

# Taurine increases cell proliferation and generates an increase in $[Mg^{2+}]_i$ accompanied by ERK 1/2 activation in human osteoblast cells

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**Abstract** Taurine has been reported to influence bone metabolism, and its specific transport system, the taurine transporter, is expressed in osteoblasts. The mean  $[Mg^{2+}]_i$  was  $0.51 \pm 0.01$  mM in normal culture media. Taurine caused an increase in  $[Mg^{2+}]_i$  by  $0.72 \pm 0.04$  mM in human osteoblast (HOB) cells. This increment in  $[Mg^{2+}]_i$  was inhibited significantly by PD98059, nifedipine, lidocaine, and imipramine. Taurine was also shown to stimulate the activation of ERK 1/2. This taurine-stimulated ERK 1/2 activation was inhibited by PD98059. In the present study, taurine was shown to increase cell proliferation and generate an increase in  $[Mg^{2+}]_i$  accompanied by ERK 1/2 activation in HOB cells.

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**Keywords:** Taurine; Intracellular magnesium; ERK 1/2; Human osteoblast cell; PD98059

## 1. Introduction

Taurine, 2-aminoethanesulfonic acid, is the principal free  $\beta$ -amino acid in mammals, and is also detected in high concentrations in the brain, heart, eye, muscle, and liver. It can be synthesized from cysteine acid or hypotaurine, which is derived from methionine and cysteine within the body [1]. A variety of physiological roles have been suggested for taurine, including calcium modulation, membrane stabilization, the intracellular regulation of osmosis, and the regulation of protein phosphorylation [2–5]. Thus, changes in the net cellular taurine content may exert a dramatic impact on cell function. Taurine has been shown to influence bone metabolism, and its specific transport system, the taurine transporter, is expressed in osteoblasts

[6,7]. Furthermore, taurine promotes the expression of connective tissue growth factor (CTGF) in osteoblasts via the ERK signal pathway [8]. Moreover, taurine has been determined to inhibit experimental bone resorption and the formation and survival of osteoclasts [9].

In association with bone metabolism, the results of a number of studies have shown that divalent cations, including  $Mg^{2+}$ , perform a pivotal role in bone remodeling and skeletal development [10,11].  $Mg^{2+}$  exists in macronutrient quantities in bone (0.5–1% bone ash), and dietary  $Mg^{2+}$  deficiencies have been implicated as a risk factor for osteoporosis. The effect of selective dietary  $Mg^{2+}$  depletion has been studied extensively in the rat. Impaired bone growth, reduced bone formation, increased bone resorption, osteoporosis, and increased skeletal fragility have all been observed [12–16]. Recently, Kim et al. [17] reported that the augmentation of intracellular magnesium ( $[Mg^{2+}]_i$ ) in  $H_9C_2$  cells is related to the activation of the ERK signal pathway.

The present study attempted to determine the effects of taurine treatment on cell proliferation, intracellular  $Mg^{2+}$  regulation, and the activation of mitogen-activated protein kinase (MAPK) in human osteoblast (HOB) cells.

## 2. Materials and methods

### 2.1. Materials

Dulbecco's modified eagle's medium nutrient mixture F-12 HAM (DMEM-F12 HAM) media were purchased from Sigma (St. Louis, MO). Fetal calf serum was acquired from Gibco (Grand Island, NY). Mag-fura-2AM was obtained from Molecular Probes (Eugene, OR). The MAPK inhibitor (PD98059), taurine, calcium channel blocker (nifedipine), sodium channel blocker (lidocaine, imipramine) and other materials were purchased from Sigma.

### 2.2. Cell culture

HOB cells were purchased from ATCC (No.CRL-11372). The HOB cell line was grown on 50 mL tissue culture flasks in DMEM-F12 HAM media: this was supplemented with 10% fetal calf serum, 5 mM L-glutamine, 50 U/mL of penicillin, and 50  $\mu$ g/mL of streptomycin in a humidified 5%  $CO_2$ –95% air environment at 37 °C.

For the performance of the fluorescent studies, confluent cells were washed three times in phosphate-buffered saline (PBS) containing 5 mM ethylene glycol-bis ( $\beta$ -aminoenyl ether)  $N,N,N',N'$ -tetraacetic acid (EGTA); they were then trypsinized and seeded onto glass coverslips. Aliquots of the harvested cells were permitted to settle on sterile

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**Abbreviations:** MAPK, mitogen-activated protein kinase; ERK 1/2, extracellular signal-regulated kinase 1/2; HOB, human osteoblast; LDH, lactate dehydrogenase;  $[Mg^{2+}]_i$ , intracellular  $Mg^{2+}$  concentration

glass coverslips in 100-mm Corning tissue culture dishes, and the cells were grown to subconfluence over 1–2 days in supplemented media, as was described above.

### 2.3. Lactate dehydrogenase (LDH) measurements

HOB cells were grown on 6-well plates ( $8 \times 10^4$ /well), and cultured for 48 h in DMEM-F12 HAM media containing 10% fetal calf serum. The HOB cells were either left untreated, or treated with 10 mM taurine, or 20 mM taurine, for 48 h. Culture media were collected and utilized to measure LDH activity via a chemical colorimetric method. LDH activity was determined by measuring the level of pyruvic acid with a spectrophotometer (SpectraMax fluorometer with SoftMax Programme Molecular Probes, USA), and the absorbance was determined at a wavelength of 490 nm.

### 2.4. The MTT test

The centrifuged deposits of control or treated cells were washed twice with PBS and were then treated with approximately 50  $\mu$ L of the MTT solution (0.5 mg/mL PBS); the tubes, after mixing of the contents by side-tapping, were incubated at 37 °C for 3 h. The tubes containing MTT-cell mixtures were centrifuged ( $400 \times g$  for 10 min) to deposit the cells, the supernatant MTT solution was pipetted out and then acid-isopropanol (95 mL isopropanol with 5 mL 3 N HCl) was added to the colored cell deposit. After stirring of the acid-alcohol-treated deposit with a glass rod, the mixture was allowed to react for 5 min, followed by centrifugation at  $400 \times g$  for 10 min. One hundred microliter of the purple-blue colored supernatant that contained the solubilized formazan in each sample was added to a well in a 96-well plate for spectrophotometry at 570 nm in an ELISA reader. Acid-isopropanol was used as a blank in the readings.

### 2.5. Total magnesium measurements

After 48 h, confluent cells were washed three times in PBS. After washing, the cells were scraped with a cell scraper. The harvested cells were homogenized immediately and the extract was transferred to a 1.5-mL microfuge tube, which was placed on ice. The sample was then sonicated for five pulses at a 40% duty cycle using a Sonics & Materials Ultrasonic Processor (USA), then microcentrifuged for 10 min at 10000 rpm. The supernatant was collected and used to measure total magnesium with an atomic absorption spectrophotometer (Analab 9200, Seoul, Korea) at a wavelength of 285.2 nm.

### 2.6. Cytoplasmic $Mg^{2+}$ measurements

The coverslips were mounted in a perfusion chamber, and the intracellular concentration of free  $Mg^{2+}$  ( $[Mg^{2+}]_i$ ) was determined using the  $Mg^{2+}$ -sensitive fluorescent dye, Mag-fura-2AM (Molecular Probes). The cell-permeant acetoxyethyl ester (AM) from the dye was dissolved in dimethyl sulfoxide (DMSO) to make a stock concentration of 5 mM, and this was diluted to 2.5  $\mu$ M with mag-fura-2AM in media for 30 min at 37 °C. The cells were then washed three times in buffered salt solution containing (in mM): 145 NaCl, 4.0 KCl, 0.8  $K_2HPO_4$ , 0.2  $KH_2PO_4$ , 1.0  $CaCl_2$ , 5.0 glucose, and 20 *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)/Tris (hydroxymethyl) aminomethane (Tris) at a pH of 7.4. The HOB cells were incubated for an additional 30 min to allow for complete deesterification, after which the cells were washed once in this buffer solution prior to measuring the fluorescence.

Epifluorescence microscopy was employed to monitor the changes in Mag-fura-2AM within the single HOB cells. The chamber (0.5 mL) was mounted on an inverted Nikon Diaphot-TMD microscope equipped with a FluorX100 objective, and the fluorescence was monitored within a single cell under oil immersion over the course of the study. The fluorescence was recorded at 1-s intervals with a dual-excitation wavelength spectrophotometer (Delta-scan, Photon Technologies, Princeton, NJ) with the excitation wavelengths for mag-fura-2 at 340 and 380 nm (the chopper speed was set at 100 Hz), and an emission wavelength of 495 nm. All of the experiments were conducted at 23 °C with continuous exchange of the bathing solution (1 mL/min). Media changes were conducted without interrupting the recording.

The  $[Mg^{2+}]_i$  was calculated from the ratio of the fluorescence at the two excitation wavelengths as described previously, using a dissociation constant ( $K_d$ ) of 1.4 mM, for the Mag-fura-2/ $Mg^{2+}$  complex.

The  $R_{max}$  for mag-fura-2 was determined via the addition of 50 mM  $MgCl_2$  in the absence of  $Ca^{2+}$ , and the  $R_{min}$  was obtained via the removal of  $Mg^{2+}$  and the addition of 100 mM EDTA, at a pH of 7.2.

### 2.7. Western blot analysis of the MAPK (ERK 1/2)

After incubation, the medium was aspirated and the cells were washed three times in 100  $\mu$ L of ice-cold PBS SDS sample buffer (2.5 mM Tris-HCl, 2% SDS, 10% glycerol, 50 mM DTT, and 0.1% bromophenol blue, pH 6.8). The cells were homogenized immediately and the extract was transferred to a 1.5-mL microfuge tube, which was placed on ice. The sample was then sonicated for five pulses at 40% duty cycle using a Sonics & Materials Ultrasonic Processor (USA), then microcentrifuged for 30 min at 14000 rpm. Thirty microliters of the supernatant was loaded onto 7.5% SDS-PAGE gel. After conducting electrophoresis, the protein was electrotransferred to a Hybond-ECL nitrocellulose membrane. The membrane was blocked with Tris-buffered saline (20 mM Tris and 140 mM NaCl, pH 7.6) containing 0.1% Tween20 (TBST) and 5% milk for 2 h at room temperature. The membrane was then incubated overnight with monoclonal phosphospecific p44/42 MAPK antibody, which was the primary antibody, at a 1:1000 dilution in TBST with 5% milk at 4 °C. The blot was then washed three times for 10 min each in TBST (three times for 5 min each) and the bands were detected using ECL with exposure to X-OMAT AR film. The blots were quantified via laser scanning densitometry (Bio-Rad, USA).

### 2.8. Statistical analysis

The results were expressed as the means  $\pm$  standard error of the mean (S.E.M.). The data were analyzed via the Student's *t* test and repeated-measures of analyses of variance (ANOVA) followed by the Bonferroni test. A probability of less than 0.05 was considered to be statistically significant.

## 3. Result

### 3.1. Effect of taurine on proliferation and lactate dehydrogenase (LDH) of HOB cells

In the control HOB cell culture, cellular proliferation gradually increased until day 3. Microscopic observations indicated that the cell growth pattern of taurine treatment was far higher than that of the controls (Fig. 1A). It was proofed in the MTT test (Fig. 1B). In order to determine whether taurine exerts any cytotoxic effects on HOB cells, LDH assays were conducted by measuring the level of pyruvic acid with a spectrophotometer. As is shown in panel C of Fig. 1, LDH activities were reduced by  $5 \pm 0.3\%$  and  $10 \pm 0.2\%$  as the result of treatment with 10 and 20 mM taurine in these cells, respectively.

### 3.2. Effect of taurine on total magnesium concentration and $[Mg^{2+}]_i$ in HOB cells

Taurine (10 and 20 mM) elicited a transient increase in total magnesium in HOB cells. In the HOB cells, the mean total magnesium content was determined to be  $116 \pm 0.30 \mu$ M/mg protein ( $n = 10$ ). As is shown in Fig. 2A, exposure to 10 mM taurine ( $128 \pm 0.15 \mu$ M/mg protein) and 20 mM taurine ( $141 \pm 0.56 \mu$ M/mg protein) generated a significant increase in total magnesium contents.

In the HOB cells, the mean  $[Mg^{2+}]_i$  at the extracellular  $Mg^{2+}$  concentration ( $[Mg^{2+}]_o$ , 1 mM) buffer was determined to be  $0.51 \pm 0.01$  mM ( $n = 26$ ). Taurine generated a dose-dependent increase in  $[Mg^{2+}]_i$ . Fig. 2B shows typical changes in the levels of  $[Mg^{2+}]_i$  following exposure to taurine (10 and 20 mM). The value of  $[Mg^{2+}]_i$  ( $0.54 \pm 0.02$  mM) after exposure to 10 mM taurine was slightly increased from the control ( $0.51 \pm$

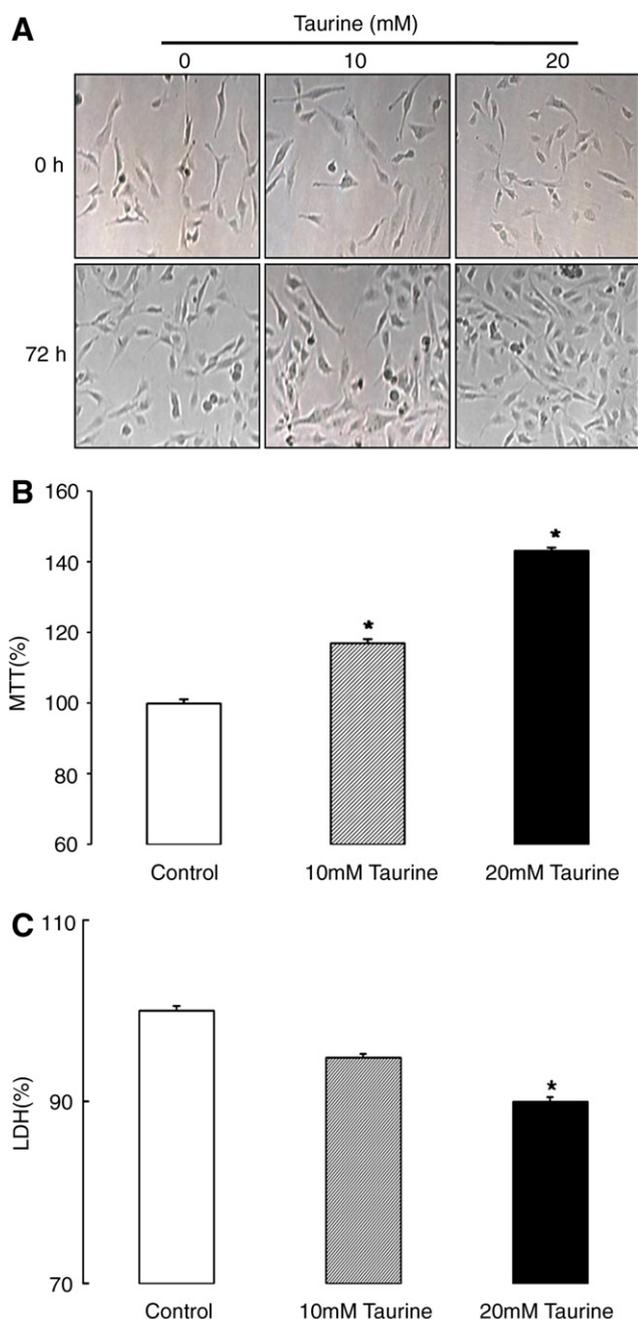


Fig. 1. Effects of taurine (10 and 20 mM) on cell proliferation and lactate dehydrogenase activity in HOB cells. HOB cells plated in six wells were treated for 48 h with the indicated doses of taurine. Cell morphology was assessed via microscopy ( $\times 200$ , A). Cell viability was determined in accordance with lactate dehydrogenase activity and MTT test. The viability of control cells was set at 100%, and viability relative to the control was expressed. The experiments were conducted at least in triplicate (B,C). \* $P < 0.05$  vs. control.

0.01 mM), and 20 mM taurine generated a significant increase in  $[Mg^{2+}]_i$  ( $0.72 \pm 0.04$  mM) in the HOB cells. The increase in  $[Mg^{2+}]_i$  induced by 20 mM taurine was inhibited significantly by 10  $\mu$ M PD98059 ( $0.51 \pm 0.02$  mM) as a specific ERK 1/2 antagonist, 10  $\mu$ M nifedipine ( $0.53 \pm 0.01$  mM) as a calcium channel blocking agent, and 10  $\mu$ M lidocaine ( $0.53 \pm 0.02$  mM) and 10  $\mu$ M imipramine ( $0.50 \pm 0.02$  mM) as fast sodium channel inactivators (Fig. 3B). The increment of  $[Mg^{2+}]_i$  induced by 20 mM taurine remained unaltered in the presence

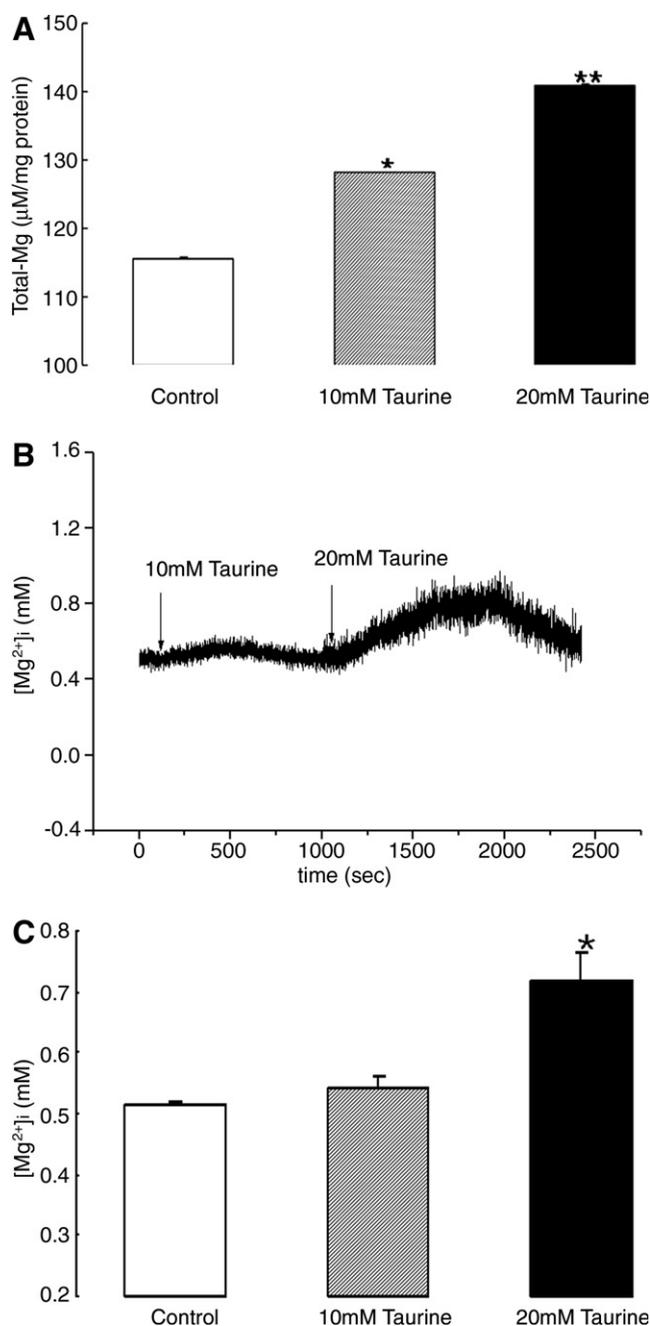


Fig. 2. Effects of taurine (10 and 20 mM) on the total and intracellular magnesium concentrations in HOB cells. The HOB cells were either left untreated (control), or treated with taurine (10 and 20 mM) for 24 h. Total magnesium was measured via atomic absorption spectrophotometry (A); typical recording (B) and data summary (C) of the intracellular free magnesium concentration ( $[Mg^{2+}]_i$ ) induced by 10 and 20 mM taurine in HOB cells. The data are reported as the means  $\pm$  S.E. of eight different preparations. \* $P < 0.05$  vs. control. \*\* $P < 0.01$  vs. control.

of extracellular 1 mM  $Mg^{2+}$  or in the absence of extracellular magnesium (Fig. 3A).

### 3.3. Effects of taurine on p38 MAP kinase and ERK 1/2 in cultured HOB cells

The phosphorylation levels of ERK1/2 and p38 MAPK were determined by conducting Western blotting after the cells were

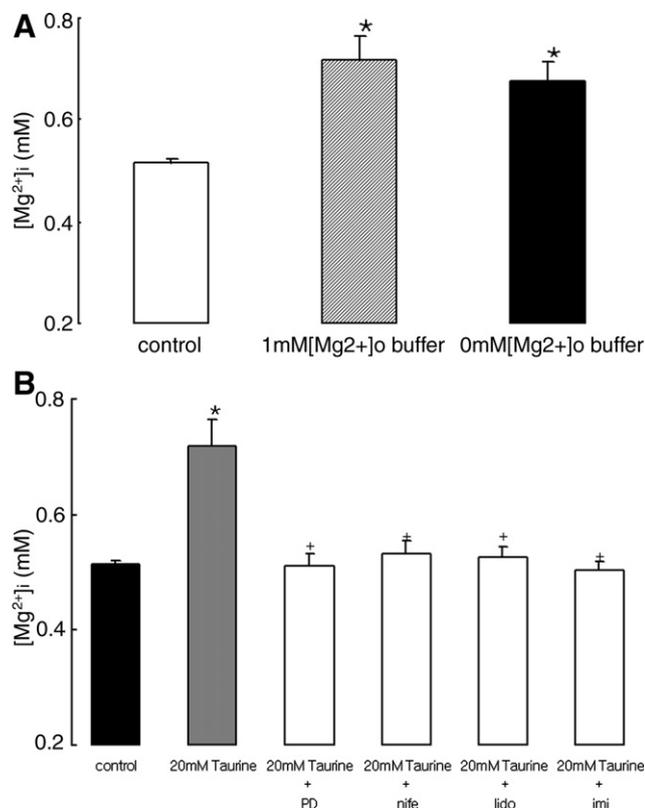


Fig. 3. The mechanisms of taurine on increments of intracellular magnesium concentration in HOB cells. The change in  $[Mg^{2+}]_i$  ( $n = 5$ ) induced by 20 mM taurine in the  $[Mg^{2+}]_o$ -free buffer was similar to the 1 mM  $[Mg^{2+}]_o$  buffer (A). \* $P < 0.05$  vs. control. Data summary (B) of the intracellular free magnesium concentration ( $[Mg^{2+}]_i$ ) induced by 20 mM taurine in HOB cell. PD, 10  $\mu$ M PD98059; nife, 10  $\mu$ M nifedipine; lido, 10  $\mu$ M lidocaine; imi, 10  $\mu$ M imipramine. The samples obtained after the first 5 min were expressed as the means  $\pm$  S.E. of 26 different preparations. \* $P < 0.05$  vs. control, + $P < 0.05$  vs. 20 mM taurine.

exposed to taurine alone or pretreated for 24 h with 10  $\mu$ M PD98059. Fig. 4 shows the typical changes in total- and phosphorylated-ERK 1/2 following exposure to 20 mM taurine in HOB cells. Using densitometry, the % variations of the taurine treated groups ( $n = 4$ ) compared with the non-treated groups (100%) were  $548.3 \pm 39.6$  (total-ERK 1/2),  $426.0 \pm 33.4$  (phosphorylated-ERK 1/2),  $100.7 \pm 0.1$  (total-p38 MAP kinase),  $170.0 \pm 7.7$  (phosphorylated-p38 MAP kinase). Taurine induced a significant activation of ERK1/2. Pretreatment with PD98059 inhibited the levels of taurine-stimulated ERK1/2 phosphorylation.

#### 4. Discussion

Taurine, 2-aminoethanesulfonic acid, is known to be involved in bone metabolism. As a result of this study, taurine was determined to induce cell proliferation in human osteoblast (HOB) cells. Recently, Yuan et al. [7] reported that the taurine transporter is expressed within osteoblasts, which perform an important function in the maintenance of a high taurine concentration in tissues, and taurine also promotes osteoblast differentiation. Also, the results of their previous study showed that taurine, an abundant free amino acid which is present in bone tissue, increases the secretion of CTGF in a

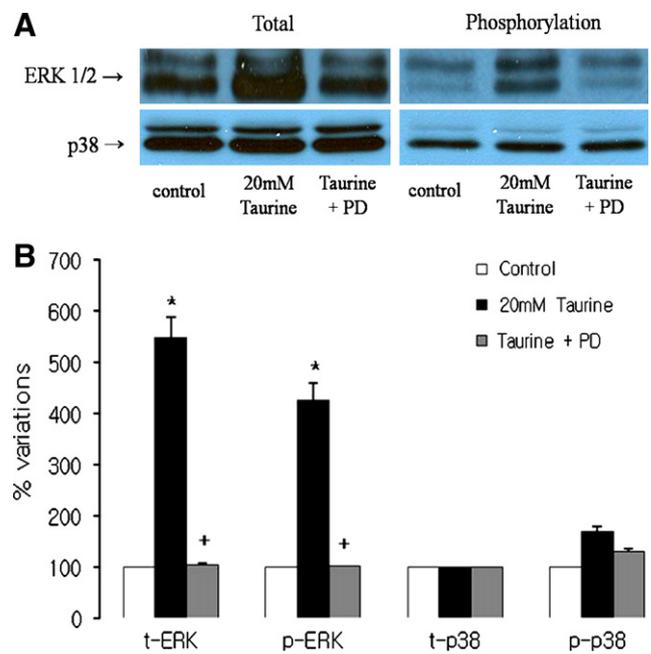


Fig. 4. Effects of taurine on p38 MAP kinase and ERK 1/2 in cultured HOB cells. Immunoprecipitation of ERK1/2 of HOB cells with no treatment, treated with taurine alone, or treated with taurine plus PD98059. HOB cells were treated with 20 mM taurine for 24 h prior to the addition of 10  $\mu$ M PD98059, and then harvested for the determination of MAPK levels. This picture shows the typical changes in total- and phosphorylated-ERK 1/2 after exposure to 20 mM taurine (A). The blots were quantified by laser scanning densitometry. The data are reported as means  $\pm$  S.E. of four different preparations (B). \* $P < 0.01$  vs. control.

dose- and time-dependent manner in the MC3T3-E1 murine osteoblast line, and that this effect is mediated by the ERK pathway. These findings further show that osteoblasts can function as a direct target of taurine [8]. Interestingly, taurine augmented cell proliferation in HOB cells at 10 and 20 mM of taurine treatment (Fig. 1). We determined that increased cell viability in HOB cells as the result of taurine treatment is related to increments in  $Mg^{2+}$ . Taurine treatment caused an increase in total and intracellular magnesium concentrations in HOB cells. In the previous study, it was suggested that the modification of biomaterials with a divalent cation ( $Mg^{2+}$ ) induces an increase in the adhesion of osteoblasts to the altered substrata via an integrin-mediated mechanism [18]. In the last analysis, the augmentation of magnesium levels as the result of taurine treatment contributes to an increase in the viability of HOB cells.

Taurine generated a dose-dependent increase in  $[Mg^{2+}]_i$ . We attempted to characterize the specific mechanisms of taurine-induced  $[Mg^{2+}]_i$  increment in HOB cells. Although the mechanisms underlying  $Mg^{2+}$  transport have yet to be isolated or purified, experimental evidence supports the notion that the intrusion of  $Mg^{2+}$  occurs via two distinct routes: an  $Na^+$ -dependent pathway and an  $Na^+$ -independent pathway [19]. In the majority of cases, the intrusion of  $Mg^{2+}$  across the cell membrane occurs via an  $Na^+$ -dependent mechanism [20–23]. Although not structurally defined, experimental data indicate that this transporter is a  $Na^+/Mg^{2+}$  exchanger [24], with a stoichiometry that varies from  $1Na^+$  for  $1Mg^{2+}$  to  $3Na^+$  for  $1Mg^{2+}$ , according to the cell type or the experimental conditions evaluated [24–26]. On the other hand, the presence of a

putative Na<sup>+</sup>-dependent Mg<sup>2+</sup> transporter has been assessed in a variety of mammalian cells [27–30]. This transporter is commonly inhibited by both lidocaine and imipramine. Our results indicate that the Na<sup>+</sup>-dependent increase in [Mg<sup>2+</sup>]<sub>i</sub> as the result of taurine treatment is inhibited by lidocaine or imipramine in HOB cells. Therefore, we suggest that the Na<sup>+</sup>-dependent Mg<sup>2+</sup> influx pathway exploited by taurine in HOB cells is a novel pathway, which is sensitive to lidocaine and imipramine (Fig. 3B). The Na<sup>+</sup>-independent pathway appears to extrude Mg<sup>2+</sup> in exchange for extracellular Ca<sup>2+</sup> [21,22] or Mn<sup>2+</sup> [31,32], or in cotransport with anions [33]. However, the modality of the activation of this pathway remains largely uncharacterized. In isolated porcine thick ascending limb cells, Dai and Quamme [34] previously reported that Mg<sup>2+</sup> transport was inhibited by Ca<sup>2+</sup> channel blockers, although it was also inhibited by nitrendipine, which indicates that the Mg<sup>2+</sup> influx pathway is a channel evidencing close homology with known Ca<sup>2+</sup> channels. In particular, nifedipine and its associated dihydropyridines are clearly relevant to Mg<sup>2+</sup> efflux channels. Thus, the taurine-induced augmentation of [Mg<sup>2+</sup>]<sub>i</sub> in HOB cells was inhibited by nifedipine (Fig. 3B).

The MAP kinase cascade is an important signaling system by which cells can transduce extracellular stimuli into intracellular responses [35,36]. An important observation presented herein is that a specific ERK 1/2 antagonist (PD98059) [37] can attenuate significantly the taurine-induced increase in the [Mg<sup>2+</sup>]<sub>i</sub> levels of cultured HOB cells, which suggests that ERK 1/2 activation in the HOB cell performs a function in the taurine-induced increase in the levels of [Mg<sup>2+</sup>]<sub>i</sub>. Recently, Yuan et al. reported that taurine stimulates the expression of CTGF in osteoblasts through the activation of the ERK signal pathway. Also, Park et al. reported that taurine rapidly activated ERK2, and its activation occurred even at a concentration of 0.01 mM, which suggests that osteoblasts are extremely sensitive to taurine [6]. After all, taurine-induced osteoblast proliferation depends on the activation of ERK 1/2. On the other hand, we may have mentioned previously that taurine induced an increase in the total and intracellular magnesium concentrations in the HOB cells. The increment of intracellular magnesium as the result of taurine treatment contributes to an increase in the proliferation of HOB cells. Recently, Kim et al. [17] reported that the augmentation of intracellular magnesium ([Mg<sup>2+</sup>]<sub>i</sub>) in H<sub>9</sub>C<sub>2</sub> cells may be related to the activation of the ERK signal pathway. Therefore, taurine may exert an osteoblast proliferation effect directly, by increasing the levels of [Mg<sup>2+</sup>]<sub>i</sub> and/or via ERK1/2 activation.

Taken together, these results appear to indicate that taurine operates directly on cultured HOB cells; it generates cell proliferation, in part, via an increase in [Mg<sup>2+</sup>]<sub>i</sub>, accompanied by ERK 1/2 activation.

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