Amino Acids (2007) 32: 425–430
DOI 10.1007/s00726-006-0380-4
Printed in The Netherlands

Amino Acids

Taurine promotes connective tissue growth factor (CTGF) expression in osteoblasts through the ERK signal pathway

L.-Q. Yuan, Y. Lu, X.-H. Luo, H. Xie, X.-P. Wu, and **E.-Y. Liao**

Institute of Metabolism and Endocrinology, The Second Xiang-Ya Hospital, Central South University, Changsha, Hunan, China

Received April 25, 2006
Accepted June 19, 2006
Published online August 29, 2006;

Summary. Taurine is found in bone tissue, but its function in skeletal tissue is not fully understood. The present study was undertaken to investigate regulation of gene expression of connective tissue growth factor (CTGF), and the roles of mitogen-activated protein kinases (MAPKs) in murine osteoblast MC3T3-E1 cells treated with taurine. Western blot analysis showed taurine stimulated CTGF protein secretion in a dose- and time-dependent manner. Taurine induced activation of extracellular signal-regulated kinase (ERK), but not p38 and c-*jun* N-terminal Kinase (JNK), in osteoblasts. Furthermore, pretreatment of osteoblasts with the ERK inhibitor PD98059 abolished the taurine-induced CTGF production. These data indicate that taurine induces CTGF secretion in MC3T3-E1 cells mediated by the ERK pathway, and suggest that osteoblasts are direct targets of taurine.

Keywords: Taurine – Osteoblast – Connective tissue growth factor – Mitogen-activated protein kinase

Introduction

Taurine (2-aminoethanesulphonic acid) is the major free β-amino acid in mammals, and is widely distributed in mammalian plasma as well as cells. Various physiological roles have been suggested for taurine, including calcium modulation, membrane stabilization, intracellular regulation of osmosis, and regulation of protein phosphorylation (Huxtable, 1992; Lombardini, 1994; Schaffer et al., 1994; Militante and Lombardini, 2003). Taurine is necessary for normal development, and defects in growth, tissue differentiation and immune development occur when taurine is deficient (Sturman, 1993; Schuller-Levis and Park, 2003). Taurine has been reported to influence bone metabolism and its specific transport system, the taurine transporter, is expressed in osteoblasts (Park et al., 2001; Yuan et al., 2006). However, the mechanism of taurine regulation of bone metabolism has not been elucidated.

Connective tissue growth factor (CTGF) is a member of the CCN (CTGF, Cyr61, and nephroblastoma overexpressed) family of growth factors, which is characterized by significant sequence homology and the conservation of all 38 cysteine residues. CTGF is a secreted, extracelluar matrix-associated protein that exhibits diverse cellular functions depending upon the cell type including adhesion, proliferation, migration, differentiation, matrix production, and survival (Moussad and Brigstock, 2000). CTGF is expressed in many tissues, with the highest levels in the kidney and brain. To date, CTGF mRNA expression and/or protein production has been demonstrated in endothelial cells (Bradham et al., 1991; Shimo et al., 1998), fibroblasts (Igarashi et al., 1993; Steffen et al., 1998), chondrocytes (Nakanishi et al., 1997), vascular smooth muscle cells (Lin et al., 1998), and osteoblasts (Nishida et al., 2000; Parisi et al., 2006; Xu et al., 2000). Recent studies have demonstrated that CTGF is expressed in bone tissue and has diverse modulatory functions including influences on osteoblast proliferation and differentiation (Safadi et al., 2003). The mechanism by which taurine is involved in the regulation of osteoblast differentiation is not known, however it may involve regulation of CTGF expression.

Our recent study revealed that the taurine transporter is expressed in osteoblasts, suggesting that osteoblasts are direct targets of taurine (Yuan et al., 2006). Other researchers have found that bone tissue contains high concentrations of taurine (Huxtable, 1992; Park et al., 2001). These findings suggest that taurine may directly influence the metabolism of bone. On one hand, taurine regulates osteoblast metabolism and increases osteoblast differentiation (Park et al., 2001). On the other hand,

taurine has been reported to inhibit experimental bone resorption and osteoclast differentiation (Koide et al., 1999; Kum et al., 2003). The present study was undertaken to examine the effects of taurine on CTGF expression in osteoblasts and to investigate the cell signaling pathway involved in this effect.

Materials and methods

Chemicals

Taurine was obtained from Sigma Chemical Company (St. Louis, MO, USA). Anti-ERK, p-ERK, p38, p-p38, JNK and p-JNK antibody, anti-goat CTGF polyclonal antibody, anti-mouse and goat IgG peroxidase conjugate antibodies were purchased from Santa Cruz Biotechnology Inc (Waltham, MA, USA). PD98059, SB203580, and SP600125 were purchased from Calbiochem (San Diego, CA, USA). All chemicals were of the purest grade commercially available.

Cell culture and taurine treatment

The MC3T3-E1 mouse osteoblast cell line was obtained from American Type Culture Collection (ATCC, Rockville, MD) and cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco BRLCorp., Grand Island, NY, USA) supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 100 IU/ml penicillin-G, and 100 mg/ml streptomycin. Cells were incubated at 37 °C in a 95% air and 5% CO_2 atmosphere until 80–90% confluent and passaged by means of 0.2 mol trypsin/1 mM EDTA (Sigma) in phosphate-buffered saline (PBS). Passages 15 to 20 were used in the experiments. There were no significant differences in biologic function and characterizations in those passages.

For taurine treatment, MC3T3-E1 cells were plated in 25 cm^2 flasks in DMEM containing 10% FBS. After 4 d culture, cells were subsequently treated with vehicle (serum-free DMEM) or 1, 10, or 20 mM taurine for 24 h in serum-free DMEM. Parallel cultures were exposed to 10 mM mannitol as a control for hyperosmolarity induced by 10 mM taurine treatment. Cultures were also exposed to fresh serum-free DMEM containing 10 mM taurine for 12–48 h as a time course. To study the effects of inhibitors, cells were pretreated with PD98059 for 2 h prior to 10 mM taurine treatment. Conditioned osteoblast culture media and the cell monolayers were collected and stored frozen at −70 °C until assayed by total protein determination and immunoblot analysis.

Detection of CTGF in conditioned media of cultured osteoblasts by immunoblot analysis

Medium aliquots from MC3T3-E1 cell cultures were precipitated with 10% trichloroacetic acid. Equal amounts of protein were mixed with an equal volume of 2 × SDS sample buffer, boiled for 10 min, and subjected to 10% SDS-PAGE. After electrophoresis, the SDS-PAGE separated proteins were transferred to a nitrocellulose membrane (Amersham Pharmacia Biotech). Membrane was blocked with 2.5% non-fat milk in PBS for 2 h and incubated with anti-goat-CTGF primary antibody (2 mg/ml) overnight at 4 °C. The membrane was reprobed with rabbit anti-goat IgG conjugated with horseradish peroxidase for 1 h. Blots were processed using an ECL kit (Santa Cruz) and exposed to film.

Detection of mitogen-activated protein kinase (MAPK) activation

To investigate the effects of taurine on the MAPK signaling pathway, immunoblot analysis was performed as described above. Proteins were transferred to a nitrocellulose membrane. The membrane was incubated with anti-phospho-ERK, anti-ERK, anti-phospho-p38, anti-p38, anti-phospho-JNK, and anti-JNK primary antibodies. Then the membrane was reprobed with the specific secondary antibodies, respectively. Blots were processed using an ECL kit and exposed to the film.

Statistics

The various experimental values were obtained from three experiments. 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

Effects of taurine on CTGF protein production in conditioned medium

Western blot analysis revealed that taurine increased the levels of CTGF protein production in conditioned medium

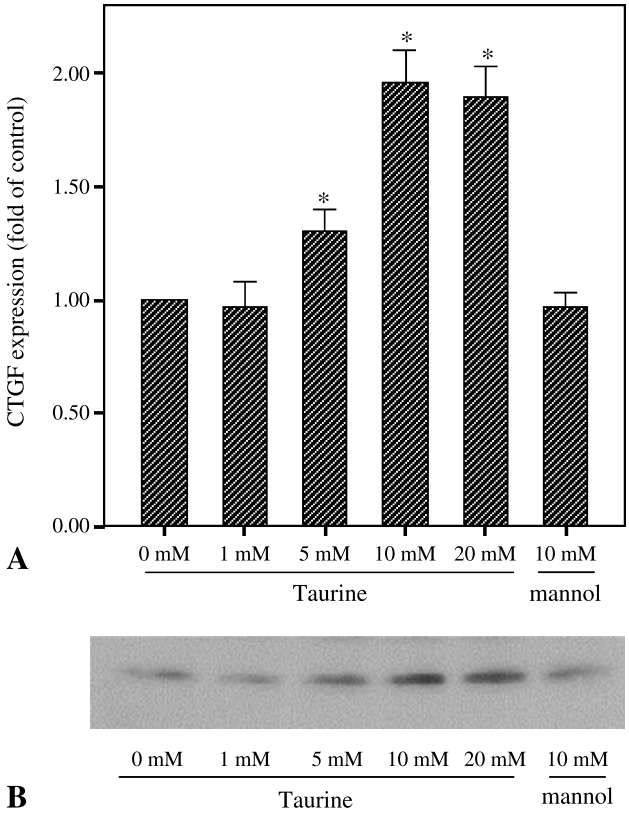

Fig. 1. Dose dependent CTGF protein secretion in response to taurine in cultured murine osteoblast line MC3T3-E1. Cells were exposed to fresh serum-free medium with or without various concentrations of taurine for 24 h. 10 mM Mannitol was used as a control of hyperosmolarity, which is equal to 10 mM taurine treatment. Subsequently western blot analysis was performed using an anti-CTGF antibody as described in Materials and methods. **A** Dose response of taurine on CTGF protein levels in cultured MC3T3-E1 cells. **B** A representative western blot from three independent experiments is shown. The bar represents the mean ± SD ($n = 3$; $^*P < 0.05$ vs. control)

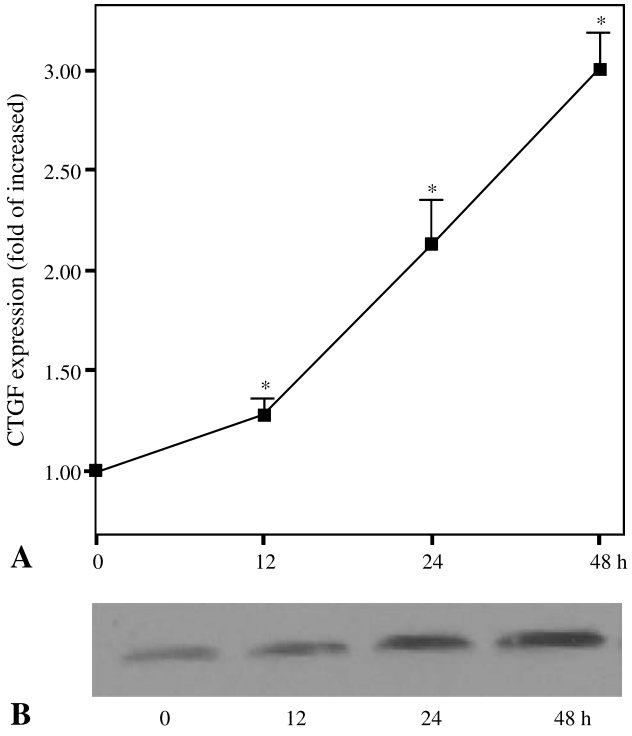

Fig. 2. Time course analysis for CTGF protein expression in response to taurine in cultured murine osteoblast MC3T3-E1. Cells were exposed to fresh serum-free medium with or without 10 mM of taurine for 12–48 h. Subsequently western blot analysis was performed as described in Materials and methods. **A** Time response of taurine on CTGF protein secretion in cultured MC3T3-E1 cells. **B** A representative western blot analysis from three independent experiments is shown. The bar represents the mean ± SD ($n = 3$; $^*P < 0.05$ vs. control)

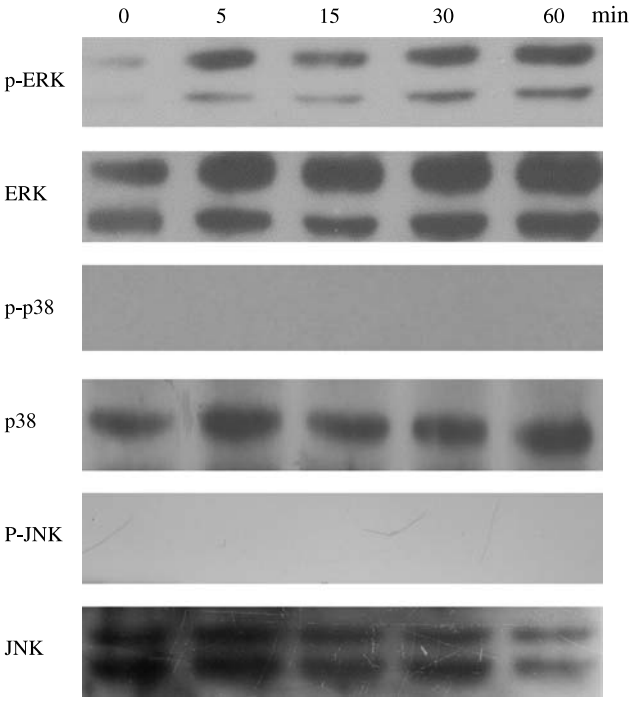

Fig. 3. Effects of taurine on ERK, p38, and JNK1/2 activation in murine osteoblast MC3T3-E1. Cell lysates were subjected to western blot and incubated with ERK, P38, and JNK1/2 antibodies. Representative results for cells exposed to 10 mM taurine for 5–60 min are shown

in a dose- and time-dependent manner (Figs. 1, 2). The antibody to CTGF recognizes the 38-kDa form. After 24 h of culture, the intensity of 38-kDa band at 5 mM taurine concentration was greater than that of control. At 10 mM taurine exposure, the intensity of 38-kDa bands appeared obviously compared with control. After 20 mM taurine exposure, the bands increased dramatically but were lower than 10 mM taurine exposure. At 12, 24 and 48 h, the intensity of 38-kDa band at 10 mM taurine concentration was significantly increased compared with control.

Taurine activated ERK signaling pathways in MC3T3-E1 cells

Taurine had little effect on p38 and JNK phosphorylation, whereas it enhanced the levels of phosphorylated ERK. This effect occurred within 5 min of exposure, and phosphorylated ERK remained activated for 60 min after taurine treatment (Fig. 3). These data demonstrated that taurine activated the ERK signaling pathways in MC3T3-E1 cells.

The effect of signal transduction inhibitors on MC3T3-E1 cells CTGF expression

MAPK kinases are key factors in CTGF expression (Rodriguez-Vita et al., 2005; Leivonen et al., 2005). Therefore, we examined whether the increase of CTGF in taurine-treated MC3T3-E1 cells was mediated via the activation of MAPK kinase signal pathway. We examined the expression of CTGF protein production in the presence and/or absence of 10 μM PD98059 (a specific inhibitor of the ERK/MAPK kinase), 10 μM SB203580 (a specific inhibitor of p38 kinase), and 20 μM SP600125 (JNK inhibitor II) in taurine treated MC3T3-E1 cells. Taurine-mediated CTGF secretion was reduced by PD98059 (Fig. 4) but not by presence of SB203580 and SP600125. Taken together, these results indicate that

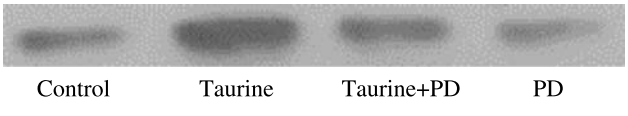

Fig. 4. ERK signaling pathways mediate taurine-induced osteoblast expression. Cells were incubated with PD98059 for 2 h prior to treatment with 10 mM taurine. Conditioned medium was subjected to western blot

taurine-enhanced MC3T3-E1 cells CTGF protein production is mediated through the ERK signaling pathway.

Discussion

Recent studies have suggested that taurine is involved in bone metabolism, although the mechanism has not been resolved. Our previous study showed that the taurine transporter is expressed in osteoblasts, which plays an important role in maintaining a high concentration of taurine in tissues, and taurine promots osteoblast differentiation (Yuan et al., 2006). The present study demonstrated that taurine, an abundant free amino acid present in bone tissue, increases CTGF secretion in a dose- and time-dependent manner in the murine osteoblast line MC3T3-E1, and that this effect is mediated by the ERK pathway. These findings further reveal that osteoblasts can be a direct target of taurine.

Recently, taurine has been identified in bone, but the precise concentration of taurine contained in bone is not known at present. According to the fact that taurine accumulates in the bone with a rate similar to that of the liver or kidney (Lubec et al., 1997; Terauchi et al., 1998), Park et al. (2001) speculated that taurine content in bone may be similar to those of liver or kidney (2–11 mM/kg tissue). Based on previous studies, the effects of taurine on the stimulation of CTGF synthesis were physiologically relevant in our experiment. Although 20 mM taurine could promote CTGF secretion, its effect was less than the effect of 10 mM taurine.

Numerous studies have confirmed that taurine is found intracellularly in vivo (Chan-Palay et al., 1982; Satsu et al., 2002; Nakamura et al., 2006). Lobo et al. (2000) suggested that taurine was localized in myofilaments, dense bodies, mitochondria, plasma membrane and nuclei in vascular and visceral smooth muscle cells. Our previous study demonstrated that the taurine transporter is expressed in osteoblasts as a functional protein, promoting active uptake of [^{3}H] taurine (Yuan et al., 2006). In the present study, applying taurine to cells in vitro represents a physiological paradigm for the study of taurine function. The present experiments may partly explain the physiological function of taurine in osteoblasts. Future studies should be performed to elucidate the physiologic function of taurine in osteoblasts.

Our work does not directly address the function of CTGF in osteoblasts, but previous reports have revealed important interactions between CTGF peptides and regulators of osteoblast cell growth and differentiation. Numerous studies have confirmed that CTGF was expressed in osteoblasts in vivo and in vitro. Nishida et al. (2000) have shown that CTGF augments type I collagen gene expression, alkaline phosphatase activity, osteopontin and osteocalcin production in osteoblastic cell lines (Saos-2 and MC3T3-E1 cells), and also stimulates collagen synthesis and matrix mineralization in MC3T3-E1 cells in vitro. Safadi et al. (2003) found that local delivery of rCTGF into the bone marrow cavity elicited a strong osteogenic response associated with increased angiogenesis. The phenotype of the recently generated CTGF null mouse provides support for the role of CTGF in chondrogenesis, endochondral ossification, and osteogenesis. *Ctgf*$^{-/-}$ mice die shortly after birth due to respiratory failure caused by severe malformation of the rib cage and skeletal defects. In these mice, bones are abnormally shaped with increased thickness of the hypertrophic zone of growth plate cartilage and impaired bone formation and mineralization, resulting in osteopenia (Ivkovic et al., 2003). However, over-expression of CTGF in transgenic mice decreases bone density and produces dwarfism. It is possible that the decreased bone density in these mice may be secondary to an abnormality in endochondral bone formation induced by over-expression of CTGF in the growth plate (Nakanishi et al., 2001). These data establish a functional connection between CTGF and osteoblast development and function. The present experiment shown taurine could upregulate CTGF expression in osteoblast. Therefore, we suppose that taurine promoting osteoblast differentiation effect may partly induced by taurine stimulating CTGF secretion. However, this hypothesis should be confirmed by further experiments.

We evaluated MAPK signaling events to gain further insight into the mechanisms by which taurine promotes CTGF production in osteoblasts. Our results were in agreement with previous studies, showing that taurine treatment can activate the ERK/MAPK pathway. Park et al. (2001) found that even at a concentration of 0.1 mM, taurine stimulated ERK phosphorylation within 1 min, and the stimulatory action of taurine was blocked by an ERK inhibitor. Yasutomi et al. (2002) found that taurine increased the nuclear localization of Cbfa1, and this effect was mediated through the ERK signal pathway. Numerous studies have found that MAPK signal pathway is involved in CTGF expression (Rodriguez-Vita et al., 2005; Leivonen et al., 2005). In our study, we confirmed the role of this signaling pathway in mediating the effects of taurine on CTGF protein expression. The ERK inhibitor PD98059 abrogated the effects of taurine on the induction of CTGF gene expression, whereas the p38 inhibitor SB203580 and JNK inhibitor SP600125 did not

alter osteoblast CTGF production and release. These findings are consistent with recent results showing that ERK is involved in activation of the CTGF promoter by TGF-β in skin fibroblasts (Leask et al., 2003) and gingival fibroblasts (Leivonen et al., 2005).

In conclusion, the present study has provided evidence that taurine stimulates CTGF expression in osteoblasts through activation of the ERK signal pathway. An increased level of CTGF in the bone microenvironment may be relevant to the actions of taurine on bone formation. Our demonstration that taurine upregulates the expression of CTGF in osteoblasts suggests that there is a direct link between taurine and bone homeostasis.

Acknowledgements

This work was supported by Grant-30572078 from the National Natural Science Foundation of China, and Hunan Provincial Outstanding Younger Foundation of P.R. China (03JJY1005).

References

Bradham DM, Igarashi A, Potter RL, Grotendorst GR (1991) Connective tissue growth factor: a cysteine-rich mitogen secreted by human vascular endothelial cells is related to the SRC-induced immediate early gene product CEF-10. J Cell Biol 114: 1285–1294

Chan-Palay V, Lin CT, Palay S, Yamamoto M, Wu JY (1982) Taurine in the mammalian cerebellum: demonstration by autoradiography with [^{3}H]taurine and immunocytochemistry with antibodies against the taurine-synthesizing enzyme, cysteine-sulfinic acid decarboxylase. Proc Natl Acad Sci USA 79: 2695–2699

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

Igarashi A, Okochi H, Bradham DM, Grotendorst GR (1993) Regulation of connective tissue growth factor gene expression in human skin fibroblasts and during wound repair. Mol Biol Cell 4: 637–645

Ivkovic S, Yoon BS, Popoff SN, Safadi FF, Libuda DE, Stephenson RC, Daluiski A, Lyons KM (2003) Connective tissue growth factor coordinates chondrogenesis and angiogenesis during skeletal development. Development 130: 2779–2791

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

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

Leask A, Holmes A, Black CM, Abraham DJ (2003) Connective tissue growth factor gene regulation. Requirements for its induction by transforming growth factor-beta 2 in fibroblasts. J Biol Chem 278: 13008–13015

Leivonen SK, Hakkinen L, Liu D, Kahari VM (2005) Smad3 and extracellular signal-regulated kinase 1/2 coordinately mediate transforming growth factor-beta-induced expression of connective tissue growth factor in human fibroblasts. J Invest Dermatol 124: 1162–1169

Lin J, Liliensiek B, Kanitz M, Schimanski U, Bohrer H, Waldherr R, Martin E, Kauffmann G, Ziegler R, Nawroth PP (1998) Molecular cloning of genes differentially regulated by TNF-alpha in bovine aortic endothelial cells, fibroblasts and smooth muscle cells. Cardiovasc Res 38: 802–813

Lobo MV, Alonso FJ, Martin del Rio R (2000) Immunocytochemical localization of taurine in different muscle cell types of the dog and rat. Histochem J 32: 53–61

Lombardini JB (1994) The inhibitory effects of taurine on protein phosphorylation: comparison of various characteristics of the taurine-affected phosphoproteins present in rat retina, brain and heart. Adv Exp Med Biol 359: 9–17

Lubec B, Ya-hua Z, Pertti S, Pentti T, Kitzmuller 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

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

Moussad EE, Brigstock DR (2000) Connective tissue growth factor: what's in a name? Mol Genet Metab 71: 276–292

Nakamura H, Yatsuki J, Ubuka T (2006) Production of hypotaurine, taurine and sulfate in rats and mice injected with L-cysteinesulfinate. Amino Acids 31: 27–33

Nakanishi T, Kimura Y, Tamura T, Ichikawa H, Yamaai Y, Sugimoto T, Takigawa M (1997) Cloning of a mRNA preferentially expressed in chondrocytes by differential display-PCR from a human chondrocytic cell line that is identical with connective tissue growth factor (CTGF) mRNA. Biochem Biophys Res Commun 234: 206–210

Nakanishi T, Yamaai T, Asano M, Nawachi K, Suzuki M, Sugimoto T, Takigawa M (2001) Overexpression of connective tissue growth factor/hypertrophic chondrocyte-specific gene product 24 decreases bone density in adult mice and induces dwarfism. Biochem Biophys Res Commun 281: 678–681

Nishida T, Nakanishi T, Asano M, Shimo T, Takigawa M (2000) Effects of CTGF/Hcs24, a hypertrophic chondrocyte-specific gene product, on the proliferation and differentiation of osteoblastic cells in vitro. J Cell Physiol 184: 197–206

Parisi MS, Gazzerro E, Rydziel S, Canalis E (2006) Expression and regulation of CCN genes in murine osteoblasts. Bone 38: 671–677

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

Rodriguez-Vita J, Ruiz-Ortega M, Ruperez M, Esteban V, Sanchez-Lopez E, Plaza JJ, Egido J (2005) Endothelin-1, via ETA receptor and independently of transforming growth factor-beta, increases the connective tissue growth factor in vascular smooth muscle cells. Circ Res 97: 125–134

Safadi FF, Xu J, Smock SL, Kanaan RA, Selim AH, Odgren PR, Marks SC Jr, Owen TA, Popoff SN (2003) Expression of connective tissue growth factor in bone: its role in osteoblast proliferation and differentiation in vitro and bone formation in vivo. J Cell Physiol 196: 51–62

Satsu H, Kobayashi Y, Yokoyama T, Terasawa E, Shimizu M (2002) Effect of dietary sulfur amino acids on the taurine content of rat tissues. Amino Acids 23: 447–452

Schaffer SW, Ballard C, Azuma J (1994) Mechanisms underlying physiological and pharmacological actions of taurine on myocardial calcium transport. Adv Exp Med Biol 359: 171–180

Schuller-Levis GB, Park E (2003) Taurine: new implications for an old amino acid. FEMS Microbiol Lett 226: 195–202

Shimo T, Nakanishi T, Kimura Y, Nishida T, Ishizeki K, Matsumura T, Takigawa M (1998) Inhibition of endogenous expression of connective

tissue growth factor by its antisense oligonucleotide and antisense RNA suppresses proliferation and migration of vascular endothelial cells. J Biochem (Tokyo) 124: 130–140

Steffen CL, Ball-Mirth DK, Harding PA, Bhattacharyya N, Pillai S, Brigstock DR (1998) Characterization of cell-associated and soluble forms of connective tissue growth factor (CTGF) produced by fibroblast cells in vitro. Growth Factors 15: 199–213

Sturman JA (1993) Taurine in development. Physiol Rev 73: 119–148

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

Xu J, Smock SL, Safadi FF, Rosenzweig AB, Odgren PR, Marks SC Jr, Owen TA, Popoff SN (2000) Cloning the full-length cDNA for rat connective tissue growth factor: implications for skeletal development. J Cell Biochem 77: 103–115

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: 114P–115P

Yuan LQ, Xie H, Luo XH (2006) Taurine transporter is expressed in osteoblasts. Amino Acids 31: 157–163

Authors' address: Er-Yuan Liao, Institute of Metabolism and Endocrinology, The Second Xiang-Ya Hospital, Central South University, Changsha, Hunan 410011, P.R. China,
Fax: 086-0731-5361472; E-mail: eyliao2003@21cn.com