The Change of Taurine Transport in Osteocytes by Oxidative Stress, Hypertonicity and Calcium Channel Blockers

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Abstract – Taurine is the most abundant amino acid in many tissues and is found to be enhancing the bone tissue formation or inhibits the bone loss. Although it is reported that taurine reduces the alveolar bone loss through inhibiting the bone resorption, its functions of taurine and expression of taurine transporter (TauT) in bone have not been identified yet. The purpose of this study is to clarify the uptake mechanism of taurine in osteoblast using mouse osteoblast cell lines. In this study, mouse stromal ST2 cells and mouse osteoblast-like MC3T3-E1 cells as osteoblast cell lines were used. The activity of taurine uptake was assessed by measuring the uptake of [3H]taurine in the presence or absence of inhibitors. TauT mRNA was detected in ST2 and MC3T3-E1 cells. [3H]Taurine uptake by these cells was dependent on the presence of extracellular calcium ion. The [3H]taurine uptake in ST2 cells treated with 4 mM calcium was increased by 1.7-fold of the control which was a significant change. In contrast, in Ca++-free condition and L-type calcium channel blockers (CCBs), taurine transport to osteocyte was significantly inhibited. In oxidative stress conditions, [3H]taurine uptake was decreased by TNF-α and H2O2. Under the hyperosmotic conditions, taurine uptake was increased, but inhibited by CCBs in hyperosmotic condition. These results suggest that, in mouse osteoblast cell lines, taurine uptake by TauT was increased by the presence of extracellular calcium, whereas decreased by CCBs and oxidative stresses, such as TNF-α and H2O2.

Key words: Taurine, Taurine transporter, ST2 cell, MC3T3-E1 cell, Taurine uptake, TNF-α, Hyperosmotic condition, Calcium channel blockers

INTRODUCTION

Taurine (2-aminoethanesulfonic acid) is conditionally essential amino acid for children, which is present in a variety of tissue and exhibits many important physiological functions such as stabilization of membranes, osmoregulation, antioxidation, and detoxification (Huxtable, 1992). In bone cells, taurine is also found in high concentration (Lubec et al., 1997), and is known to help in enhancing the bone tissue formation, which was observed by increased matrix formation and collagen synthesis (Park et al., 2001). Besides stimulating the bone tissue formation, it is suggested that being incorporated in bone tissue, taurine plays an important role in bone metabolism by inhibiting experimental bone resorption, osteoclast formation, and osteoclast survival (Koide et al., 1999), however, this mechanism was not clarified yet.

Bones, in the state of disease, are risky to be exposed to osmotic pressure stress or inflammatory cytokines such as TNF-α. For the patients with diabetes mellitus, they are likely to be in high osmotic pressure condition since the level of glucose in the blood is high. In the condition of high osmotic pressure, it is reported that mRNA expression of TauT, betaine transporter (BGT-1) and sodium/myo-inositol co-transporter (SMIT) is changed and uptake of taurine, betaine and myo-inositol is elevated in many tissues (Warskulat et al., 1997; Takeuchi et al., 2000; Shioda et al., 2002; El-Sherbeny et al., 2004; Warskulat et al., 2004). Moreover, there are some papers that the mRNA expression of TauT and BGT-1 and the uptake of taurine and betaine into the kidney elevated under high osmotic pressure (Uchida et al., 1992; Yamauchi et al., 1992). However, it is not yet proved that...
how the uptake of taurine is changed when bone is exposed to the state of high osmotic pressure.

Calcium, which plays an important role in bone formation, may elevate uptake of taurine. However, the mechanism of Ca++, taurine uptake to the bone cells is not yet known. How these two factors are related to bone formation in osteoblast cell lines is the main concern in this study.

The purpose of this study is to clarify the mechanism of taurine uptake in osteoblast. In this study, we used mouse stromal ST2 cells and mouse osteoblast-like MC3T3-E1 cells. The stroma cell line ST2, which is derived from mouse bone marrow, differentiated into osteoblast-like cells in response to ascorbic acid (Otsuka et al., 1999). We investigated the expression of TauT and the effect of various factors on taurine uptake in these osteoblastic cells.

MATERIALS AND METHODS

Reagent

$[^{3}H]$Taurine ($[^{3}H]$), 30.3 Ci/mmole were purchased from NEN Life Science (Boston, MA, USA). Tumor necrosis factor-$\alpha$ (TNF-$\alpha$), human recombinant (Escherichia coli) solution, 10 $\mu$g/mL was purchased from Boehringer Mannheim Biochemica (Mannheim, Germany) and lipo polysaccharide (LPS) from Salmonella minnesota R595 (Re) was obtained from List Biological Laboratories (Campbell, CA, USA). All other chemicals were of reagent grade and commercially available.

Cell culture

ST2 cells and MC3T3-E1 cells were grown routinely in tissue culture dishes at 37°C under 5% CO$_2$ and 95% air. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY) 100 U/mL benzylpenicillin, 100 $\mu$g/mL streptomycin sulfate (Gibco, Grand Island, NY), 10% fetal bovine serum (Gibco, Grand Island, NY). The osmolality of the normal culture medium was about 280 mOsm/kg. Hyperosmotic culture medium (380 mOsm/kg) was prepared by adding 100 mM raffinose to normal culture medium.

$[^{3}H]$Taurine uptake study by culture ST2 and MC3T3-E1 cells

The $[^{3}H]$taurine uptake was performed through the previous report (Kang et al. 2002). ST2 cells ($1 \times 10^5$ cells/well) and MC3T3-E1 cells ($1.5 \times 10^5$cells/well) were cultured at 37°C for 2 days on rat tail collagen type-I coated 24-well plates (Becton Dickinson)and washed with 1 mL extracellular fluid (ECF) buffer consisting of 122 mM NaCl, 25 mM NaHCO$_3$, 3mM KCl, 1.4 mM CaCl$_2$, 1.2 mM MgSO$_4$, 0.4 mM K$_2$HPO$_4$, 10 mM D-glucose and 10 mM Hepes (pH 7.4) at 37°C. Uptake was initiated by applying 200 $\mu$L ECF buffer containing 0.5 $\mu$Ci $[^{3}H]$taurine for water adhesion at 37°C in the presence or absence of inhibitors. After appropriate time periods, uptake was terminated by removing the applied solution and cells were immersed in ice-cold ECF buffer. The cells were then solubilized in 750 $\mu$L 1 M NaOH. An aliquot (50 $\mu$L) was taken for protein assay using a DC protein assay kit (Bio-Rad, Hercules, CA, USA) with bovine serum albumin as a standard. The remaining solution (500 $\mu$L) was mixed with 5 mL scintillation cocktail (Hionic-fluor, Packard, Meriden, CT, USA) for measurement of radioactivity in a liquid scintillation counter (LS6500, Beckman, Fullerton, CA, USA).

Reverse transcription-polymerase chain reaction (RT-PCR) analysis

Expression of rat taurine transporter, rat glyceraldehyde 3-phosphate dehydrogenase (G3PDH), by osteoblast cell lines was analyzed by RT-PCR analysis. The sequences of the taurine transporter (TauT) primers were as follows: sense, 5'-CTACCGGTCCATCGTCATCGT-GT CC-3'; antisense, 5'-AAGTGAAGTTGGCG-GCGCTAAG GGA-3'. Total RNA was isolated by the acid phenol procedure using ISOGEN (Wako Pure Chemical Industries, Ltd., Tokyo, Japan) according to the manufacturer's protocol. The first standard cDNA reaction was performed using ReverTra Ace M-MLV reverse transcriptase (Rever Tra Ace, Toyobo Co., Ltd., Osaka, Japan). RT-PCR was performed with TAKARA Ex TaqTM (Takara Shuzo Co., Ltd., Shiga, Japan) according to the manufacturer's instructions. The PCR was carried out using the following protocol. After an initial melting temperature of 85°C for 5 min, primers were added as a hot start method. There was 1 min of denaturation at 94°C, 1 min of annealing at 57°C or 59°C, and 1 min of extension at 72°C for repeated cycles of amplification, followed by a final extension at 72°C for 10 min. The PCR products were analyzed on a 5% acrylamide gel, stained with ethidium bromide, and visualized under ultraviolet light.

Statistical analysis

All data represent mean±SEM values. An unpaired, two-tailed Student's t-test was used to determine the significance of differences between two group means and p<0.05 was considered statistically significant.
RESULTS

Effect of calcium ion on [3H]taurine uptake

The effect on taurine uptake into osteoblast cells was examined at the 5 min time point under calcium-free condition (Fig. 1). Under calcium-free condition, [3H]taurine uptake was significantly reduced by 57.7% and 30.4% in ST2 cells and MC3T3-E1 cells, respectively. The uptake of [3H]taurine was not affected in the presence of 2.8 mM (2-fold) calcium ion by ST2 cells and MC3T3-E1 cells. On the other hand, the uptake of [3H]taurine was increased in the presence of 4 mM calcium ion by ST2 cells, but not affected by MC3T3-E1 cells. Taurine uptake was regulated by Ca++ in progenitor osteoblast cells but, less in osteoblast cells.

Effect of TNF-α, LPS and H2O2 on [3H]taurine uptake

The effect of TNF-α, LPS and H2O2 treatment on [3H]taurine uptake was examined in osteoblast cell lines and results are summarized in Table I. Treatment with 10 ng/mL LPS for 24 h resulted in a significant increase in [3H]taurine uptake. On the other hand, 20 ng/mL TNF-α pre-incubation, for up to 4 h, showed increment and then decreased after 8 h in the [3H]taurine uptake by ST2 cells. However, in MC3T3-E1 cells, uptake increased till 8 hours, and began to decrease as 12 hours had passed.

Effect on hyperosmotic conditions on [3H]taurine uptake by osteoblast cell lines

The effect of hyperosmotic conditions on taurine transport activity was examined using osteoblast cell lines exposed to hypertonic culture medium (380 mOsm/kg) for up to 24 h. As shown in Fig. 3, TauT mRNA was increased up to 8 h in ST2 cells, but in MC3T3-E1 cells, resulted in an increase until 4 hours and decreased after 8 h. Under hyperosmotic conditions, though [3H]taurine uptake decreased from 1h to 12 h, increased at 24 h in ST2 and MC3T3-E1 cells (Fig. 4).

Effect of calcium channel blockers on [3H]taurine uptake

To examine the effect of calcium inhibitor on [3H]taurine uptake, we used the L-type calcium channel blockers, nifedipine and verapamil. The uptake of [3H]taurine at 5 min significantly inhibited in the presence of 100 µM nifedipine and 100 µM verapamil compared with control (Fig. 5). Also, the effect of calcium inhibitor on hyperosmotic-induced [3H]taurine uptake, 100 µM nifedipine and 100 µM verapamil inhibited the hyperosmotic-induced [3H]taurine uptake compared with hyperosmotic conditions (Fig. 6).

DISCUSSION

To characterize taurine transport by the osteoblast cells, [3H]taurine uptake was performed using ST2 and MC3T3-E1 cells. Under Ca++ free condition, [3H]taurine uptake was significantly reduced in ST2 and MC3T3-E1 cells by 57.7% and 30.4 %, respectively. On the other hand, the uptake of [3H]taurine increased in the presence of 4 mM Ca++ in ST2 cells, but not affected by MC3T3-E1 cells. As reported, taurine uptake in rat retina of original cell host was not affected under 0.1 mM Ca++ (Militante et al., 1999). However, in this study, taurine uptake in ST2 cell was reduced in low level of Ca++ concentration while increased in 4mM Ca++ (2.8 times under the normal conditions) (Fig. 1). There needs to be more reviews on the relationship between taurine and Ca ++ concentration. Taurine also has an effect on Ca++ uptake. Ca++ uptake is activated in many tissues such as heart, brain and retina, and maintains homeostasis as reported (Della et al., 2002; El Idrissi et al., 2004). It is also known that calcium plays an important role in bone formation. As taurine conductances were inhibited by L-type calcium channel blocker in rat astrocytes (Li G. et al., 2002), it is possible that change of extracellular Ca ++ concentration also affects the taurine uptake in osteoblast. Therefore, we examined the effect of Ca++ or L-type calcium channel blocker for the [3H]taurine uptake by osteoblast cells. The effect of taurine transporter on the extracellular taurine concentration under calcium depletion increased the evoked taurine release in rat striatum (Molchanova et al.,

### Table I. The effect of oxidative stress on [3H]taurine uptake by ST2 and MC3T3-E1 cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Uptake of [3H]taurine (% of control)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>ST2 cells</td>
</tr>
<tr>
<td>Control</td>
<td>100 ± 4</td>
</tr>
<tr>
<td>TNF-α</td>
<td>78.7 ± 3.7”</td>
</tr>
<tr>
<td>H2O2</td>
<td>13.9 ± 1.3”</td>
</tr>
<tr>
<td>LPS</td>
<td>109 ± 2’</td>
</tr>
</tbody>
</table>

[3H]Taurine (41.3 nM) uptake was at 37°C for 5min in the presence of taurine transporter substrates such as 20 ng/mL TNF-α, 100 µM H2O2, 10 ng/mL LPS. Each value represents the mean ± SEM (n=4).

*p<0.05, **p<0.01, ***p<0.001; significantly different from control.
Moreover, the uptake of $[^3H]$taurine was significantly inhibited in the presence of nifedipine and verapamil, which are L-type calcium channel blockers, compared with control in ST2 and MC3T3-E1 cells. Some reported that taurine excretion in astrocyte cell of rat is inhibited by Ca++ and calmodulin inhibitor ((Li G. et al., 2002). From this study, it is supposed that inhibiting taurine uptake by calcium inhibitor is from inhibiting TauT expression. That is also because calcium inhibitor blocks reaction of Ca++ and calmodulin.

Cytokine is one of the factors modulating taurine uptake. TNF-$\alpha$, an inflammatory cytokine, increases expression of TauT mRNA and intracellular taurine uptake (Kang et al., 2002; Mochizuki et al., 2002). Pre-treatment TNF-$\alpha$ increases taurine uptake more in TR-BBB13 than in rat astrocyte and human intestinal Caco-2 cells (Chang et al., 2001). In this study, taurine uptake was decreased 24 hours after pre-treatment TNF-$\alpha$ in ST2 and MC3T3-E1 cell hosts. And so, the changes of uptake for 24 hours after pre-treatment were investigated (Fig. 2). As the result, $[^3H]$taurine uptake in ST2 cell was increased 1 hour after pre-treatment TNF-$\alpha$ but began to decrease after that. In MC3T3-E1 cell, it was increased for 8 hours, decreased after that. Moreover, TNF-$\alpha$ pre-treatment obviously decreased $[^3H]$taurine uptake (22% decrease in ST2 cell, 48% decrease in MC3T3-E1 cell). As there is a report that TNF-$\alpha$ stimulation induced apoptosis in MC3T3-E1 cell (Pavalko et al., 2003), in this experiment, it can be considered that taurine uptake was relatively decreased with apoptosis being induced even though taurine uptake increased as time pass.

LPS, known as an endotoxin, is reported to stimulate production of TNF-$\alpha$ and increases taurine uptake in rat astrocyte. From this experiment, taurine uptake in ST2 and MC3T3-E1 cells was increased by 24 hours after LPS pre-treatment. It is known that LPS stimulates production of many kinds of cytokines inducing TNF-$\alpha$ (Chung and Benveniste, 1990). Also, in this study, there was no increase of taurine uptake in ST2 and MC3T3-E1 cell hosts. Therefore, it is considered that the uptake of taurine increased by LPS was not influenced by TNF-$\alpha$ produced from LPS, but other cytokines or another factor. Taurine (100 $\mu$g/ml) suppressed the formation of these osteoclast-like cells in the presence of LPS, IL-1$\alpha$ or PGE2 in mouse marrow cultures. Although IL-1$\alpha$ elongated the survival of the osteoclast-like cells, taurine blocked the supportive effect of IL-1$\alpha$ on osteoclast survival (Koide et al., 1999). In contrast, LPS decreased taurine uptake significantly in macrophage cell line, RAW 264.7 cell (Kim et al., 2003). This study suggested that LPS induced down regulation of taurine uptake in murine macrophage might be mediated via the action of nitric oxide. Therefore, the mechanism study of changes of taurine uptake by LPS is needed to be further elucidated.

Oxidative stress is another factor of modulating taurine uptake. It is known that $H_2O_2$ or cumene hydroperoxide which is a kind of active oxygen decreases taurine uptake (Wersinger et al., 2001). Thus, $[^3H]$taurine uptake

![Fig. 1. Effect of calcium ion on $[^3H]$taurine uptake ; $[^3H]$Taurine (41.3 nM) uptake was at 37°C for 5 min under of Ca++ free and calcium ion. Ca++ was replaced with Mg++. Each value represents the mean ± SEM (n=4). *P<0.01, **P<0.001; significantly different from control.](image1)

![Fig. 2. $[^3H]$Taurine uptake effect after TNF-$\alpha$ preincubation for 24 h in ST2 and MC3T3-E1 cells. Each point represents the mean ± SEM (n=4). *P<0.01, **P<0.001; significantly different from the time=0.](image2)
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in ST2 cell obviously decreased by 36 % compared to control by 24 hours after H2O2 pre-treatment. There is also a report that oxidative stress inhibits ion channel, such as Na+ channel, Cl- channel and Ca++ channel (Kourie, 1998). Taurine uptake is related to TauT that is dependent on Na+ and Cl-, and that in ST2 cells and MC3T3-E1 cells is dependent on Ca++ too. Therefore, we can suggest that decrease of taurine uptake by H2O2 pre-treatment is because of the concentration in Na+, Cl- and Ca++ by inhibiting ion channels.

Taurine is also known to act as an organic osmolyte. Organic osmolytes are involved in regulatory mechanisms of cell volume decrease and increase (Warskulat et al., 1997). It is reported that osmolyte uptake and osmolyte transporter mRNA are increased by hyperosmotic exposure in several tissues (Warskulat et al., 1997; Satsu et al., 1999; Shioda et al., 2002). However, the hyperosmotic responses to taurine uptake in osteoblast cells have not been previously investigated. Therefore the effect of hyperosmotic conditions (by addition of 100 mM raffinose) on [3H]taurine uptake activity was examined. Under hyperosmotic conditions, [3H]taurine uptake was increased at 24 hours in both ST2 and MC3T3-E1 cells, though decreased up to 12 hours (Fig. 4). To determine whether the expression of TauT mRNA was induced or not by hyperosmotic conditions, the semi-quantitative analysis was performed. The TauT mRNA level was markedly higher in ST2 and MC3T3-E1 when exposed to hyperosmotic conditions (Fig. 3). It was obvious that TauT mRNA expression level and taurine uptake was increased under hyperosmotic condition in these osteoblast cell lines as well as other tissues. Taurine uptake by pre-treatment for 24 hours shows more decrease than that for 12 hours, and it is because of decrease in TauT expression. However, there are needs for more reviews for other factors that may have the effect on decrease of uptake.

Next, we examined whether hyperosmotic-induced [3H]taurine uptake was inhibited by L-type calcium channel blockers (Fig. 6). Nifedipine and verapamil also inhibited

Fig. 3. Time-dependent change in TauT mRNA levels in ST2 and MC3T3-E1 exposed to hyperosmotic conditions. Cells were treated with 100 mM raffinose. Each point represents the mean ± SEM (n=3).

Fig. 4. Time-course of the effect in hypertonic conditions for 24 hrs on [3H]taurine uptake at ST2 and MC3T3-E1 cells. Cells were treated with 100 mM raffinose. Each point represents the mean ± SEM (n=4). *P<0.01, **P<0.001; significantly different from the time=0.
the hyperosmotic-induced [3H]taurine uptake compared with control. It is reported that taurine uptake induced by hyperosmotic pressure is inhibited by Ca++/calmodulin-dependent protein kinase II inhibitor in human Caco-2 cell (Satsu et al., 2004). In this study, decrease in taurine uptake by Ca++ channel blockers is supposed that, channel blocker inhibits the effects of Ca++ on CaM as Ca++-dependent taurine uptake and then TauT expression is decreased. The effect of Ca++ and calcium channel blocker on taurine uptake showed similar behavior between ST2 and MC3T3-E1 cells although some difference was observed. Therefore, our results suggested that Ca++ regulated taurine uptake in osteoblast cells.

Moreover, taurine uptake in osteoblast cell lines was regulated by Ca++ and the L-type calcium channel blocker, although some differences were observed between ST2 and MC3T3-E1 cells. Furthermore taurine uptake in osteoblast cells was regulated by hyperosmotic conditions accompanied by the change in expression level of TauT mRNA. In this condition taurine uptake was inhibited by calcium inhibitors. These results suggested that taurine uptake into the osteoblast cells contribute to bone formation is regulated by various factors. Also, although there are some differences compared to ST2 and MC3T3-E1 cells, it is considered that there may be some relationship with bone formation or bone absorption modulated by diverse factors such as Ca++, H2O2 and LPS.

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