Amino Acids (2010) 39:89–99
DOI 10.1007/s00726-009-0380-2

ORIGINAL ARTICLE

Taurine inhibits osteoclastogenesis through the taurine transporter

Ling-Qing Yuan · Wei Liu · Rong-Rong Cui · Dan Wang · Ji-Cai Meng · Hui Xie · Xian-Ping Wu · Hou-De Zhou · Ying Lu · Er-Yuan Liao

Received: 15 July 2009 / Accepted: 20 October 2009 / Published online: 14 November 2009

Abstract Several studies have suggested a direct link between taurine and bone homeostasis. However, the mechanisms of taurine on the regulation of bone metabolism have not been elucidated. Using a coculture of osteoblasts and bone marrow cells as a model for the study of osteoclastogenesis, RANKL-stimulated RAW264.7 cells and M-CSF- and RANKL-induced bone marrow macrophages were investigated to elucidate the possible roles of taurine in osteoclastogenesis. Taurine inhibited osteoclastogenesis in the coculture of osteoblasts and bone marrow cells, but did not influence the expression of OPG and RANKL in osteoblasts. The taurine transporter (TAUT) expressed by RAW264.7 and bone marrow macrophages exhibited typical taurine uptake activity. Taurine directly reduced osteoclastogenesis in RANKL-stimulated RAW264.7 cells and M-CSF- and RANKL-induced bone marrow macrophages, while TAUT siRNA relieved this effect. Our study demonstrated that taurine directly inhibited osteoclastogenesis through the taurine transporter. Taken together, these data suggest that taurine plays a direct role in bone homeostasis by inhibiting osteoclastogenesis.

L.-Q. Yuan and W. Liu contributed equally to this work.

L.-Q. Yuan · W. Liu · R.-R. Cui · D. Wang · J.-C. Meng · H. Xie · X.-P. Wu · H.-D. Zhou · Y. Lu · E.-Y. Liao (✉)
Institute of Metabolism and Endocrinology, The Second Xiang-Ya Hospital, Central South University, #86 Middle Renmin Road, 410011 Changsha, Hunan, People's Republic of China
e-mail: allenylq@hotmail.com

R.-R. Cui
Department of Pediatrics, The Second Xiang-Ya Hospital, Central South University, 410011 Changsha, Hunan, People's Republic of China

Keywords Taurine · Taurine transporter · Osteoclastogenesis · siRNA

Introduction

Homeostasis of bone density is elaborately maintained via two phases of bone remodeling: bone resorption by osteoclasts and bone formation by osteoblasts (Parfitt 1987; Rodan 1992). Multinucleated osteoclasts facilitate bone remodeling by resorbing bone matrices and play an important role in calcium homeostasis (Zaidi et al. 2003). Osteoclasts arise from the differentiation of osteoclast precursors belonging to the monocyte/macrophage lineage, and osteoprotegerin (OPG) and nuclear factor-kappa B ligand (RANKL) produced by osteoblasts and stromal cells are well-documented potent regulators of osteoclast development (Hofbauer and Heufelder 2001; Khosla 2001).

Taurine (2-aminoethanesulfonic acid), the major free β-amino acid in mammals, is widely distributed in mammalian plasma as well as tissues. Various physiological roles of taurine have been suggested, including calcium modulation, membrane stabilization and the regulation of intracellular osmosis and protein phosphorylation. Taurine is necessary for normal cell differentiation and immune maturation (Huxtable 1992; Pasantes-Morales et al. 1998). The cellular content of taurine is moderated by a balance between the active uptake of taurine through the saturable Na^+-dependent taurine transporter TAUT and its passive release via a volume-sensitive taurine-leak pathway. Intracellularly, taurine is present at millimolar concentrations, whereas it occurs at a concentration of 20–100 nM in plasma; this suggests that TAUT plays an important role in maintaining a high concentration of taurine in tissues and cells (Wright et al. 1986; Lambert 2004).

In the last decade, a large number of experimental studies have indicated that taurine is localized in the matrices of the bone and emerges as an element in the regulation of bone metabolism (D'Eufemia et al. 2007; Lubec et al. 1997; Terauchi et al. 1998; Gupta and Kim 2003; Gupta et al. 2005; Cheong and Chang 2009; Choi and DiMarco 2009). On one hand, taurine can reduce alveolar bone resorption in experimental animals (Koide et al. 1999), while on the other, many in vitro studies have demonstrated the direct action of taurine on osteoblasts and osteoclasts (Yasutomi et al. 2002; Park et al. 2001; Kum et al. 2003). Our recent studies showed that TAUT was expressed in osteoblasts, and taurine promoted osteoblast differentiation and stimulated connective tissue growth factor expression in osteoblasts through ERK/MAPK activation (Yuan et al. 2006; Yuan et al. 2007). Moreover, a recent study demonstrated that both alendronate and taurine inhibit bacteria-stimulated osteoclastogenesis in vitro (Kum et al. 2003). However, the mechanism underlying the effect of taurine on osteoclastogenesis remains unclear. The aim of this study was to evaluate the precise role of taurine in osteoclastogenesis and to determine the function of TAUT in this process.

Materials and methods

Reagents

Taurine and 1α, 25-dihydroxyvitamin D_3 (1, 25 vitD) were purchased from Sigma (St. Louis, MO, USA). Recombinant murine macrophage colony-stimulating factor (M-CSF) and recombinant murine RANKL were purchased from R&D systems (Minneapolis, MN, USA). [^{3}H]Taurine (specific activity, 30.0 Ci/mmol) was obtained from Amersham International (Buckinghamshire, UK). TAUT polyclonal affinity-purified IgG was purchased from Alpha Diagnostic Intl. (St. Antonio, TX, USA). All animal experiments in this study were performed with the approval of the Animal Care Committee of the Second Xiangya Hospital of Central South University.

Coculture of mouse primary osteoblasts and bone marrow cells

Mouse osteoblasts and bone marrow cells were cocultured as described previously (Dolder et al. 2006; Kwak et al. 2004). Briefly, primary osteoblasts were obtained from the calvariae of neonatal ICR mice using 0.1% collagenase and 0.2% dispase for 3 days in α-MEM supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA, USA), 100 U/mL penicillin and 100 μg/mL streptomycin. Bone marrow cells were flushed out from the femora and tibias of 6–8-week-old male ICR mice by using α-MEM containing Hanks' salt. Bone marrow cells (6×10^4) and osteoblasts (8×10^3) were seeded into each well of 48-well plates and cultured in α-MEM supplemented with antibiotics, 10% FBS, 10 nM 1, 25 vitD, 50 μM mercaptoethanol and 50 mg/L ascorbate. Taurine was either absent or present in the culture medium. At 5–6 days of culture, the cells were stained for tartrate-resistant acid phosphatase (TRACP), a marker enzyme of osteoclasts, by using a commercial TRACP-staining kit (product 387-A; Sigma). The cells were fixed with 1% (vol/vol) formalin/PBS for 10 min and stained for acid phosphatase by using 10 mg/mL naphthol AS-BI phosphatase as a substrate in the presence of acetate tartrate buffer (50 mM sodium acetate, 40 mM potassium-sodium tartrate) at a pH of 5.0. The product was then coupled with Fast garnet GBC salt. The cells were observed under a microscope, and the $TRACP^+$ multinucleated cells containing more than three nuclei were identified as osteoclasts and counted.

Culturing osteoclasts from bone marrow macrophages and RAW264.7 cells

Osteoclast differentiation was induced in bone marrow-derived macrophage (BMMs) preparations. Bone marrow cells were cultured for 24 h in α-MEM supplemented with 10% FBS. The nonadherent cells were collected and cultured for 3 days in α-MEM supplemented with 10% FBS and 50 ng/mL M-CSF. The cells thus obtained were considered as bone marrow-derived macrophages. For osteoclastogenesis, the cells were further incubated for 5–6 days in a medium containing 30 ng/mL M-CSF and 50 ng/mL RANKL.

RAW264.7 mouse macrophage/monocytes were purchased from ATCC (Manassas, VA, USA). They were cultured in DMEM supplemented with 1.5 g/L sodium bicarbonate (JRH Biosciences, Lenexa, KS, USA) and 10% FBS. To stimulate osteoclastogenesis, 50 ng/mL of RANKL was added to this medium. The cells were cultured for 5–7 days, and the medium and the added RANKL were changed every 3 days.

Reverse transcription PCR and real-time PCR

To study the expression of TAUT mRNA in RAW264.7 cells and bone marrow macrophages, reverse transcription PCR was performed as described previously (Yuan et al. 2006; Liao et al. 2007). Total RNA was prepared by using the TRIzol Reagent (GIBCO-BRL) as described in the manufacturer's manual, and it was treated with RNase-free DNase I to prevent genomic DNA contamination. A first-strand synthesis kit (Invitrogen) was used to generate full-length cDNA from 2 μg of total RNA. The PCR primers for

mouse TAUT were 5′-GCCACATACTACCTATTCCA-3′ and 5′-CAGCAGCATACAGTCCCT-3′, yielding a 555-bp fragment. For mouse β-actin, the PCR primers were 5′-GAAGAGCTATGAGCTGCCTG-3′ and 5′-CACAGAGTACTTGCGCTCAG-3′, yielding a 307-bp fragment. The amplification was performed as follows: after initial denaturation at 94°C for 5 min, 30 cycles of PCR were performed at 94°C for 45 s, followed by annealing for 30 s and extension at 72°C for 45 s (10 min in the last cycle). The identities of the PCR products were confirmed by direct sequencing using an automatic DNA sequencer (PE Applied Biosystems).

For determining OPG and RANKL expression in osteoblasts treated with taurine, real-time quantitative PCR analysis was done using Roche Molecular LightCycler (Roche Applied Science, Indianapolis, IN, USA) as previously described (Luo et al. 2006). Osteoblasts were seeded in 24-well plates (2×10^4 cells/well). After 4 days of culture, the cells were treated for 48 h with the vehicle (serum-free α-MEM), 1, 5 or 10 mM taurine or 10^{-7} M 1, 25 vitD as a positive control (Kim et al. 2006; Kondo et al. 2004) in serum-free α-MEM. Total RNA from the cultured cells was isolated using TRIzol reagent, and reverse transcription was performed using 1.0 μg total RNA and a reverse transcription system (Promega). Amplification reactions were set up in 25-μL reaction volumes containing amplification primers and SYBR Green PCR Master Mix (PE Applied Biosystems). For each amplification reaction, 1 μL of cDNA was used. Preliminary experiments were carried out for the optimization of primer concentration. The primer sequences are detailed as previously described (Rucci et al. 2007). The PCR primers were as follows: for OPG, 5′-AGTCCGTGAAGCAGGAGT-3′ and 5′-CCATCTGGACATTTTTTGCAAA-3′; for RANKL, 5′-CCAAGATCTCTAACATGACG-3′ and 5′-CACCATCAGCTGAAGATAGT-3′; and for GAPDH, 5′-CACCATGGAGAAGGCCGGGG-3′ and 5′-GACGGACACATTGGGGGTAG-3′.

PCR amplifications were performed, and calibration curves were run in parallel and in triplicate with each analysis. Each sample was analyzed six times during each experiment, and the experiments were carried out at least twice. The amplification data were analyzed using the sequence detector system software (PE Applied Biosystems). Relative quantification was calculated by normalizing the test crossing thresholds (C_t) with the GAPDH-amplified control C_t. The results were normalized to GAPDH and expressed as a percentage of the control.

Western blot analysis

Undifferentiated and differentiated RAW264.7 cells were rinsed twice with 1 mM EDTA in PBS and lysed with a lysis solution containing 50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 1% Triton X-100, 0.02% sodium azide, 10 mM EDTA, 10 μg/mL aprotinin and 1 μg/mL aminoethyl benzenesulfonyl fluoride (Sigma). The cells were scraped from the flasks with a rubber policeman and transferred to microcentrifuge tubes. The viscosity of the sample was reduced by brief sonication, and the insoluble material was removed by centrifugation for 5 min. The protein concentrations were determined using the Bradford protein assay. A 100-μg aliquot of protein from each cell lysate was mixed with 2× SDS gel-loading buffer and immediately boiled for 5 min. The lysate was electrophoresed on SDS gel, transferred onto a PVDF membrane and immunoblotted with the anti-TAUT polyclonal antibody (1:1,000) or anti-actin monoclonal antibody (1:1,000; Sigma). The ECL detection kit (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used for detection.

Immunocytochemistry

Mouse bone marrow macrophages were isolated as described above and seeded in 24-well plates in α-MEM containing 10% FBS, 30 ng/mL M-CSF and 50 ng/mL RANKL. After 5 days, the cells were fixed with 1% paraformaldehyde in PBS for 20 min at RT and washed with PBS. Nonspecific binding sites were blocked by treatment with 5% normal goat serum (Sigma) for 30 min at 4°C. The cells were then incubated in a pool of anti-TAUT antibodies for 1 h at 4°C. After washing with PBS, the cells were incubated with 1 μg/mL FITC-conjugated rabbit anti-goat IgG for 30 min at 4°C. The nuclei were counterstained with DAPI mounting solution. After washing with PBS, the cells were examined by fluorescence microscopy and selected fields were photographed.

Taurine uptake measurement

Taurine uptake activity was measured as previously described (Liao et al. 2007; Kim et al. 2003). The RAW264.7 cells (2×10^4 cells/mL) that were grown in six-well polyethylene dishes (9.6 cm^2/well) for 6 days with or without RANKL (50 ng/mL) were harvested. After the culture medium was removed, the cells were washed twice at 37°C with the uptake medium (20 mmol/L HEPES, 140 mmol/L NaCl, 5.4 mmol/L KCl, 1.8 mmol/L $CaCl_2$, 0.8 mmol/L $MgSO_4$, and 5 mmol/L glucose) and equilibrated in 2 mL of taurine uptake buffer at 37°C. Taurine uptake was initiated by adding an uptake buffer containing 1 μCi [^{3}H]taurine and increasing the concentrations of the unlabeled substances. After incubation for 20 min, the cells were rapidly washed with cold PBS containing 0.05% sodium azide and lysed with 0.1 M NaOH. The radioactivity of the lysate was measured by liquid scintillation spectrometry. The sixth well was used for the estimation of

the average protein content (grams of protein per well) by using the Bradford protein assay. The results were normalized with the protein content of the control.

Cell viability test

The viability of the bone marrow macrophages and RAW264.7 cells was determined by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test (Mosmann 1983). Bone marrow macrophages and RAW264.7 cells were incubated for the indicated times at a density of 10^5 cells/well in 96-well plates with or without taurine at a final volume of 0.1 mL. They were washed with PBS three times, and MTT (50 mg/mL) was added to the medium for 4 h at 37°C. Further, the supernatant was removed, and the formazone crystals were dissolved using dimethyl sulfoxide (DMSO). The absorbance was read at 570 nm by using a Micro-ELISA reader (Bio-Tek, Winooski, VT, USA).

RNA interference for TAUT

RNA interference was used to downregulate the expression of TAUT in RAW264.7 cells and bone marrow cells as previously described (Liu et al. 2009). Two pairs of small interfering RNAs (siRNAs) were synthesized by Genesil Biotechnology Co., Wuhan, China. The sequences of the sense mouse TAUT siRNAs used were GATCTGTCCTTT GTTCTCTTT. Control siRNAs were synthesized by Genesil, Inc. For gene knockdown experiments, RAW264.7 cells and bone marrow cells were plated in 60 mm-diameter dish and cultured for 24 h in medium without antibiotics. Cells were transfected with siRNAs (0.4 nmol/well) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After 24 h of culture, cells were retransfected with siRNAs and then recultured for another 48 h. Protein expression was analyzed by immunoblot analysis.

Statistics

All data are expressed as the mean ± SD of triplicate or quadruplicate determinations. Student's *t* test was used for the evaluation of significance.

Results

Taurine inhibited osteoclastogenesis in a coculture of osteoblasts and bone marrow cells without affecting OPG and RANKL expression

We first examined the effect of taurine on osteoclastogenesis by coculturing osteoblasts and bone marrow cells. Figure 1 shows a microscopic view of TRACP staining. Multinucleate $TRACP^+$ cells containing more than three nuclei were identified as osteoclasts and counted. During the coculture of osteoblasts and bone marrow cells for 6 days, taurine decreased the formation of $TRACP^+$ multinucleated osteoclasts in a dose-dependent manner.

Figure 2 shows that osteoblast expression of the OPG mRNA and the RANKL mRNA were not affected by taurine during osteoclastogenesis, while the positive control 1, 25 vitD significantly downregulated OPG mRNA expression and upregulate RANKL mRNA expression, indicating that taurine might directly inhibited osteoclastogenesis via a system other than the OPG/RANKL signal system in vitro.

Taurine directly inhibited osteoclastogenesis in RAW264.7 cells and bone marrow macrophages

Our previous study generated osteoclasts by stimulating murine RAW264.7 macrophage-like cells with 50 ng/mL RANKL. After 6 days of culture, numerous multinucleated giant cells were formed. These cells strongly expressed osteoclast-specific markers, including TRACP, cathepsin K, calcitonin receptor and matrix metalloproteinase-9 (MMP-9); moreover, they formed resorption pits on a calcium phosphate-coated plate (Xiao et al. 2005).

To test the possibility that taurine directly affected osteoclast formation, we examined the impact of taurine on osteoclastogenesis from RAW264.7 cells stimulated with RANKL. As shown in Fig. 3, the number of osteoclasts formed from cells treated with RANKL and taurine were considerably less than that formed from cells treated with RANKL alone. This indicated that taurine could directly inhibit RANKL-induced RAW264.7-cell differentiation into mature osteoclasts.

To confirm this finding in normal cells, we generated osteoclasts from bone marrow macrophages in the presence of M-CSF and RANKL. We observed that bone marrow macrophage-derived osteoclasts were more efficiently generated in the presence of 30 ng/mL M-CSF and 50 ng/mL RANKL. Taurine also significantly inhibited bone marrow macrophage differentiation into osteoclasts (Fig. 3c).

We ruled out the possible inhibitory effect of taurine toxicity on osteoclastogenesis by an MTT assay, in which taurine had no influence on the viability of RAW264.7 cells and bone marrow macrophages (data not shown).

Osteoclasts expressed functional TAUT

We observed the differentiation of RAW264.7 cells and bone marrow macrophages to identify TAUT expression during osteoclastogenesis. RT-PCR with these

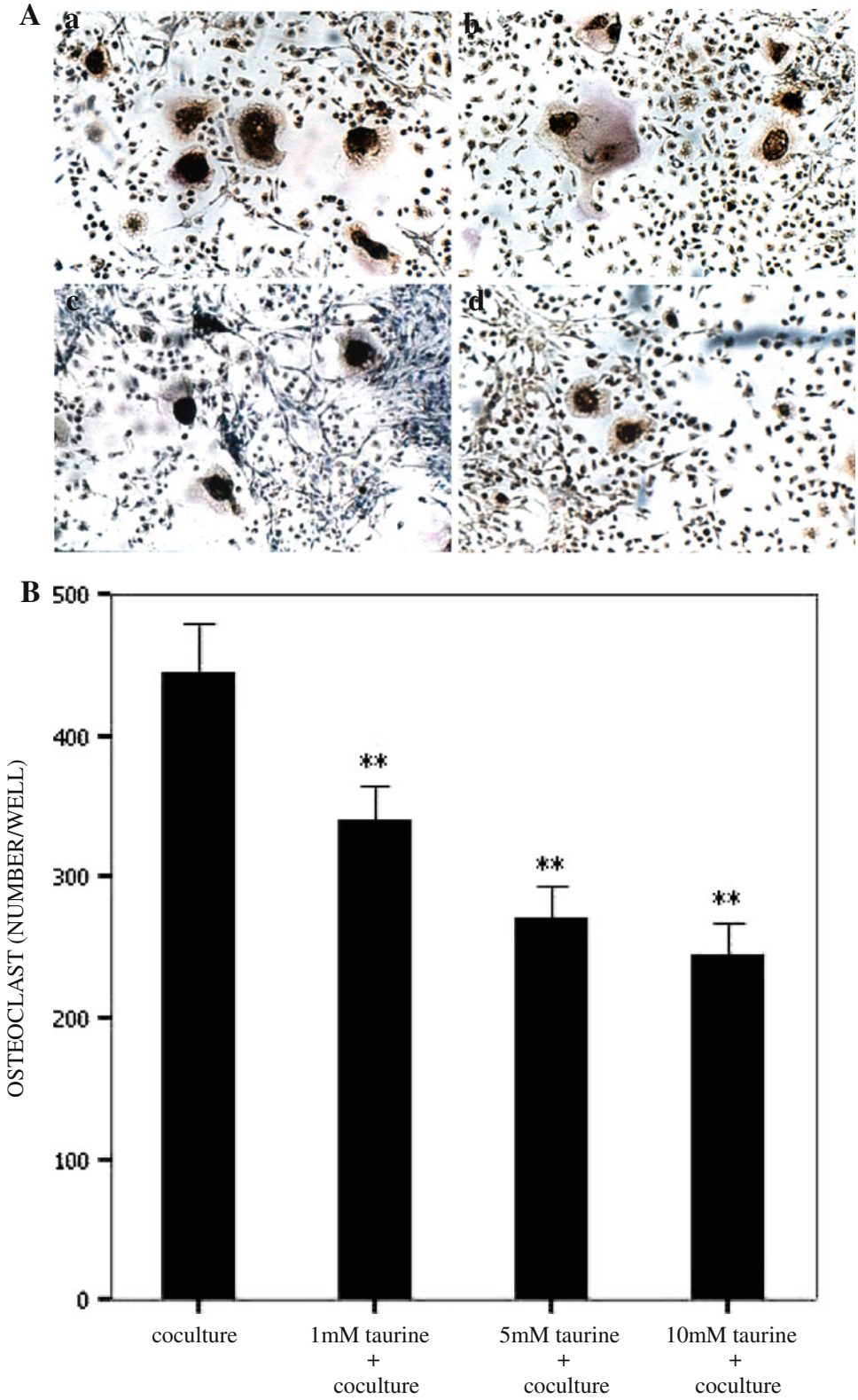

Fig. 1 Taurine inhibits osteoclastogenesis in a coculture of osteoblasts and bone marrow cells. Osteoblasts and bone marrow cells were cocultured in the presence of $1\alpha,25(OH)_2D_3$ and taurine. After 6 days of culture, multinucleated $TRACP^+$ cells were identified as osteoclasts and counted. **A** Representative microscopic view of the effects of taurine on osteoclastogenesis in a coculture of osteoblasts and bone marrow cells at a magnification of 200×. *a* Control. *b* Treatment with 1 mM taurine. *c* Treatment with 5 mM taurine. *d* Treatment with 10 mM taurine. **B** Inhibitory effect of taurine on osteoclastogenesis. Cells were treated with control (media alone); 1, 5 or 10 mM taurine. Taurine at a concentration of 1–10 mM significantly inhibited osteoclastogenesis. The bar represents the mean ± SD ($n = 3$; $^{**}p < 0.01$ vs. control)

TAUT-specific primers amplified products of the sizes expected for cDNA synthesized from TAUT transcripts in RAW264.7 cells and bone marrow macrophages. No products were observed when the RT was omitted during synthesis of cDNA (Fig. 4a). DNA sequence analysis of each PCR product revealed 100% homologies with the mus

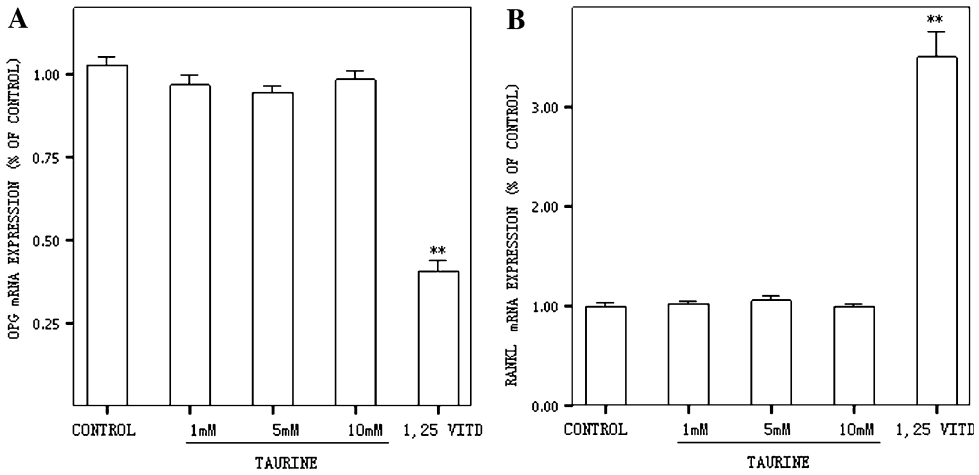

Fig. 2 Effects of taurine on OPG and RANKL mRNA expression in osteoblasts. Cells were exposed for 48 h to 1–10 mM taurine or 10^{-7} M $1\alpha,25(OH)_2D_3$ as a positive control. RANKL and OPG mRNA expression was determined by real-time quantitative PCR. Results are expressed as percentage of the control. Our results showed that taurine had no effect on OPG mRNA and RANKL mRNA expression in osteoblasts, while the positive control $1\alpha,25(OH)_2D_3$ significantly decreased OPG mRNA and increased RANKL mRNA expression. The *bar* represents the mean ± SD ($n = 3$; $^{**}p < 0.01$)

musculus retina and human placental TAUT cDNA sequences, respectively. TAUT proteins were detected in low abundance at the undifferentiated stage, and their expression increased after RANKL stimulation (Fig. 4b, c); this was further reconfirmed by the immunohistochemical analysis of bone marrow macrophage-derived osteoclasts (Fig. 5).

To determine whether the osteoclast-expressed TAUT was functional, we performed [^{3}H]taurine uptake assays in RANKL-induced RAW264.7 cells. Differentiated osteoclasts transported [^{3}H]taurine in a concentration-dependent manner, indicating that TAUT can be saturated (Fig. 6).

TAUT knockdown by siRNA interference abolished the inhibitory effect of taurine on osteoclastogenesis

The functional expression of TAUT in osteoclasts suggested that it could play a role in the differentiation of this cell type. Therefore, we determined whether the TAUT knockdown using siRNA could prevent the inhibitory effect of taurine on osteoclastogenesis. As shown in Fig. 7a, treatment with siRNA-TAUT, but not siRNA control, blocked the expression of TAUT protein in RAW264.7 cells. It showed the TAUT knockdown efficiency in RAW264.7 cells by RNA interference, while [^{3}H]taurine uptake function of TAUT was decreased by TAUT siRNA (Fig. 6). To further verify the TAUT function in taurine-inhibited osteoclastogenesis, we used the TAUT knockdown by siRNA interference. As shown in Fig. 7, taurine inhibited the formation of TRACP$^+$ osteoclasts from RANKL-induced RAW264.7 cells, while TAUT siRNA significantly abolished this effect of taurine.

To confirm this finding in normal cells, we generated osteoclasts from bone marrow macrophages in the presence of M-CSF and RANKL. TAUT siRNA significantly abolished the inhibitory effect of taurine on osteoclastogenesis in this case as well (data not shown).

Discussion

Taurine was isolated from the bile of the ox (*Bos taurus*) a century and a half ago. It has been only considered as a metabolic product of sulfur-containing amino acids. Research on taurine in the modern era has suggested its involvement in various physiological processes, including calcium modulation, membrane stabilization and the regulation of intracellular osmosis and protein phosphorylation (Militante and Lombardini 2003; Pasantes-Morales et al. 1998; Huxtable 1992). Recent studies demonstrated that taurine supplementation played a positive role in bone metabolism (Cheong and Chang 2009; Choi and DiMarco 2009). In addition to these effects, our data provided evidence for another role played by taurine, i.e., in osteoclastogenesis. The transcription and translation of TAUT in osteoclasts was one of the key factors in the regulation of the differentiation of bone marrow macrophages and RAW264.7 cells. Blocking TAUT with siRNA resulted in the prevention of the direct inhibitory effect of taurine on osteoclastogenesis. The effect of taurine-inhibiting osteoclastogenesis was independent of the OPG/RANKL system.

Recently, taurine was identified in bone, but its precise function has not been fully understood. Cheong et al.

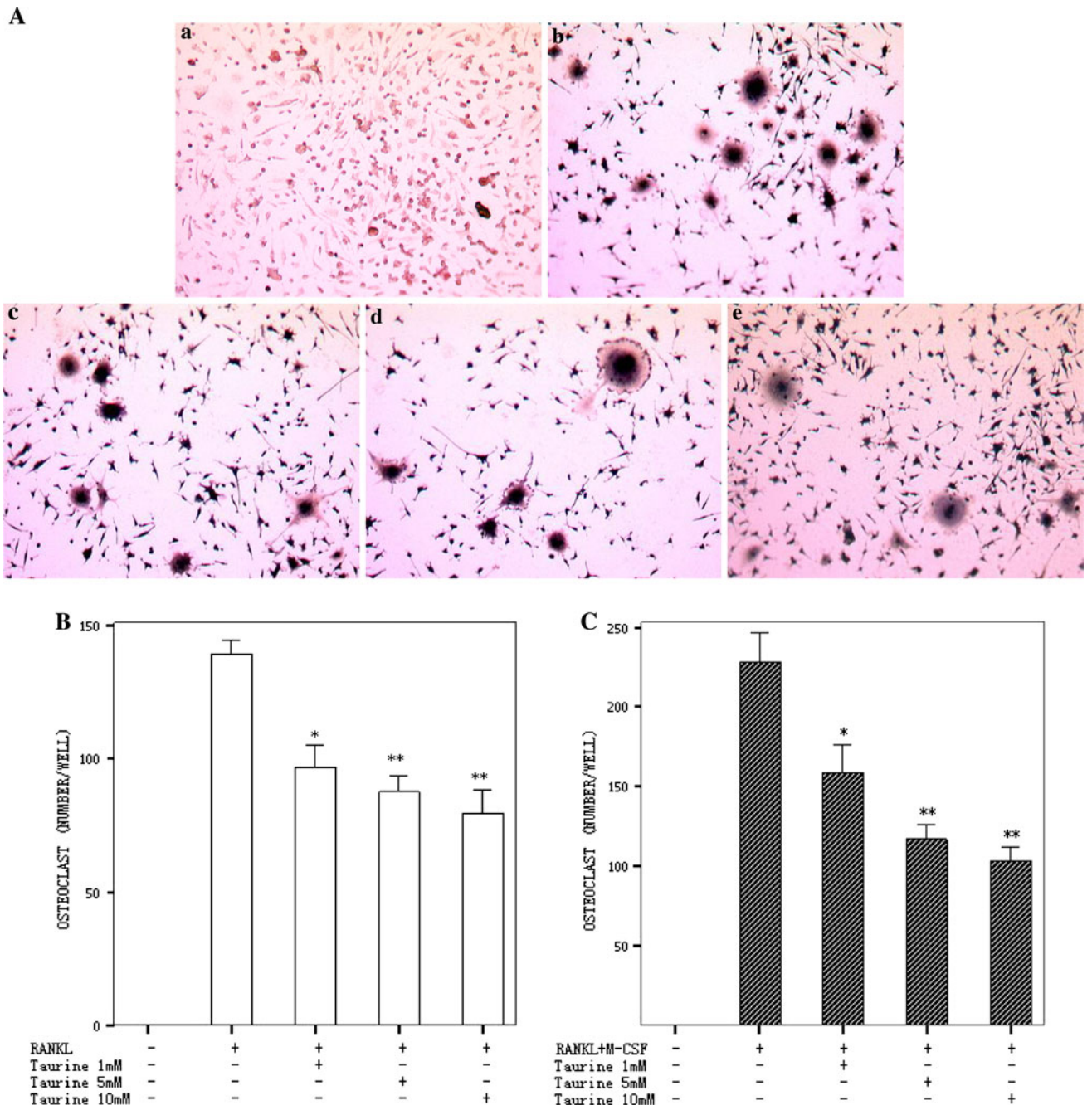

Fig. 3 Taurine directly inhibited osteoclastogenesis from RANKL-induced RAW264.7 cells and M-CSF and RANKL-induced bone marrow macrophages. **A** RAW264.7 cells were grown in the presence of 50 ng/mL RANKL with or without taurine. After 6 days of culture, the multinucleated $TRACP^+$ cells were identified as osteoclasts and counted. Representative microscopic view of the effects of taurine on osteoclastogenesis in the RANKL-induced RAW264.7 cells at a magnification of 200×. *a* Control. *b* Osteoclasts from RANKL-induced RAW264.7 cell. *c* Osteoclasts from RANKL-induced RAW264.7 cell treated with 1, 5 or 10 mM taurine, respectively. **B** Inhibitory effect of taurine on osteoclastogenesis from RANKL-induced RAW264.7 cells. The control group could not form $TRACP^+$ cells. All other groups were treated with 50 ng/mL RANKL. Cells were treated with 1, 5 or 10 mM taurine. Taurine significantly decreased osteoclastogenesis from the RANKL-induced RAW264.7 cells. **C** Bone marrow macrophages were grown in the presence of 30 ng/mL M-CSF and 50 ng/mL RANKL with or without taurine. After 6 days of culture, the multinucleated $TRACP^+$ cells were identified as osteoclasts and counted. There were no osteoclastogenesis in the control group without M-CSF and RANKL stimulation. Taurine significantly decreased osteoclastogenesis in M-CSF- and RANKL-induced bone marrow macrophages. The *bar* represents the mean ± SD ($n = 3$; $^*p < 0.05$, $^{**}p < 0.01$ vs. control)

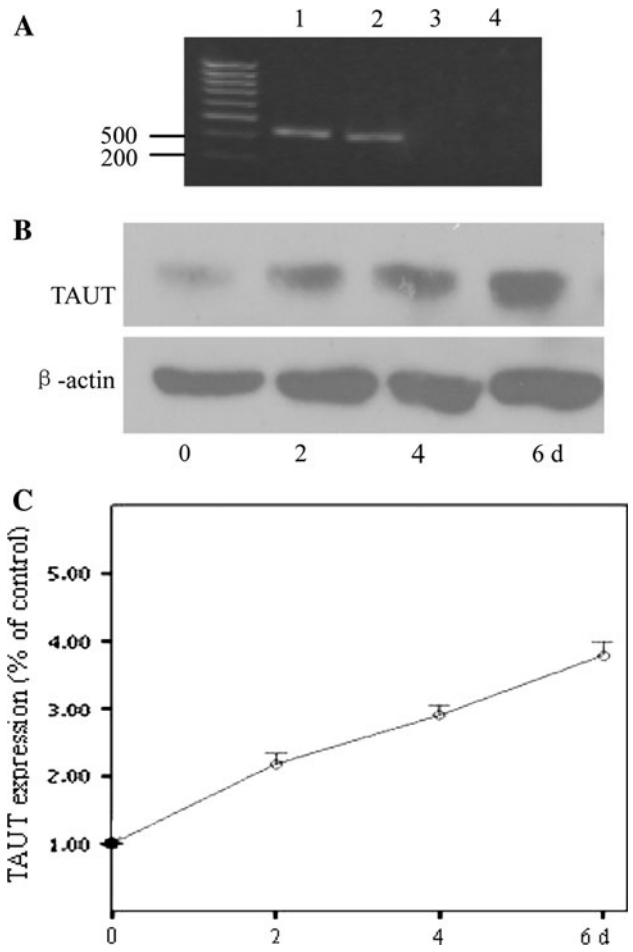

Fig. 4 Expression of TAUT mRNA and protein in osteoclasts. **a** Total RNA from primary RANKL-induced osteoclasts (*lane 1*) and bone marrow macrophages (*lane 2*) were subjected to RT-PCR. The PCR products were visualized in a 1.2% agarose gel stained with ethidium bromide. Omission of RT (*lanes 3 and 4*) was used as negative control. **b** TAUT protein expressed in undifferentiated and differentiated RAW264.7 cells. Representative results of Western blot analysis of undifferentiated and differentiated RAW264.7 cells by using a TAUT antibody. 0 day: no stimulation by RANKL; 2, 4 and 6 days: RANKL stimulation for 2, 4 and 6 days, respectively. **c** The levels of TAUT protein were increased during osteoclastogenesis. The protein expression was normalized to *β*-actin. The data represents the mean ± SD of three similar experiments

demonstrated taurine supplementation could prevent bone loss in ovariectomized rats (Cheong and Chang 2009), and Choi et al. found taurine supplementation could slightly increase femur and spine bone mineral content; however, compared with the control group the difference was not statistically significant (Choi and DiMarco 2009). In osteoblasts, taurine could stimulate osteoblasts proliferation and differentiation through ERK1/2 activation signal pathway (Park et al. 2001). In osteoclasts, taurine inhibited osteoclast formation induced by lipopolysaccharide, IL-1alpha and PGE2 (Koide et al. 1999), and repressed osteoclast differentiation mediated by *Porphyromonas gingivalis* (Kum et al. 2003). These data showed that taurine played an important role in bone metabolism. In the present study, we evaluated the role of taurine in osteoclastogenesis and determined the effect of TAUT in this process.

Osteoblast-expressed OPG and RANKL are well-documented potent regulators of osteoclastogenic activity. RANKL, a member of the tumor necrosis factor (TNF) family, stimulated osteoclastogenesis, osteoclast differentiation, activity, and survival via the receptor activator of nuclear factor κB (RANK), and the behavior of soluble RANKL (sRANKL) cleaved from RANKL is similar to that of RANKL. However, OPG, a soluble decoy receptor against RANKL and sRANKL, has been identified as a key factor in the inhibition of osteoclast differentiation and activation (Khosla 2001; Aubin and Bonnelye 2000; Suda et al. 1999; Takahashi et al. 1999). The regulation of OPG synthesis and RANKL expression causes either activation or inactivation of osteoclasts; this considerably affects bone remodeling (Lee et al. 2002; Holloway et al. 2002). Taurine inhibited osteoclastogenesis in a coculture of osteoblasts and bone marrow cells; so, we investigated whether taurine influenced osteoclastogenesis through regulating OPG and RANKL expression in osteoblasts. Our present study demonstrated that taurine did not regulate OPG and RANKL expression in osteoblasts.

After ruling out the possibility of taurine regulation on OPG and RANKL expression, we hypothesized that taurine

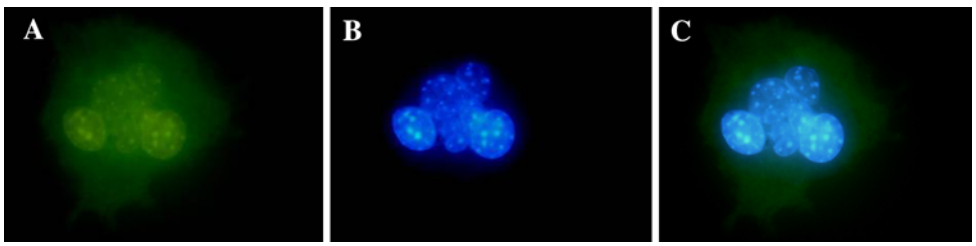

Fig. 5 Immunochemical analysis of TAUT expression in osteoclasts differentiated from M-CSF- and RANKL-induced bone marrow macrophages. **a** Osteoclasts stained with preimmune rabbit IgG and FITC-conjugated goat anti-rabbit IgG. **b** DAPI staining showed multinucleated osteoclasts. **c** Merged images

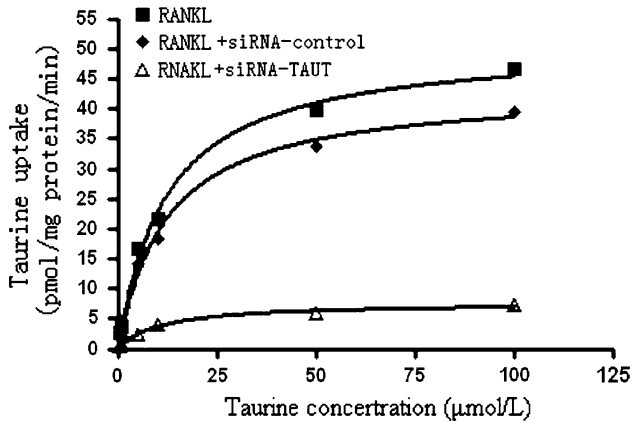

Fig. 6 TAUT is a functional protein in osteoclasts. Uptake of [^{3}H]taurine by osteoclasts (*filled squares*) and osteoclasts treated with siRNA-control (*filled diamonds*) and osteoclasts treated with siRNA-TAUT (*open triangles*)

might directly inhibit osteoclastogenesis from osteoclast precursors. Our results demonstrated that taurine could directly inhibit osteoclastogenesis in RANKL-induced RAW264.7 cells and M-CSF- and RANKL-stimulated bone marrow macrophages.

To gain further insight into the mechanism by which taurine involved osteoclastogenesis, we evaluated TAUT. Taurine was transported into the cells through a specific transporter, i.e., TAUT, which belongs to the gene family of Na^+- and/or Cl^--dependent neurotransmitter transporters (Liu et al. 1992). Human placental TAUT mRNA encodes a 620-amino acid protein with a calculated Mr of 69,853. The TAUT protein possesses 12 putative transmembrane domains and a large hydrophilic loop between transmembrane domains 3 and 4. This loop contains three potential N-glycosylation sites. Human TAUT (hTAUT) is intracellular, while the loop bearing the N-glycosylation sites is extracellular. In addition to these three glycosylation sites on the loop between transmembrane domains 3 and 4, a fourth N-glycosylation site is present on the extracellular loop between transmembrane domains 11 and 12 (Uchida et al. 1992; Jhiang et al. 1993; Ramamoorthy et al. 1994). A deficiency in TAUT expression or activity and/or changes in the cellular taurine concentration have been associated with a plethora of mammalian disorders, such as impaired skeletal muscle function (Warskulat et al. 2004), degeneration of retinal photoreceptors (Heller-Stilb et al. 2002) and abnormal development of the kidney, heart and central nervous system (Huxtable 1992). TAUT-knockout mice exhibited reduced fertility and loss of vision due to severe retinal degeneration (Warskulat et al. 2004), reduced total exercise capacity and electromyographic abnormalities (Heller-Stilb et al. 2002). However, little information is available in the literature with regard to TAUT and its effect on bone. Our previous results

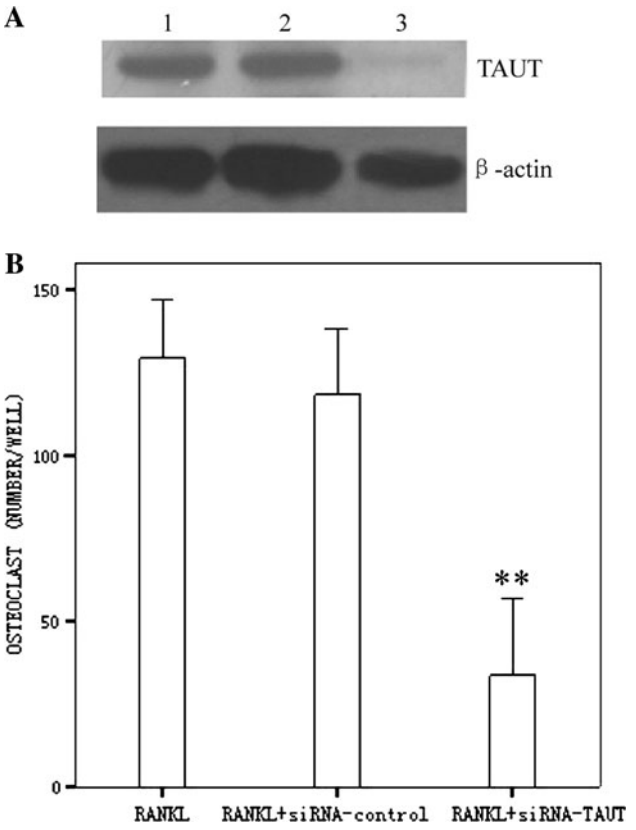

Fig. 7 TAUT siRNA downregulated TAUT expression in RAW264.7 cells and inhibited osteoclastogenesis. **a** Representative results of Western blot analysis using a TAUT antibody. Total cellular protein was subjected to immunoblot analysis using anti-TAUT and β-acting antibody. The anti-TAUT antibody identified a band at 70 kDa. *Lane 1* RAW264.7 cells; *lane 2* lysate from RAW264.7 cells treated with siRNA control; *lane 3* lysate from RAW264.7 cells treated with siRNA-TAUT. **b** TAUT siRNA significantly inhibited osteoclastogenesis. The *bar* represents the mean ± SD ($n = 3$; $^{**}p < 0.01$ vs. RANKL group)

demonstrated that TAUT was expressed in osteoblasts and that taurine may directly influence the metabolism of osteoblasts (Yuan et al. 2006, 2007). Moreover, taurine uptake by TAUT is regulated by extracellular calcium, calcium channel blockers and oxidative stresses, respectively (Kang and Kim 2008). We report that osteoclasts derived from the differentiation of RANKL-induced RAW264.7 cells can express TAUT protein, as assessed by Western blot analysis with a TAUT-specific pure IgG in vitro. Furthermore, RT-PCR performed on total RNA isolated from the osteoclasts followed by sequence analysis of the PCR products demonstrated the presence of TAUT transcripts in these cells. During osteoclastogenesis, the expression of TAUT protein was significantly increased. Moreover, immunohistochemical analysis of osteoclasts derived from M-CSF- and RANKL-induced bone marrow macrophages indicated that the TAUT protein was expressed in the osteoclasts. Our experiment also showed

the TAUT expressed in osteoclasts was functional, since it could actively uptake [^{3}H]taurine. These findings demonstrate that TAUT is transcribed and translated in both undifferentiated and differentiated osteoclasts. Further, the [^{3}H]taurine-uptake experiment confirmed that TAUT expressed in osteoclasts is functional. Moreover, TAUT siRNA abolished the TAUT expression in osteoclasts, [^{3}H]taurine uptake function and the inhibitory effect of taurine on osteoclastogenesis.

In conclusion, this study provides evidence that taurine may directly inhibit osteoclastogenesis through TAUT uptake. However, taurine does not directly affect the expression of OPG and RANKL in osteoblasts. These findings suggest a functional role of taurine in bone homeostasis that should be studied further.

Acknowledgments The National Natural Science Foundation of China (No. 30801174) and the Ph. D. Programs Foundation of the Ministry of Education of China (No. 200805331017).

References

Aubin JE, Bonnelye E (2000) Osteoprotegerin and its ligand: a new paradigm for regulation of osteoclastogenesis and bone resorption. Osteoporos Int 11:905–913

Cheong SH, Chang KJ (2009) The preventive effect of fermented milk supplement containing tomato (*Lycopersion esculentum*) and taurine on bone loss in ovariectomized rats. Adv Exp Med Biol 643:333–340

Choi MJ, DiMarco NM (2009) The effects of dietary taurine supplementation on bone mineral density in ovariectomized rats. Adv Exp Med Biol 643:341–349

D'Eufemia P, Finocchiaro R, Zambrano A, Tetti M, Ferrucci V, Celli M (2007) Reduction of plasma taurine level in children affected by osteogenesis imperfecta during bisphosphonate therapy. Biomed Pharmacother 61:235–240

Dolder S, Hofstetter W, Wetterwald A, Muhlbauer RC, Felix R (2006) Effect of monoterpenes on the formation and activation of osteoclasts in vitro. J Bone Miner Res 21:647–655

Gupta RC, Kim SJ (2003) Taurine, analogues and bone: a growing relationship. Adv Exp Med Biol 526:323–328

Gupta RC, Win T, Bitter S (2005) Taurine analogues, a new class of therapeutics: retrospect and prospect. Curr Med Chem 1247: 2021–2039

Heller-Stilb B, van Roeyen C, Rascher K, Hartwig HG, Huth A, Seeliger MW, Warskulat U, Haussinger D (2002) Disruption of the taurine transporter gene (taut) leads to retinal degeneration in mice. FASEB J 16:231–233

Hofbauer LC, Heufelder AE (2001) Role of receptor activator of nuclear factor-kappa B ligand and osteoprotegerin in bone cell biology. J Mol Med 79:243–253

Holloway WR, Collier FM, Aitken CJ, Myers DE, Hodge JM, Malakellis M, Gough TJ, Collier GR, Nicholson GC (2002) Leptin inhibits osteoclast generation. J Bone Miner Res 17:200–209

Huxtable RJ (1992) Physiological actions of taurine. Physiol Rev 72:101–163

Jhiang SM, Fithian L, Smanik P, McGill J, Tong Q, Mazzaferri EL (1993) Cloning of the human taurine transporter and characterization of taurine uptake in thyroid cells. FEBS Lett 318:139–144

Kang YS, Kim SJ (2008) The change of taurine transport in osteocytes by oxidative stress, hypertonicity and calcium channel blockers. Biomol Therap 16:219–225

Khosla S (2001) Minireview: the OPG/RANKL/RANK system. Endocrinology 142:5050–5055

Kim HW, Kim JH, An HS, Park KK, Kim BK, Park T (2003) Myo-inositol restores the inflammation-induced down-regulation of taurine transport by the murine macrophage cell line, RAW 264.7. Life Sci 73:2477–2489

Kim S, Yamazaki M, Zella LA, Shevde NK, Pike JW (2006) Activation of receptor activator of NF-kappaB ligand gene expression by 1, 25-dihydroxyvitamin D3 is mediated through multiple long-range enhancers. Mol Cell Biol 26:6469–6486

Koide M, Okahashi N, Tanaka R, Kazuno K, Shibasaki K, Yamazaki Y, Kaneko K, Ueda N, Ohguchi M, Ishihara Y, Noguchi T, Nishihara T (1999) Inhibition of experimental bone resorption and osteoclast formation and survival by 2-aminoethanesulphonic acid. Arch Oral Biol 44:711–719

Kondo T, Kitazawa R, Maeda S, Kitazawa S (2004) 1 alpha, 25 dihydroxyvitamin D3 rapidly regulates the mouse osteoprotegerin gene through dual pathways. J Bone Miner Res 19:1411–1419

Kum KY, Park JH, Yoo YJ, Choi BK, Lee HJ, Lee SJ (2003) The inhibitory effect of alendronate and taurine on osteoclast differentiation mediated by *Porphyromonas gingivalis* sonicates in vitro. J Endod 29:28–30

Kwak HB, Lee SW, Li YJ, Kim YA, Han SY, Jhon GJ, Kim HH, Lee ZH (2004) Inhibition of osteoclast differentiation and bone resorption by a novel lysophosphatidylcholine derivative, SCOH. Biochem Pharmacol 67:1239–1248

Lambert IH (2004) Regulation of the cellular content of the organic osmolyte taurine in mammalian cells. Neurochem Res 29:27–63

Lee SE, Woo KM, Kim SY, Kim HM, Kwack K, Lee ZH, Kim HH (2002) The phosphatidylinositol 3-kinase, p38, and extracellular signal-regulated kinase pathways are involved in osteoclast differentiation. Bone 30:71–77

Liao XB, Zhou XM, Li JM, Tan ZP, Liu LM, Zhang W, Tan H, Lu Y, Yuan LQ (2007) Taurine transporter is expressed in vascular smooth muscle cells. Amino Acids 33:639–643

Liu QR, Lopez-Corcuera B, Nelson H, Mandiyan S, Nelson N (1992) Cloning and expression of a cDNA encoding the transporter of taurine and b-alanine in mouse brain. Proc Natl Acad Sci USA 89:12145–12149

Liu YS, Lu Y, Liu W, Xie H, Luo XH, Wu XP, Yuan LQ, Liao EY (2009) Connective tissue growth factor is a downstream mediator for peptin-induced proliferation and differentiation in human osteoblasts. Amino Acids [Epub ahead of print]

Lubec B, Ya-hua Z, Pertti S, Pentti T, Kizmuller E, Lubec G (1997) Distribution and disappearance of the radiolabeled carbon derived from L-arginine and taurine in the mouse. Life Sci 60:2373–2381

Luo XH, Guo LJ, Xie H, Yuan LQ, Wu XP, Zhou HD, Liao EY (2006) Adiponectin stimulates RANKL and inhibits OPG expression in human osteoblasts through the MAPK signaling pathway. J Bone Miner Res 21:1648–1656

Militante JD, Lombardini JB (2003) Taurine stimulation of calcium uptake in the retina: mechanism of action. Adv Exp Med Biol 526:547–554

Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 65:55–63

Parfitt AM (1987) Bone remodeling and bone loss: understanding the pathophysiology of osteoporosis. Clin Obstet Gynecol 30:789–811

Park S, Kim H, Kim SJ (2001) Stimulation of ERK2 by taurine with enhanced alkaline phosphatase activity and collagen synthesis in

osteoblast-like UMR-106 cells. Biochem Pharmacol 62:1107–1111

Pasantes-Morales H, Quesada O, Moran J (1998) Taurine: an osmolyte in mammalian tissues. Adv Exp Med Biol 442:209–217

Ramamoorthy S, Leibach FH, Mahesh VB, Han H, Yang-Feng T, Blakely RD, Ganapathy V (1994) Functional characterization and chromosomal localization of a cloned taurine transporter from human placenta. Biochem J 300:893–900

Rodan GA (1992) Introduction to bone biology. Bone 13:S3–S6

Rucci N, Rufo A, Alamanou M, Teti A (2007) Modeled microgravity stimulates osteoclastogenesis and bone resorption by increasing osteoblast RANKL/OPG ratio. J Cell Biochem 100:464–473

Suda T, Takahashi N, Udagawa N, Jimi E, Gillespie MT, Martin TJ (1999) Modulation of osteoclast differentiation and function by the new members of the tumor necrosis factor receptor and ligand families. Endocr Rev 20:345–357

Takahashi N, Udagawa N, Suda T (1999) A new member of tumor necrosis factor ligand family, ODF/OPGL/TRANCE/RANKL, regulates osteoclast differentiation and function. Biochem Biophys Res Commun 256:449–455

Terauchi A, Nakazaw A, Johkura K, Yan L, Usuda N (1998) Immunohistochemical localization of taurine in various tissue of the mouse. Amino Acids 15:151–160

Uchida S, Kwon HM, Yamauchi A, Preson AS, Marumo F, Handler JS (1992) Molecular cloning of the cDNA for an MDCK cell Nat and Cl-dependent taurine transporter that is regulated by hypertonicity. Proc Natl Acad Sci USA 89:8230–8234

Warskulat U, Flogel U, Jacoby C, Hartwig HG, Thewissen M, Merx MW, Molojavyi A, Heller-Stilb B, Schrader J, Haussinger D (2004) Taurine transporter knockout depletes muscle taurine levels and results in severe skeletal muscle impairment but leaves cardiac function uncompromised. FASEB J 18:577–579

Wright CE, Tallan HH, Lin YY, Gaull GE (1986) Taurine: biological update. Annu Rev Biochem 55:427–453

Xiao XH, Liao EY, Zhou HD, Dai RC, Yuan LQ, Wu XP (2005) Ascorbic acid inhibits osteoclastogenesis of RAW264.7 cells induced by receptor-activated nuclear factor kappaB ligand (RANKL) in vitro. J Endocrinol Invest 28:253–260

Yasutomi C, Nakamuta H, Fujita T, Takenaga T, Koida M (2002) Anti-osteopenic effect of taurine: possible involvement of activated MEK-ERK-Cbfa1 signaling. Nippon Yakurigaku Zasshi 120:114–115

Yuan LQ, Xie H, Luo XH, Wu XP, Zhou HD, Lu Y, Liao EY (2006) Taurine transporter is expressed in osteoblasts. Amino Acids 31:157–163

Yuan LQ, Lu Y, Luo XH, Xie H, Wu XP, Liao EY (2007) Taurine promotes connective tissue growth factor (CTGF) expression in osteoblasts through the ERK signal pathway. Amino Acids 32:425–430

Zaidi M, Blair HC, Moonga BS, Abe E, Huang CL (2003) Osteoclastogenesis, bone resorption, and osteoclast-based therapeutics. J Bone Miner Res 18:599–609