANKL and M-CSF alone (*C*t) in this cell model (Figure 2A and B). These effects were significantly enhanced in the presence of 3 mM Pi.

IS reduces the bone-resorbing activity of PBMC-derived osteoclasts

After counting, human OCL cells were washed out of bone slices. Bone resorption appeared as characteristic pits on the surface of the bone slices. By evaluating the bone resorption surface area under the various conditions, we found that ISu and ISm concentrations significantly and dose dependently decreased the bone-resorbing activity of OCL cells compared with the *C*t and ISn conditions (Figure 3A). We found that effects of ISu and ISm were enhanced in the presence of 3 mM Pi. The IS's effects on the bone-resorbing activity of OCL were also assessed by measuring the amount of CTX in the supernatants. We observed a dose-dependent decrease in the amounts of CTX released under ISu and ISm conditions compared with the medium-only and ISn conditions (Figure 3B). We did not observe significantly greater inhibition when Pi was added to ISu, relative to the effects of ISu alone. This may be due to the high observed variability of CTX release from one experiment to another.

OATs are directly involved in the effects of IS on OCL differentiation

In order to explore the molecular mechanism through which IS might inhibit the differentiation of RAW 264.7 cells into OCLs, we assessed the role of OATs. By using different concentrations of the OAT inhibitor probenecid (1, 10 and 100 μM), we were able to block the inhibitory effect exerted by ISu (Figure 4) and ISm (data not shown) on OCL-like differentiation in a dose-dependent manner. Cells cultured in the presence of uraemic or maximal concentrations of IS and 100 μM probenecid displayed a similar differentiation rate to that seen in the *C*t, whereas probenecid alone did not modulate OCL differentiation. These results suggest that IS exerts its effects after OAT-mediated entry into cells.

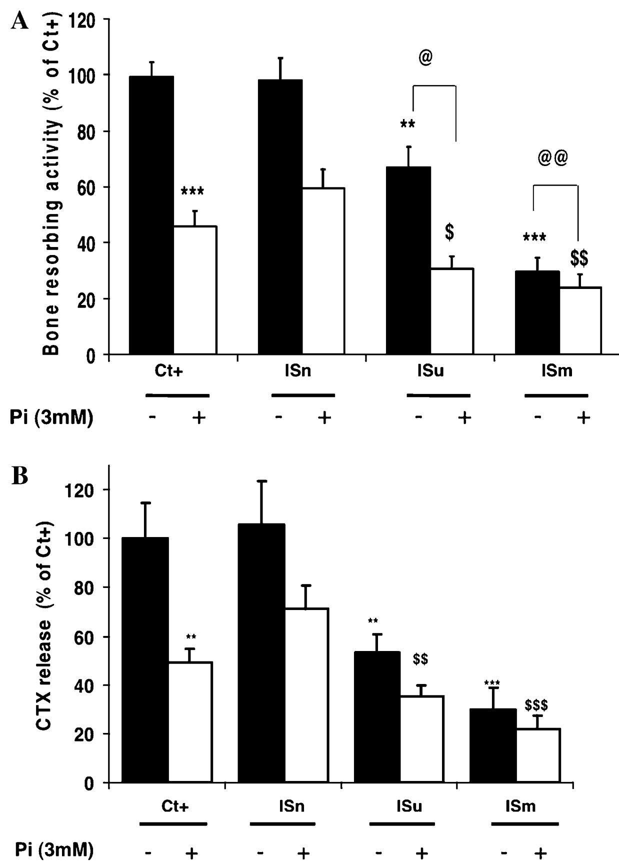

Fig. 3. IS inhibits RANKL-induced bone resorption. (**A**) After removing OCL cells, the bone slices were stained with haematoxylin red and toluidine blue to visualize the resorption lacunae (pits). For each condition, we assessed the total pit surface area. (**B**) Quantitative assay of C-terminal of type I collagen telopeptide (CTX) degradation products in PBMC culture supernatants. The results are quoted as a percentage of Ct+ and represent the mean ± SEM of four independent experiments. **P < 0.01, ***P < 0.001 compared with bone resorption when PBMCs were cultured in *C*t+. $P < 0.05, $$P < 0.01 and $$$P < 0.001 compared with cells cultured in the presence of 3 mM Pi. @P < 0.01 and @@P < 0.001 IS alone compared with IS + 3 mM Pi.

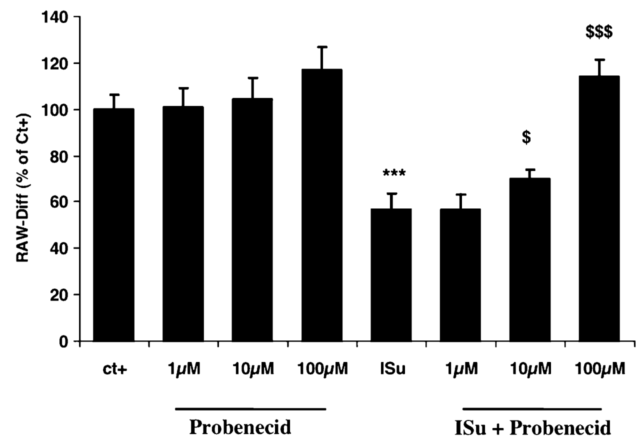

Fig. 4. The OAT inhibitor probenecid blocks IS's inhibition of RAW 264.7 cell differentiation. RAW 264.7 cells were cultured for 5 days in the presence of RANKL (30 ng/mL) and 0, 1, 10 and 100 μM probenecid in the presence or absence of IS. The number of TRAP+ multinucleated osteoclasts derived from RAW 264.7 cells were counted in each well. The results are quoted as a percentage of *C*t+ and represent the mean ± SEM of two independent experiments. ***P < 0.001 versus *C*t+. $$P < 0.01 and $$$P < 0.001 compared with cells cultured in the presence of ISu.

IS inhibits the activation of RANKL protein kinases

The activation states of ERK1/2, p38, JNK and Akt were determined by western blotting with specific antibodies against the phosphorylated forms of these enzymes. Concerning the effect of IS itself, weak activation of ERK1/2, JNK, p38 and Akt was observed at the ISm concentration. As expected, the addition of RANKL activated all four kinases. Under these experimental conditions, we showed that IS inhibited ERK1/2, JNK, p38 and Akt phosphorylations in a dose-dependent manner (Figure 5).

IS inhibits RANKL-induced DNA-binding activities of NF-κB and AP-1

We next evaluated the effect of IS on the DNA-binding activities of NF-κB and AP-1 transcriptional factors by using the ELISA TransAM® method. We observed that IS alone did not exert any effect on the DNA-binding activities of these two transcriptional factors in RAW 264.7 cells. As previously reported, incubation with RANKL increased the DNA-binding activities of both transcriptional factors. We then observed that increasing uraemic concentrations of IS led to a significant dose-dependent inhibition of AP-1-binding activity (Figure 6A). Significant inhibition of NF-κB DNA-binding activity was observed only at the ISm concentration (Figure 6B). Probenecid at 500 μM (but not 100 μM) was able to prevent the inhibitory effect exerted by ISm on AP-1-binding activity (P < 0.001 for ISm alone versus ISm + probenecid, in the presence of RANKL).

Discussion

Using two well-established cellular models of OCL differentiation, we demonstrated that the addition of IS at uraemic and maximum concentrations dose dependently inhibited not only OCL differentiation from human and mouse monocyte/macrophage models but also bone-resorbing activity. Interestingly, these effects were significantly enhanced by the presence of 3 mM Pi. Molecular investigations showed that IS dose dependently inhibits the activities of protein kinases Akt, JNK, p38 and ERK1/2 and the DNA-binding activity of AP-1—all key proteins involved in RANKL-induced OCL differentiation. We were able to implicate the OATs in these effects by using probenecid, a pharmacological inhibitor of these transporters suggesting that IS might need to be taken up by monocyte/macrophage precursors to inhibit differentiation.

Our data emphasize the fact that several uraemic toxins could affect OCL differentiation and function. In fact, we had already observed *in vitro* that the uraemic toxin Pi at uraemic concentration directly inhibited both OCL differentiation and bone-resorbing activity induced by RANKL and M-CSF [4]. In the present study, we observed that the uraemic toxin IS has comparable effects on OCL differentiation and function. Moreover, we demonstrated that the

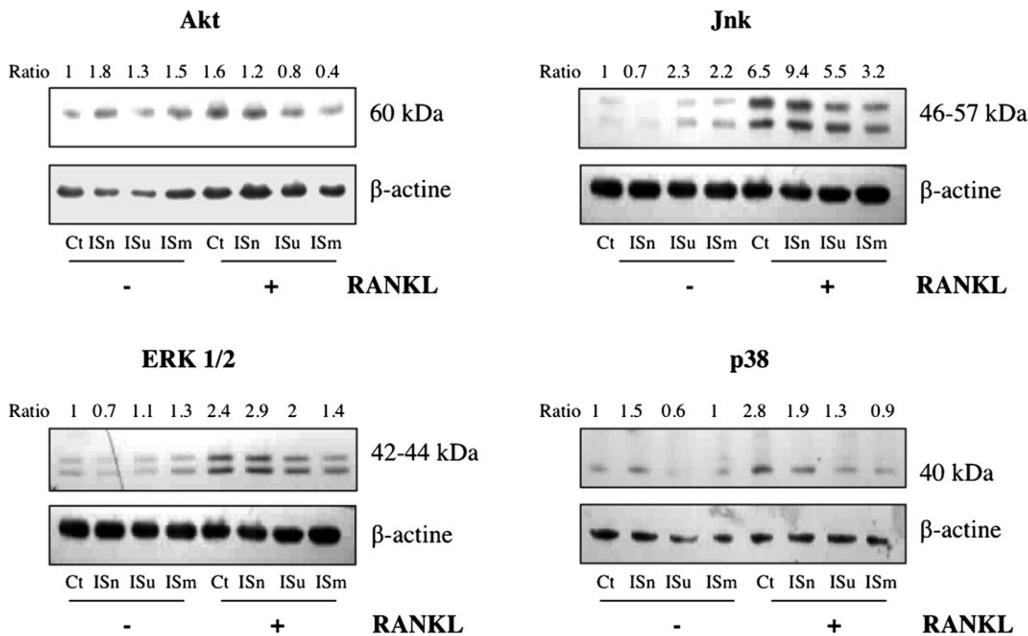

Fig. 5. Effect of IS on RANKL-induced phosphorylation of the signalling kinases ERK, p38, JNK and Akt. The cells were pre-incubated for 24 h in the differentiation medium αMEM supplemented with 10% FCS. RANKL 100 ng/mL was added for 10 min before the preparation of cellular extracts. The IS was added just before the RANKL, in order to reach the indicated concentrations. The phosphorylation state of the kinases was determined by immunoblot analysis. The results are representative of three independent experiments. Protein expression was quantified and data are expressed as the phosphorylated protein/β-actin ratio. The *C*t condition in the absence of RANKL was considered as 1.

effects of these two uraemic toxins (i.e. IS and Pi) were additive. Other uraemic toxins may have opposite effects. Recently, it has been shown that the uraemic toxin beta(2)-microglobulin stimulates osteoclastogenesis via up-regulation of tumour necrosis factor-alpha and interleukin-1 expression [10]. However, it seems that the uraemic milieu has rather inhibitory effects on both OCL differentiation and bone-resorbing activity. Indeed, when we compared the impact of pooled uraemic serum from CKD haemodialysis patients with that of healthy control serum (10% of healthy control serum or FCS), we observed that PBMC differentiation into OCLs and bone-resorbing activity were greatly decreased [11]. A note of caution should be added since the final concentrations in the present experimental conditions by far exceed free IS concentrations *in vivo*. Unfortunately, HSA did affect our model by itself inducing the differentiation of RAW 264.7 cells into OCL-like cells. These phenomena prevented us from gaining a clear picture of protein-binding effects. However, one could advocate that the situation may be different between *in vitro* and *in vivo*. Indeed, the short-term exposure *in vitro* to high IS concentrations in the present study may mimic the long-term exposure in patients to lower IS concentrations.

Bone remodelling depends not only on osteoblast activity but also on the OCLs' bone-resorbing activity. Hence, disturbance of osteoblast and/or OCL activities can induce bone metabolism disorders. Along with CKD progression, the kidney's functions gradually become unable to maintain systemic mineral homeostasis, resulting in the various abnormalities of bone and vascular physiology observed in CKD-MBD (CKD associated with mineral and bone disorders) [12]. The various bone disorders observed in CKD patients are probably depending not only on the serum concentration in PTH but also on the concentrations of various uraemic toxins. It has been suggested that when compared with healthy patients, dialysed CKD patients require two to three times more PTH to maintain a normal bone turnover rate probably to counterbalance direct effects of uraemic toxins on bone in these patients [13]. It is noteworthy that in a rat model of CKD with low bone turnover, Iwasaki *et al.* [5] showed that serum IS rises while bone turnover decreases with the impairment in renal function. Oral administration of indole-absorbing Kremezin® prevents both the progression of renal failure and the decrease in bone formation [5]. Accumulation of IS in CKD patients could be at least one of the factors that induce skeletal resistance to PTH by its deleterious effects on osteoblasts and OCLs. Recently, Goto *et al.* [14] reported that IS correlated negatively with two serum markers of bone formation (alkaline phosphatase and bone-specific alkaline phosphatase) independently of intact PTH but not with a serum marker of bone resorption (TRAP 5b). However, serum markers may not be ideal to test bone turnover and bone histomorphometric data are needed. Hence, by affecting both osteoblast and OCL activities, IS may promote low bone turnover diseases outcomes (such as ABD) observed in CKD patients.

Our data confirm and extend previous works showing that IS acts as a bone toxin by affecting both osteoblast and OCL activities. More work is required to understand how IS enters the cell to exert its effects and to determine whether OATs play a role in entry. The relevance of these findings must, however, be confirmed by further studies in a clinical setting.

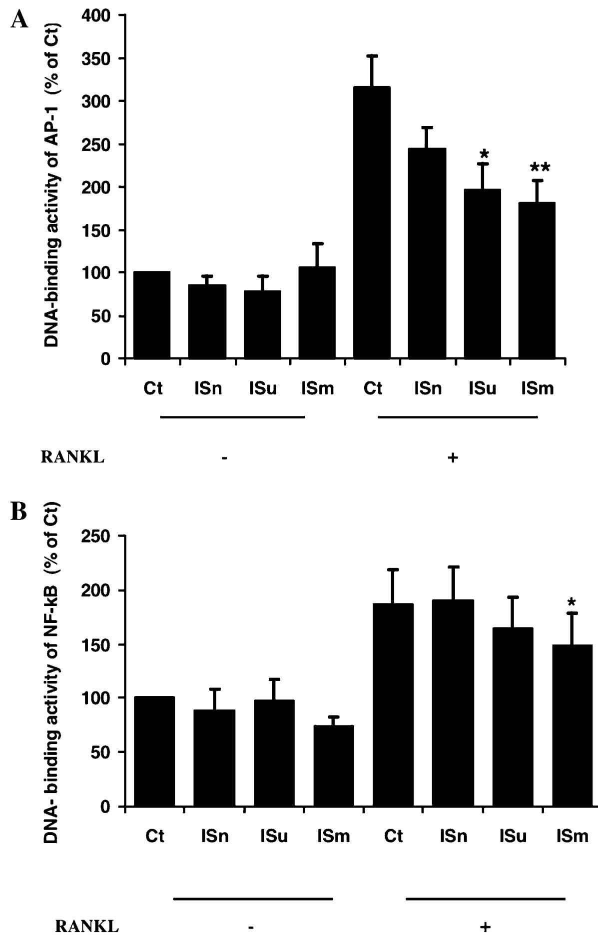

Fig. 6. The effect of IS on the RANKL-induced DNA-binding activity of the transcriptional factors NF-κB and AP-1. RAW 264.7 cells were pre-incubated for 24 h in αMEM differentiation medium supplemented with 10% FCS. RANKL 50 ng/mL was added for 30 min before the preparation of nuclear extracts. The IS was added just before the RANKL, in order to reach the indicated concentration. The DNA-binding activity was determined with NF-κB-p65 and AP-1-cjun TransAM® ELISA kits from Active Motif. The results (means ± SEM) are quoted as a percentage of the *C*t experiment in the absence of RANKL. (**A**) Effects of IS on NF-κB DNA-binding activity in the presence or absence of 50 ng/mL of murine RANKL. (**B**) Effects of IS on AP-1 DNA-binding activity in the presence or absence of 50 ng/mL murine RANKL. Data are represented as a percentage of the Ct condition in the absence of RANKL and represent the mean ± SEM of three independent experiments ± SEM. *P < 0.05, **P < 0.01 and ***P < 0.001 when compared with *C*t condition with RANKL.

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Authors' contributions: Anaïs Mozar, Loïc Louvet and Ziad Massy participated in the conception and design of the study and acquisition of the data; they analysed and interpreted the data and drafted the first and final version of the paper. Saïd Kamel, Michel Brazier and Romuald Mentaverri participated in the conception and design of the study and interpretation of the data and revised the manuscript for content. Corinne Godin participated in the acquisition and interpretation of data and revised the manuscript for content.

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Conflict of interest statement. A.M., L.L., C.G., R.M., M.B., S.K., Z.A.M. authors declare no conflict of interest.

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