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Taurine transporter is expressed in vascular smooth muscle cells

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Summary. The regulation of vascular smooth muscle cells (VSMCs) function by taurine has been a subject of increasing interest and investigation, and taurine is taken up into cells through a specific transporter system, the taurine transporter (TAUT). In the present study, we examined the expression of TAUT in VSMCs and the kinetic parameters of the uptake process of TAUT in VSMCs. RT-PCR and western blot demonstrated that the mRNA and protein of TAUT was expressed in VSMCs in vitro. Immunohistochemistry using antibody for TAUT revealed the expression of this protein in rat thoracic aorta. The maximal [^{3}H]taurine uptake rate in VSMCs was 37.75 ± 3.13 pmol/min per mg of protein, with a K_m value of 5.42 ± 0.81 μM. Thus, VSMCs are able to express a functional taurine transporter. The regulation and detailed function of taurine and TAUT in VSMCs remain unclear, but our findings suggest a functional role for them in VSMCs metabolism.

Keywords: Taurine transporter – Vascular smooth muscle cells – Kinetic analysis

Introduction

Taurine (2-aminoethanesulphonic acid) is a sulfur-containing free β-amino acid in mammals. Though this amino acid is not incorporated into proteins and is known to participate in only very few metabolic reactions, various evidences show that taurine might participate in a variety of cellular functions and even be an essential nutrition during development. Various physiological roles have been suggested for taurine, including calcium modulation, membrane stabilization, intracellular osmosis regulation, and protein phosphorylation regulation (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.

The cellular taurine content is a balance between active uptake of taurine via the saturable, Na^+- and Cl^--dependent taurine transporter (TAUT), and passive release through a volume-sensitive taurine leak pathway. In the intracellular space taurine is present in millimolar concentrations, whereas taurine is found at the concentration of 20–100 nM in plasma (Huxtable et al., 1992; Wright et al., 1986), suggesting that TAUT plays an important role in maintaining a high concentration of taurine in tissues. Deficiency in TAUT expression/activity and/or changes in the cellular taurine content has been associated with a plethora of mammalian disorders such as destruction of skeletal muscle function (Warskulat et al., 2004), degeneration of retinal photoreceptors (Heller-Stilb et al., 2002) and abnormal development of kidney, heart and central nervous system (Huxtable, 1992). Recently, a TAUT knockout mouse has been established (Heller-Stilb et al., 2002). The animals exhibited retinal degeneration and a marked impairment of reproduction. These phenomena suggest that TAUT is a functional protein in maintaining cell physiological function in vitro and in vivo.

Evidences have suggested that taurine play an important role in vascular smooth muscle cells (VSMCs) function by inhibition of VSMCs proliferation and prevention of beta-glycerophosphate-induced calcification in VSMC (Zhang et al., 1999; Li et al., 2004). These findings indicate that TAUT may be expressed in VSMCs. The present works investigate the expression of TAUT in VSMC and characterize the function of TAUT expressed in VSMCs.

Materials and methods

Materials

[^{3}H]Taurine (specific activity 30.0 Ci/mmol) was obtained from Amersham International (Buckinghamshire, UK). TAUT polyclonal affinity-purified

IgG was purchased from ADI (Alpha Diagnostic Intl., Inc, USA). Male Sprague–Dawley rats (weighing 200–250 g) were purchased from the animal center, the second Xiangya hospital of central south university. 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.

Cell culture

Rat VSMCs were acquired by an explant method described by Campbell and Campbell (1993). Briefly, medial tissue was separated from segments of rat thoracic aorta. Small pieces of tissue (1–2 mm^3) were placed in a 10-cm culture dish and cultured for several weeks 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 μg/ml of streptomycin, at 37 °C, in a humidified atmosphere containing 5% CO_2. Cells migrating from the explants were digested using trypsin and collected, and maintained in DMEM containing 10 mM sodium pyruvate supplemented with 15% FBS (culture medium). Vascular smooth muscle cells were passaged every 3–4 days, and experiments were performed between passages 3 and 6 from the primary culture. Immunocytochemical examination showed positive staining in all cells for α-smooth muscle actin. For the positive control, primary rat cardiac myocyte was prepared as described before (Sadoshima and Izumo, 1993).

Western blotting

Monolayers of VSMCs and rat cardiac myocyte in 25 cm^2 culture flasks were rinsed twice with 1 mM EDTA in PBS and lysed with triton lysis buffer (50 mM Tris–HCl, pH 8.0, containing 150 mM NaCl, 1% triton X-100, 0.02% sodium azide, 10 mM EDTA, 10 μg/ml aprotinin, and 1 μg/ml aminoethylbenzenesulfonyl fluoride (Sigma) and then saved after centrifugation. Total protein content was determined by the BCA method. Fifty micrograms of total protein from each sample were loaded onto a polyacrylamide gel (Bio-Rad) and separated by electrophoresis. The protein transfer was performed by the semidry method (Bio-Rad) onto polyvinylidene difluoride (PVDF) membrane. The blot was blocked with 2.5% nonfat milk in PBS for a minimum of 1 h. The membrane was incubated with 1 μg/ml rabbit anti-TAUT polyclonal antibody in PBS at room temperature for 3 h. The membrane was washed three times in PBS and incubated with corresponding horseradish peroxidase (HRP)-conjugated secondary antibody (Amersham International) for 1 h. Detection was performed with a chemiluminescence detection kit according to the manufacturer's protocol (ECL Detection, Amersham International).

Reverse-transcriptase polymerase chain reaction (RT-PCR) assay

Total RNA of VSMCs was extracted with use of standard techniques. For the positive control, the RNA from cultured rat cardiac myocytes was isolated at the same time. Isolated RNA was then quantified by use of an ultraviolet (UV) spectrophotometer (DU-640, Beckman, USA). Reverse transcription to cDNA was accomplished by priming 2 μg of total RNA samples with MMLV and oligo (dT) 15 primer. The products were then used for the following PCR amplification: the PCR reaction mixture was in a 25-μl volume containing 2.5 mM dNTP 1 μl, 10 × PCR buffer (20 mM $MgCl_2$, 500 mM KCl, 1.5 M Tris–HCl, pH 8.7), 2.5 μl cDNA, 200 nM of the TAUT-paired primers and 1.25 unit of Taq DNA polymerase. For mouse TAUT, the PCR primers were 5′ GCCACATACTACCTATTCCA 3′ and 5′ CAGCAGCATACAGTCCCT 3′, yielding a 555 bp fragment, which was according to our previous experiment (Yuan et al., 2006). The following PCR cycles were used: After an initial denaturation at 94 °C for 5 min, PCR was run for 30 cycles at 94 °C for 30 sec, 52 °C for 30 sec and 72 °C for 40 sec, and then 72 °C for 5 min. As an internal control for each PCR reaction, β-actin mRNA was also amplified with each sample. A total of 200 nM of actin primers and cDNA 2 μl was amplified under the same reaction conditions. All PCR products were loaded onto a 1.5% agarose–Tris–acetate–EDTA gel before electrophoresis, and products were visualized on ethidium bromide staining. The UV illumination photos then underwent computerized densitometric analysis. The PCR product was purified using the Qiagen Plasmid kit (Qiagen Inc., Chatsworth, CA), and sequenced bidirectionally employing the same primer pairs used for PCR by means of an automated sequencer (AB3730, Applied Biosystems, Foster City, CA) in the DNA Sequence Facility of Invitrogen, using dideoxy terminator Taq technology.

Immunohistochemistry for the TAUT in aorta tissue

For the immunohistochemistry examination, rat thoracic aorta was obtained from Sprague–Dawley rats. Before staining, tissue sections were baked for 1 h at 65 °C and deparaffinized through xylene and graded ethanol (100, 95, and 70%, two times at 2 min each). Sections were rinsed in phosphate-buffered saline (PBS), pH 7.4, and blocked for 15 min in 10% normal goat serum diluted in PBS. The primary antibody used was rabbit polyclonal affinity-purified IgG anti-TAUT (raised against a 20 amino acid-peptide near the carboxy-terminal region of TAUT) or 0.1 M PBS instead of primary antibodies for negative controls. Primary antibodies were incubated overnight for 18 h at 4 °C. Conventional immunohistochemistry was performed using a biotinylated goat anti-rabbit secondary antibody. After extensive wash using PBS, the color reaction was developed with the AEC chromogen kit (Boster Company, WuHan, China) for about 10 min. Slides were washed with deionized water, air-dried, and counterstained.

Measurement of taurine transporter activity

Experiments on the taurine uptake by VSMCs were basically performed as previously described (Brandsch et al., 1993; Satsu et al., 1999). Briefly, VSMCs were grown to 80% confluence in six-well polyethylene dishes (9.6 cm^2 per well). The cells were washed three times by gentle aspiration/addition of 2 ml aliquots of taurine uptake buffer (HEPES 20 mmol/l, NaCl 140 mmol/l, KCl 5.4 mmol/l, $CaCl_2$ 1.8 mmol/l, $MgSO_4$ 0.8 mmol/l, and glucose 5 mmol/l) at 37 °C and equilibrated in 2 ml taurine uptake buffer at 37 °C. Uptake was initiated by adding uptake buffer containing [^{3}H]taurine (0.1 mCi/ml, 1 ml) and increasing concentrations of unlabeled substances. After incubation for 20 min, the cells were quickly washed four times with ice-cold buffer, solubilized with 0.1 mol/l NaOH, sonicated, and measured by liquid scintillation spectrometry. Following the final wash, the sixth well was used for estimation of the average protein content (g protein per well) using a Bradford protein assay.

Data analysis

Experiments were done in duplicate or triplicate and each experiment was repeated two to three times. Results are given as means ± SEM. Apparent V_{max} and K_m were determined by nonlinear regression fit to Michaelis–Menten equation using the nonlinear regression program (GraphPad Prism software).

Results

TAUT was expressed in cultured VSMCs

We confirmed the TAUT mRNA was expressed in rat VSMCs by RT-PCR using TAUT-specific intron-spanning primers. TAUT expression in rat cardiac myocyte was used as a positive control. The results showed a 555 bp

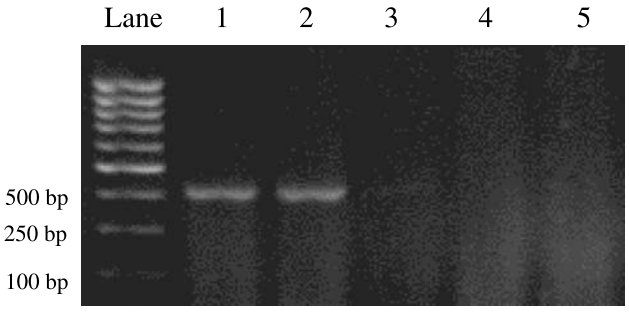

Fig. 1. Total RNA from primary rat VSMCs (*1*) and cardiac myocyte (*2*) was subjected to RT-PCR. The PCR products were visualized in a 1.2% agarose gel stained with ethidium bromide. Omission of RT (*3*) and H_2O substituted for the template (*4*) were used as negative control (*5*)

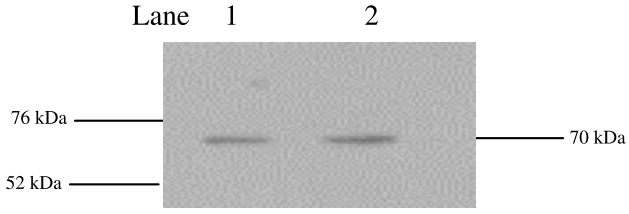

Fig. 2. Total cellular protein was subjected to immunoblot analysis using an anti-TAUT antibody. The anti-TAUT antibody identified a band at 70 kDa. *1* Rat VSMCs; *2* rat cardiac myocyte. Left margin indicates wt standards (kDa)

fragment specific to TAUT, and no bands were observed in reactions without RT or with H_2O as template (Fig. 1).

Immunoblot analysis revealed that protein expression of TAUT could be detected in rat VSMCs (Fig. 2). TAUT expression was evaluated with a polyclonal antibody raised against a peptide mapping at the carboxy-terminus of the TUAT. A dominant band of 70 kDa was identified in the immunoblots in VSMCs (lane 1), and rat cardiac myocyte (lane 2), which has been shown to express the TAUT.

We next investigated the expression of the TAUT in sections of rat thoracic aorta using immunohistochemistry. As can be seen in Fig. 3, rat ascending aorta tissue sections demonstrated specific labeling for TAUT protein in cytomembrane and cytoplasma. Thus, western blot and immunohistochemistry examination employing a specific anti-TAUT antibody revealed that these VSMCs expressed TAUT protein in vitro and in vivo.

Characteristics of the taurine transporter on VSMCs in culture

To calculate the kinetic parameters of the uptake process we examined taurine transport in VSMCs as a function of substrate concentration in the range of 0.5–100 µM (Fig. 4). The study was performed in the presence of

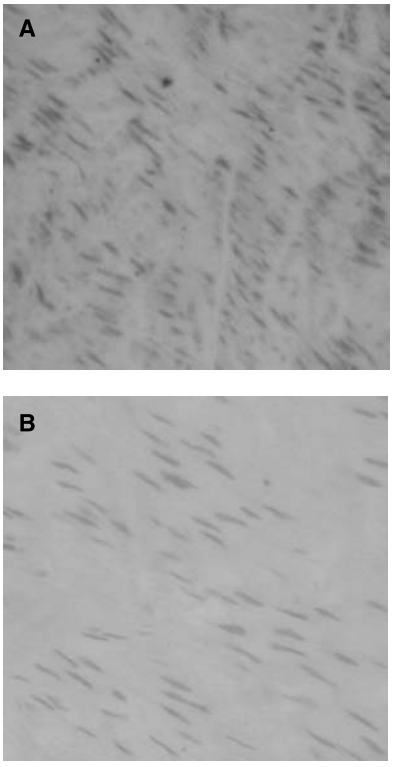

Fig. 3. Immunohistochemistry demonstrated that the cytomembrane and cytoplasm could be positively stained using TAUT antibody in rat ascending aorta (**A**). No staining was shown for the negative controls (**B**) (×200)

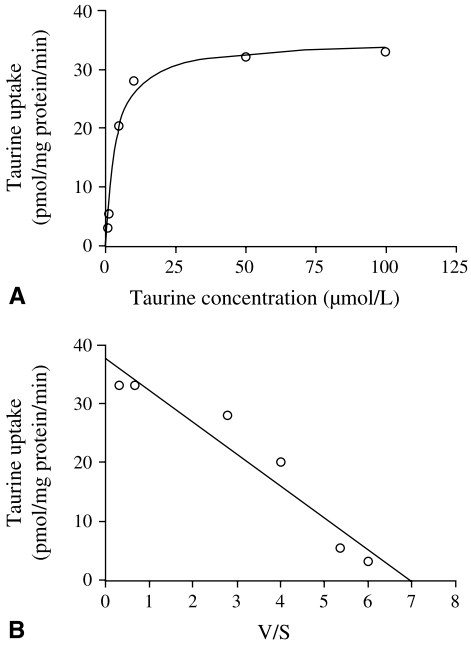

Fig. 4. **A** Uptake of [^{3}H]taurine (10 nM) was measured in the presence of increasing concentrations of unlabeled taurine (0.5, 1.0, 5.0, 10.0, 50.0, 100.0 µmol) for 20 min. The results were obtained from three different cultures. **B** Eadie–Hofstee plots

extracellular Na^+ at 37 °C for 20 min. The non-saturable diffusional component was determined by measuring the uptake of [^{3}H]taurine (10 nM) in the presence of 10 mM unlabeled taurine. This value was subtracted from the total transport rates to calculate the saturable, carrier-mediated component. The apparent affinity constant K_m (Michaelis–Menten constant) and the maximal velocity V_{max} of the Na^+-dependent taurine transporter were 5.42 ± 0.81 μM and 37.75 ± 3.13 pmol/min per mg of protein, respectively.

Discussion

We report here the first demonstration of the transcription and translation of TAUT in culture of VSMCs in vitro. We also show expression of TAUT in vivo in rat thoracic aorta. Additionally, the TAUT expressed in VSMCs is a functional protein, which can take up [^{3}H]taurine. These results have important implications toward understanding how taurine could influence the metabolism of VSMCs.

TAUT is known to be a specific transporter to taurine, whose sequence homology places it within the gene family of Na^+- and CL^--dependent neurotransmitter transporters (Smith et al., 1992; Liu et al., 1992). TAUT is a high-affinity and low-capacity sodium- and chloride-dependent transporter. Taurine transport requires at least two Na^+ ions and one Cl^- ion to transport one taurine molecule across the cell membrane. The recent application of molecular biological techniques to taurine research field led to the isolation and characterization of cDNA that encode tarurine transporters from many tissues, primary cultures, and established cell lines (Uchida et al., 1992; Smith et al., 1992; Jhiang et al., 1993; Ramamoorthy et al., 1994; Jayanthi et al., 1995; Miyamoto et al., 1996; Leibach et al., 1993; Yuan et al., 2006). The TAUT cDNA sequence encodes a protein of 620 amino acids which contains twelve potential transmembrane domains. Different TAUT protein bands in the range of 109–30 kDa have been previously demonstrated by immunoblotting. Xu et al., observed three translation products of TAUT (107, 69, and 49 kDa) expressed in the epididymis, indicative of alternate splicing of the gene. In contrast, Han et al. (1999) found only one band (70 kDa) in the canine kidney cell TAUT. Han et al. (1996) also identified a 30 kDa protein using an antibody raised against the rat taurine transporter. The variance in the molecular mass of TAUT reflects differences in post-transcriptional modifications such as glycosylation and/or phosphorylation or expression of different TAUT isoforms that need to be investigated further. And whether these low molecular proteins represent functional transporters, or spliced fractions, remains to be investigated. Our polyclonal antibody raised against the carboxy-terminus of the TAUT recognized a band of 70 kDa in VSMCs, which is identical to that seen in canine kidney cell (Han et al., 1999). The magnitude of the transporter protein expressed in VSMCs was similar to that in myocytes. The expression of taurine transporter proteins as assessed by RT-PCR analysis was also similar to the protein results. Our immunohistochemistry studies demonstrated that positive stain of TAUT protein in rat thoracic aorta VSMCs, which confirmed that TAUT is not only expressed in vitro, but also in vivo. Thus, these findings demonstrate that transcription and translation of TAUT occurs in VSMCs.

We have also begun to explore the function of TAUT expression in VSMCs. Uptake of taurine by VSMCs as a function of substrate concentration can be described by a Michaelis–Menten-type kinetics with an affinity constant of Km 5.42 ± 0.81 μM. Other authors have measured in different tissues and cells, K_m values between 1 and 232 μM, mostly in the 5–50 μM range. From the present experiment, the K_m of VSMCs is similar to that observed in rat cardiomyocytes.

The importance of taurine for VSMCs function is well documented. Zhang et al. (1999) demonstrated taurine and taurine analogues was found inhibition of rat VSMCs proliferation, and Li et al. (2004) showed taurine could prevent beta-glycerophosphate-induced calcification in cultured rat VSMCs, which suggested that taurine might be a candidate for use as a nutritional supplement to protect against vascular calcification, or even a new strategy for prevention and therapy of atherosclerosis and calcification.

Physiologically, the vessel wall is supplied with taurine from the blood. Serum concentrations of 100–200 μM have been determined (Pitkanen et al., 2003). The normal taurine concentration in animal tissues is reported to be one of the highest of any of the amino acids, attaining millimolar concentrations (25–35 mM in the heart). A system with a K_m value of 5.42 ± 0.81 μM will transport taurine under physiological conditions efficiently and might also be responsible for the transport of taurine that is used in therapeutic preparations.

Taurine may directly influence the metabolism of VSMCs, as has been described earlier in rat cardiomyocytes (Takatani et al., 2004) and heart tissue (Li et al., 2005). The mechanisms of taurine influences on the regulation of VSMCs have not been elucidated. The presently observed expression of TAUT in VSMCs provides fundamental information for this intriguing issue.

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