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ORIGINAL ARTICLE

# Taurine inhibits osteoblastic differentiation of vascular smooth muscle cells via the ERK pathway

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**Abstract** Vascular calcification develops within atherosclerotic lesions and results from a process similar to osteogenesis. Taurine is a free $\beta$-amino acid and plays an important physiological role in mammals. We have recently demonstrated that vascular smooth muscle cells (VSMCs) express a functional taurine transporter. To evaluate the possible role of taurine in vascular calcification, we assessed its effects on osteoblastic differentiation of VSMCs in vitro. The results showed that taurine inhibited the $\beta$-glycerophosphate-induced osteoblastic differentiation of VSMCs as evidenced by both the decreasing alkaline phosphate (ALP) activity and expression of the core binding factor $\alpha$1 (Cbf$\alpha$1). Taurine also activated the extracellular signal-regulated protein kinase (ERK) pathway. Inhibition of ERK pathway reversed the effect of taurine on ALP activity and Cbf$\alpha$1 expression. These results suggested that taurine inhibited osteoblastic differentiation of vascular cells via the ERK pathway.

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## Introduction

Vascular calcification, such as coronary and aortic calcification, is a common feature of advanced atherosclerotic lesions (Blumenthal 1944). It is associated with clinical complications such as myocardial infarction, impaired vascular tone, angioplasty dissection, and poor surgical outcome. Previously, vascular calcification was considered to be a passive, degenerative, end-stage process of vascular disease (Umeda 2003). Recently, however, the involvement of cellular activities in this condition has been recognized. Evidence suggests that several features of this condition are similar to those of skeletal tissue mineralization; these include the expression of alkaline phosphatase (ALP), core binding factor $\alpha$1 (Cbf$\alpha$1), osteocalcin, and osteopontin, and the presence of the bone mineral hydroxyapatite and matrix vesicles (Steitz 2001). Vascular smooth muscle cells (VSMCs), pericytes, and macrophages in the vascular wall might be involved in the process of calcification (Doherty 2002). Many experiments demonstrated that VSMCs acquired the phenotype of osteoblast-like cells and played an important role in vascular calcification. Atherogenic and inflammatory mediators regulate osteoblastic differentiation of these cells; therefore, these mediators might couple atherosclerosis and calcification. This strongly suggests that vascular calcification can be an actively regulated process.

Taurine (2-aminoethanesulphonic acid) is a sulfur-containing free $\beta$-amino acid in mammals. Various physiological roles have been suggested for taurine, including calcium modulation, membrane stabilization, intracellular

osmosis regulation, and regulation of protein phosphorylation (Huxtable 1992; Lombardini 1994; Schaffer et al. 1994; Militante and Lombardini 2003). Taurine is necessary for normal development, and its deficiency leads to defects in growth, tissue differentiation, and immune development. A worldwide epidemiological study revealed a strong inverse association between the levels of taurine excretion in a population and mortality due to ischemic heart disease. This suggested that taurine intake could be effective in preventing cardiovascular disease (Yamori et al. 2001). Murakami et al. (2002) showed that taurine prevented the progression of atherosclerosis in rabbits, indicating its probable therapeutic role in atherosclerosis. Moreover, our previous study demonstrated taurine transporter, the taurine-specific transporter, was expressed on VSMCs. Further, evidence suggested that taurine played an important role in the function of VSMCs by inhibiting their proliferation and preventing $\beta$-glycerophosphate-induced calcification in these cells (Zhang et al. 1999; Li et al. 2004). However, it is still not known how taurine prevents the calcification of VSMCs. The objective of the present study was to determine the mechanism underlying this decreased calcification of calcifying VSMCs mediated by taurine.

## Materials and methods

### Cell culture

Rat VSMCs were acquired by an explant method described by Campbell and Campbell (1993). Briefly, a fragment of rat thoracic aorta was stripped of intima and adventitia. The remaining medial layer was cut into small pieces and placed in Dulbecco's Modified Eagle's medium (DMEM), containing 4.5 g/l glucose, 10 mM sodium pyruvate, and 20% FBS supplemented with 100 U/ml of penicillin and 100 mg/ml of streptomycin, at 37°C, in a humidified atmosphere containing 5% $CO_2$. After 5–7 days, cells migrated from the explants, and explant fragments were removed after 15 days of culture. Vascular smooth muscle cells were passaged every 3–4 days, and experiments were performed between passages 3 and 6 from the primary culture. The VSMC phenotype was confirmed by positive immunostaining $\alpha$-smooth muscle actin.

VSMCs do not spontaneously calcify in culture (Balica 1997). Therefore, at $\sim$80% confluence, cells were placed into calcifying medium consisting of the growth medium described above supplemented with 10 mM $\beta$-glycerophosphate (Beck 2000). For time course experiments, the first day of culture in the calcification medium was defined as day 0. The cells at this stage were used as calcifying VSMCs for the following experiments. The media were replaced with fresh one every 2 or 3 days. The transformation to calcifying cells was characterized by the appearance of multilayer nodules undergoing calcification detected by Alizarin Red S staining. These concentrations for taurine were chosen on the basis of published literature for efficacy at physiological levels.

### Quantification of Calcium deposition

The cells were decalcified with 0.6 N HCl for 24 h. The calcium content was determined by measuring the concentrations of calcium in the HCl supernatant by atomic absorption spectroscopy. After decalcification, the cells were washed three times with PBS and solubilized with 0.1 N NaOH/0.1% SDS. The protein content was measured with a Bradford protein assay. The calcium content of the cell layer was normalized to protein content.

### Analysis of alkaline phosphatase activity

Calcifying VSMCs were washed three times with PBS, scraped into 1 ml of 10 mM Tris-Cl buffer (pH 7.6) containing 0.1% Triton-X-100 on ice, and centrifuged. The lysates were homogenized. Then, ALP activity was assayed by spectrophotometric measurement of p-nitrophenol release at 37°C. ALP activity was normalized to total protein content of the cell layer.

### Protein lysate preparation and immunoblotting

Cells lysates and nuclear extracts were prepared as previous described. Protein amounts of cell lysates, and nuclear extracts were measured by the Bradford protein assay. Equal amounts of the proteins were separated by polyacrylamide gel electrophoresis followed by transfer to a 0.45 μm Nitro-Bind membrane (Micron Separations, Inc.). Proteins of interest were then blotted using appropriate antibodies (Santa Cruz Biotechnology), biotin-streptavidin amplification, and Western blot chemiluminescence detection (NEN).

### Signaling pathway in the Taurine decreased VSMCs osteoblastic differentiation

To find out the downstream intracellular signaling pathways, we explored the relationship between extracellular signal-regulated kinases (ERK) phosphorylation of VSMCs and the taurine treatment investigated. VSMCs

were cotreated with 20 mM taurine and 10 μmol/l PD98059, which inhibits MEK, an upstream molecule of the ERK phosphorylation cascade, to see whether the cell differentiation is inhibited. Calcium deposition and ALP activity were assessed after 12 days. Cbfa-1 expression was assessed after 6 days.

### Statistics

The results of the experiments were normalized relative to total protein levels as determined by Bradford's method. The data are expressed as means ±SD. Comparisons among values of more than two groups were performed by analysis of variance (one-way ANOVA). $P$ values of less than 0.05 were considered statistically significant in all cases. All experiments were repeated at least three times, and representative experiments are shown.

## Results

### Taurine inhibits osteoblastic differentiation and mineralization of VSMCs

It has recently been shown that vascular calcification in vivo has many features in common with bone mineralization; therefore, we investigated whether bone-associated molecules were altered during taurine treatment for mineralization of VSMCs. Alkaline phosphatase is a well-established phenotypic marker of osteoblastic differentiation and a critical enzyme in calcification. Figure 1a, b shows the dose- and time-dependent effects of taurine on the ALP activity in cultured VSMCs. After 12 days of culture, the ALP activity in the calcifying VSMC group dramatically increased compared to the noncalcifying group, while taurine alone had no influence on ALP activity. The ALP activity that was induced by 5 mM taurine was lower than that of the calcifying control ($P < 0.05$). At 10 and 20 mM taurine, the ALP activity markedly decreased ($P < 0.01$), while pretreatment of cells with the ERK inhibitor PD98059 (10 μmol/l) blocked the decrease in ALP activity induced by 20 mM taurine (Fig. 1a). These data indicated that taurine-induced ALP activity is mediated by the ERK pathway. After culturing for 6 days, the ALP activity decreased slightly compared with that of the control. After 9 or 12 days in culture, the decrease in ALP activity was more apparent (Fig. 1b). The effect of taurine on calcium deposition in VSMCs was determined in parallel with the ALP activity (Fig. 1c, d).

Cbfα1 (also known as Runx2, PEBP2aA) is an important transcription factor for osteoblast differentiation and is upregulated during VSMCs calcification (Steitz 2001). Our results showed that taurine inhibited Cbfα1 expression in a dose-dependent manner during $\beta$-glycerophosphate-induced calcification of VSMCs (Fig. 2a). However, PD98059 abolished this effect of taurine (Fig. 2b).

### Taurine activated ERK signal pathway in VSMCs

Mitogen-activated protein kinase (MAPK) is well known to play an essential role in controlling cell proliferation and differentiation. ERK is the major subfamily of the MAPKs involved in VSMCs calcification (Radcliff 2005), and it plays an important role in the taurine function (Yuan 2007; Yasutomi 2002). Therefore, we examined the ERK signals induced by taurine. Taurine stimulated the activity of a specific ERK in the VSMCs after 5 min of incubation; this was determined by the increase in the phosphorylated ERK levels (Fig. 3). The ERK inhibitor inhibited both VSMC calcification (Fig. 1a, c) and the osteoblastic differentiation markers (Fig. 2b).

## Discussion

Mineralization of soft tissues occurs under pathological conditions and has detrimental consequences, particularly when it occurs in blood vessels and heart valves. Calcification of arterial plaques decreases vessel elasticity, augments plaque brittleness, and increasingly leads to plaque rupture during angioplasty procedures (Fitzpatrick 1992). It is, therefore, associated with an increased risk of myocardial infarction and death. Despite its clinical significance, the molecular mechanisms underlying the regulation of vascular calcification are unclear. However, evidence has recently emerged supporting the concept that ectopic calcification, such as bone mineralization, is a cell-regulated process. Steitz et al. (2001) reported that VSMCs undergo phenotypic transition to osteoblast-like cells in response to mineralization; this is indicated by an increase in the expression of Cbfα1, osteopontin, osteocalcin, and ALP. This study increases our understanding of vascular calcification and provides further support for skeletal-like bone formation and remodeling during vascular calcification. Moreover, the study reveals that taurine functions as a potential mediator of VSMC biology. Our data indicate that taurine exposure decreased vascular calcification in vitro by inhibiting osteoblastic differentiation of VSMCs. The supporting evidence included the following: (1) the characteristic morphological changes indicating the differentiation of VSMCs into osteoblastic cells, and (2) the downregulation of osteoblastic differentiation markers such as ALP activity and Cbfα1. This notion is further supported by

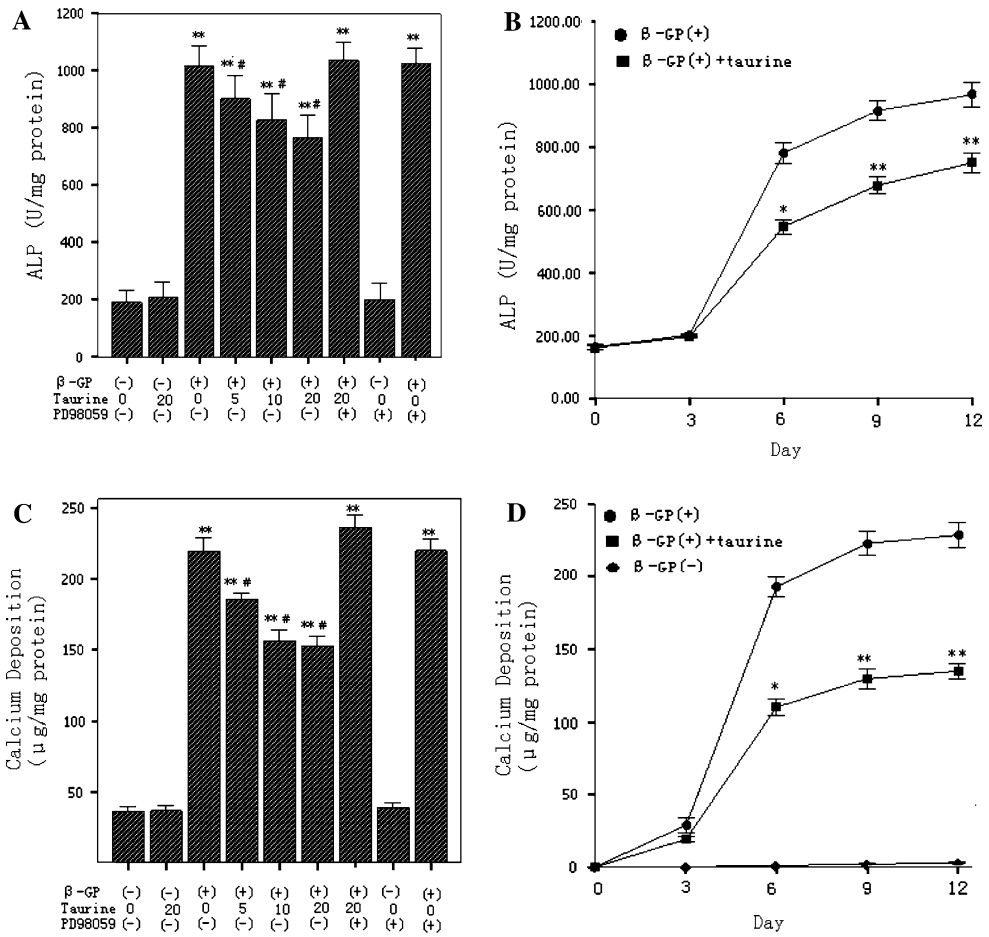


**Fig. 1** Effect of taurine on ALP activity and calcium deposition in VSMCs. **a** Dose-dependent effect of taurine on ALP activity. Cells were cultured in noncalcification or calcification medium with different concentrations of taurine. ALP activity was measured, normalized to the cellular protein contents, and presented as mean ± SD ($n = 3$; $^{**}P < 0.01$ vs. noncalcification group; $^{\#}P < 0.05$ vs. calcification control). **b** Time-dependent effect of taurine on ALP activity. The cells were cultured in calcification medium for the indicated time periods with or without 20 mM taurine as described in the "Materials and methods". ALP activity was measured, normalized to the cellular protein contents, and presented as mean ± SD ($n = 3$). The differences in comparison to calcified controls at each time point were statistically significant ($^{*}P < 0.05$, $^{**}P < 0.01$). **c** Dose-dependent effect of taurine on calcium contents. The calcium contents of the cell layers were assessed as described above and presented as mean ± SD ($n = 3$; $^{**}P < 0.01$ vs. noncalcification group; $^{\#}P < 0.05$ vs. calcification control). **d** Time-dependent effect of taurine on calcium deposition. The cells were cultured in calcification medium for indicated time periods with or without 20 mM taurine as described in "Materials and methods". The calcium contents of the cell layers were measured by the atomic absorption spectroscopy method, normalized to the cellular protein content, and presented as mean ± SD ($n = 3$). The differences in comparison to the calcified controls at each time point were statistically significant ($^{*}P < 0.05$, $^{**}P < 0.01$)

the fact that the inhibition of the ERK pathway suppressed the effects of taurine on ALP activity and Cbfα1 expression.

ALP activities were decreased by taurine treatment. These results indicated that taurine inhibited calcification, and this inhibition was associated with decreased ALP activity. In addition to the decreased expression of ALP, the expression of Cbfα1, a key regulatory transcription factor in osteoblastic differentiation, was also downregulated. Cbfα1 is a critical for the differentiation of osteoblasts and expression of bone matrix proteins. Cbfα1 knockout mice fail to form mineralized bone (Komori 1997), and the matrix Gla-protein knockout mice lose the smooth muscle markers in their arteries resulting in increased expression of Cbfα1 as they progressively mineralize their arteries (Steitz 2001). Furthermore, the expression of Cbfα1 has also been observed in the calcification of atherosclerotic plaques (Tyson 2003). Taken together, these results support the fact that Cbfα1 may be a key regulatory factor in vascular calcification. Considering these previous findings and the present results, one can deduce that taurine decreases VSMCs differentiation by downregulating Cbfa-1 expression. Therefore, it is likely that taurine inhibits the osteoblastic differentiation of

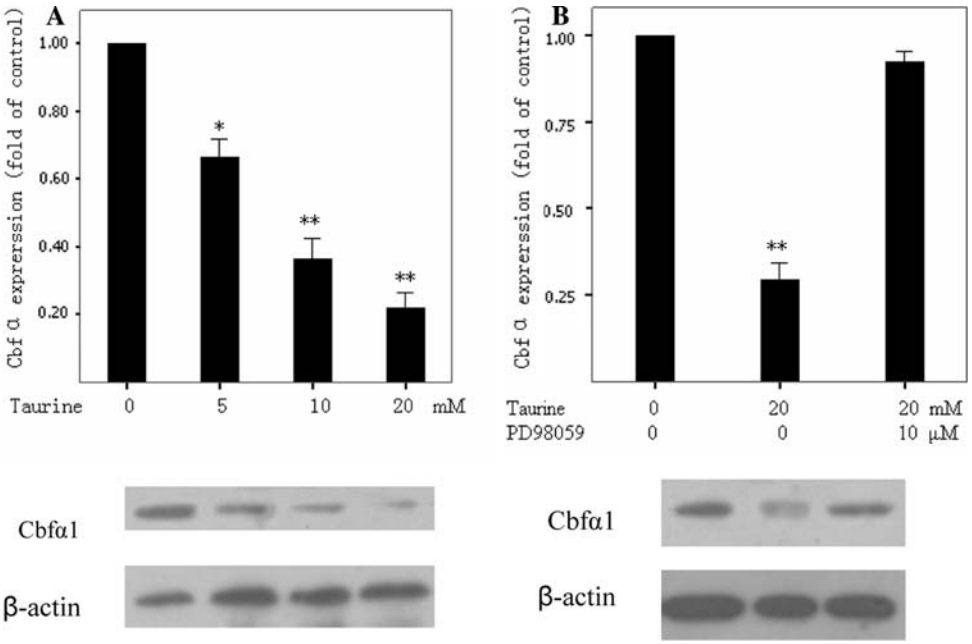


**Fig. 2** Effect of taurine on Cbfα1 expression in the VSMC culture in the calcification medium. **a** Calcifying VSMCs were treated with taurine at different concentrations for 6 days, and the cell lysates were subjected to immunoblotting. Immunoblotting was performed using specific antibodies against Cbfα-1. **b** Addition of PD98059 (10 μM) blocked the effect of taurine on Cbfα-1 expression. (*$P < 0.05$ vs. control, **$P < 0.01$ vs. control)

VSMCs by decreasing the expression of the Osf2/Cbfα1 gene, and consequently, decreasing the level of its downstream transcriptional targets such as ALP. However, it is not yet clear whether Cbfα1 is solely a transcriptional regulator and whether or not it exerts a direct effect on ALP expression.

Taurine, one of the metabolites of methionine and cysteine, has been shown to have antihypertensive and antiatherogenic effects in animal models. Epidemiological studies also revealed that taurine intake correlates inversely with the incidence of coronary heart disease (Yamori et al. 2001). The molecular mechanism underlying this protective effect is obscure, and some researchers have attributed this effect to its cholesterol-lowering properties (Murakami 1999). However, others reported that taurine could prevent atherosclerosis without significantly affecting the serum cholesterol level (Kondo 2001; Murakami 2002). Currently, taurine and its analogues are reported to suppress the proliferation of VSMCs (Zhang 1999). Further, it was reported that taurine significantly suppresses the cell proliferation induced by the platelet-derived growth factor (PDGF)-BB via protein tyrosine phosphatase (PTPase)-mediated suppression of the PDGF-$\beta$ receptor phosphorylation and by decreasing the activation of its downstream signaling molecules in VSMCs. Furthermore, Li et al. (2004) demonstrated that taurine treatment alleviated the calcification in VSMCs induced by $\beta$-glycerophosphate. Our present study showed that taurine could inhibit osteoblastic differentiation of VSMCs via ERK activation.

The taurine concentration (millimolar concentrations) in animal tissues is reported to be one of the highest compared to the other amino acids (Jacobsen 1968). Taurine had

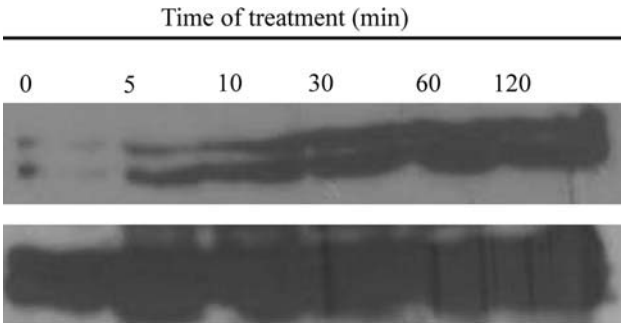


**Fig. 3** Intracellular signaling pathways mediating taurine effects. Western analyses of phosphorylated ERK in VSMCs cultures treated with 20 mM for the indicated time

minimal cytotoxic activity against rat VSMCs at concentrations between 5 and 20 mM (data not shown). Hence, we used this range of taurine concentrations to explore its effect on the cells. Our results demonstrated that the inhibitory effect of taurine on the expression of the osteoblastic differentiation markers, including ALP and Cbfα1, via the ERK signal pathway, was dose- and time-dependent. Further, taurine inhibited mineralized nodule formation in $\beta$-glycerophosphate-induced VSMCs.

Previous studies have shown that ERK was involved in the osteoblastic differentiation and mineralization of VSMCs. However, the results were contradictory. Radcliff et al. suggested that insulin-like growth factor (IGF)-I promotes the phosphorylation of ERK and phosphatidylinositol 3-kinase (PI3K) in VSMCs, and the inhibition of either the ERK or PI3K pathway reversed the IGF-I effects on osteoblastic differentiation of VSMCs. Furthermore, the overexpression of the ERK activator also mimicked IGF-I

inhibition of ALP activity. Therefore, from their experiments they concluded that IGF-I inhibited osteoblastic differentiation and vascular-cell mineralization via both the ERK and PI3K pathways (Radcliff et al. 2005). Our present study showed that taurine inhibited osteoblastic differentiation and mineralization of VSMCs via the ERK signaling pathway, which is in accordance with Radcliff's work. However, Ding et al. (2006) demonstrated that fibronectin, an important extracellular matrix protein, enhanced vascular calcification by promoting osteoblastic differentiation of VSMCs via the ERK signaling pathway. It is necessary that the relationship between ERK phosphorylation and osteoblastic differentiation of VSMCs be thoroughly investigated.

In conclusion, our findings support the fact that taurine inhibits calcification in vitro by decreasing the osteoblastic differentiation of the rat VSMCs, and this effect might be mediated by the ERK pathway, leading to a decrease in the calcification of VSMCs.

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