Note

Hypertonicity Enhances GABA Uptake by Cultured Rat Retinal Capillary Endothelial Cells

Tohru YAHARA, Masanori TACHIKAWA, Shin-ichi AKANUMA and Ken-ichi HOSOYA*
Department of Pharmaceutics, Graduate School of Medicine and Pharmaceutical Sciences, University of Toyama, Toyama, Japan

Summary: We have reported previously that taurine transporter (TauT) mediates \( \gamma \)-aminobutyric acid (GABA) as a substrate in a conditionally immortalized rat retinal capillary endothelial cell line (TR-iBRB2 cells). This study investigates how TauT-mediated GABA transport is regulated in TR-iBRB2 cells under hypertonic conditions. \([3H]GABA\) uptake by TR-iBRB2 cells exposed to 12 h- to 24 h-hypertonic culture medium was significantly greater than that of isotonic culture medium. \([3H]GABA\) uptake by TR-iBRB2 cells was \( \text{Na}^{+}\), \( \text{Cl}^{-}\), and concentration-dependent with a Michaelis-Menten (\( K_m \)) constant of 3.5 mM under isotonic conditions and \( K_m \) of 0.324 and 5.48 mM under hypertonic conditions. Under hypertonic conditions, \([3H]GABA\) uptake by TR-iBRB2 cells was more potently inhibited by substrates of TauT, such as taurine and \( \beta \)-alanine, than those of GABA transporters such as GABA, nipecotic acid, and betaine. These results suggest that an unknown high-affinity GABA transport process and TauT-mediated GABA transport are enhanced under hypertonic conditions. In conclusion, hypertonicity enhances GABA uptake by cultured rat retinal capillary endothelial cells.

Keywords: \( \gamma \)-Aminobutyric acid (GABA); taurine transporter (TauT); retinal capillary endothelial cells; hypertonicity

Introduction

\( \gamma \)-Aminobutyric acid (GABA) in the retina acts as an inhibitory neurotransmitter\(^1\) and osmolyte.\(^2\) The neurotransmitter actions of GABA in the synaptic cleft are terminated by its reuptake by either pre-synaptic neurons or nearby glial cells via GABA transporters (GATs).\(^3\) We recently found that taurine transporter (TauT/Slc6a6) transports GABA as a substrate and TauT is expressed in retinal capillary endothelial cells.\(^4,5\) These results suggest that TauT in the retinal capillary endothelial cells, which form the inner blood-retinal barrier (inner BRB), contributes to GABA uptake in the interstitial fluid of the retina when the GABA concentration is relatively high since TauT-mediated GABA transport has a lower affinity (\( K_m = \approx 2 \text{ mM} \)) than that of GATs (\( K_m = \approx 80 \mu \text{M} \)).\(^3,4\)

Severe hyperglycemia causes a hypertonic state in body fluids of patients.\(^6\) In a hypertonic environment, taurine accumulates in cells via activation of TauT as an adaptive regulation. Induction of TauT by hypertonicity is mediated by the tonicity-response element (TonE)/TonE-binding protein (TonEBP) pathway.\(^7\) Although TauT mRNA and taurine uptake in bovine aortic, rat brain, and retinal endothelial cells is known to be up-regulated by hypertonicity,\(^5,8,9\) our knowledge of this regulation of TauT-mediated GABA transport is still incomplete.

The present study investigates how TauT-mediated GABA transport is regulated in a conditionally immortalized rat retinal capillary endothelial cell line (TR-iBRB2 cells) as an in vitro model of the inner BRB\(^{10,11,12}\) under hypertonic conditions.

Methods

Reagents: 4-Amino-n-[2,3-\( ^3 \text{H} \)]butyric acid (\( [\text{H}] \text{GA-}\text{BA}, 90.0 \text{ Ci/mmol} \)) and [1,2-\( ^3 \text{H} \)]taurine (\( [\text{H}] \text{taurine}, 31.0 \text{ Ci/mmol} \)) were purchased from GE Healthcare (Little Chalfont, Buckinghamshire, UK). L-[4,5-\( ^3 \text{H}(\text{N}) \)]-leucine (\( [\text{H}] \text{L}-\text{leucine}, 59.2 \text{ Ci/mmol} \)) was purchased from Perkin-Elmer Life and Analytical Science (Boston, MA). All

Received; June 23, 2010, Accepted; September 10, 2010, J-STAGE Advance Published Date; October 1, 2010
*To whom correspondence should be addressed: Professor Ken-ichi HOSOYA, Department of Pharmaceutics, Graduate School of Medicine and Pharmaceutical Sciences, University of Toyama, 2630, Sugitani, Toyama, 930-0194, Japan. Tel. +81-76-434-7505, Fax. +81-76-434-5172, E-mail: hosoyak@pha.u-toyama.ac.jp
other chemicals were commercial products of reagent grade.

**Cell culture:** TR-iBRB2 cells (passage number 28–33) were grown routinely in rat tail collagen type I-coated tissue culture plates (BD Biosciences, Bedford, MA) and cultured at 33 °C in a humidified atmosphere of 5% CO2/air as described previously.10) The cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Nissui Pharmaceuticals, Tokyo, Japan) containing 100 U/mL benzylpenicillin potassium, 100 μg/mL streptomycin sulfate, and 10% fetal bovine serum (Moregate, Bulimbra, Australia). The permissive-temperature for TR-iBRB2 cell culture is 33 °C due to the presence of temperature-sensitive SV 40 large T-antigen.10,11) The osmolality of the isotonic culture medium was 270 mOsm/kg. Hypertonic culture medium (370 mOsm/kg) was prepared by adding 100 mM sucrose to DMEM.

**[3H]GABA uptake study by TR-iBRB2 cells:** [3H]GABA uptake was measured as described in a previous report.4,5) In brief, TR-iBRB2 cells (1.0 × 10⁵ cells/well) were cultured at 33 °C for 48 h on a rat tail collagen type I-coated 24-well plate (BD Biosciences) and washed with extracellular fluid (ECF) buffer consisting of 122 mM NaCl, 25 mM NaHCO₃, 3 mM KCl, 1.4 mM CaCl₂, 1.2 mM MgSO₄, 0.4 mM K₂HPO₄, 10 mM D-glucose, and 10 mM HEPES (pH 7.4) at 37 °C. Under hypertonic conditions, TR-iBRB2 cells were cultured for up to 24 h in hypertonic culture medium before the uptake study. Uptake was initiated by applying 200 μL ECF buffer containing 0.20 μCi [3H]GABA (11 nM) at 37 °C in the presence or absence of inhibitors. Na⁺ or Cl⁻-free ECF buffers were prepared by replacement with equimolar choline or gluconate, respectively. After a predetermined period, uptake was terminated by removing the applied solution, and cells were washed with ice-cold ECF buffer. The cells were then solubilized in 1 N NaOH, and subsequently neutralized with 1 N HCl. Cell associated radioactivity and protein content were assayed in a liquid scintillation counter (LSC5200, Aloka, Tokyo, Japan) and by using a DC protein assay kit (Bio-rad, Hercules, CA) with bovine serum albumin as a standard.

**Data analysis:** The uptake of [3H]GABA by TR-iBRB2 cells was expressed as the cell-to-medium ratio (cell/medium ratio: μL/mg protein) using the following equation 1:

\[
\text{Cell/medium ratio} = \frac{([3H] \text{ dpm in the cell per mg protein})}{([3H] \text{ dpm in the medium per } \mu L)}
\]  

(1)

The kinetic parameters for GABA uptake by TR-iBRB2 cells were obtained from the following equations 2 and 3:

\[
V = \frac{V_{\text{max1}} \times [S]}{[S] + K_{\text{m1}}} + [S] + K_{\text{m2}} + [S])
\]  

(2)

where V is the uptake rate of GABA, [S] is GABA concentration in the medium, Vmax is the maximum uptake rate, and Km is the corresponding Michaelis-Menten constant.

\[
V = \frac{V_{\text{max1}} \times [S]}{[S] + K_{\text{m1}}} + [S] + K_{\text{m2}} + [S])
\]  

(3)

where Vmax1 is the maximum uptake rate for the high-affinity process, Km1 is the corresponding Michaelis-Menten constant, Vmax2 is the maximum uptake rate for the low-affinity process, and Km2 is the corresponding Michaelis-Menten constant. To obtain kinetic parameters, the equation was fitted using the nonlinear least-square regression analysis program, MULTI.13) Unless otherwise indicated, all data represent means ± SEM. An unpaired, two tailed Student’s t-test was used to determine the significance of differences between two groups. Statistical significance of differences among means of several groups was determined by one-way analysis of variance followed by the modified Fisher’s least squares difference method.

**Results**

**Effects of hypertonic conditions on [3H]GABA uptake by TR-iBRB2 cells:** [3H]GABA uptake was performed using TR-iBRB2 cells exposed to hypertonic culture medium for up to 24 h (Fig. 1). Under hypertonic conditions, [3H]GABA uptake increased 1.47- to 1.82-fold compared with that under isotonic conditions during 12 h to 24 h preincubation. All subsequent preincubations under hypertonic conditions were conducted for 24 h.

**Characteristics of GABA transport in TR-iBRB2 cells under hypertonic conditions:** [3H]GABA uptake by TR-iBRB2 cells under hypertonic and isotonic conditions exhibited a time-dependent increase for at least 5 min with an initial uptake rate of 2.06 ± 0.15 μL/(min · mg protein) and 0.917 ± 0.049 μL/(min · mg protein).

**Fig. 1.** Effects of hypertonic conditions on [3H]GABA uptake by TR-iBRB2 cells
[3H]GABA uptake (0.20 μCi, 11 nM) by TR-iBRB2 cells was performed for 5 min at 37°C after preincubation of isotonic culture medium (open circle) or hypertonic culture medium (closed circle). Each point represents the mean ± S.E.M. (n=4). *p<0.01 significantly different from isotonic culture medium.
Fig. 2. Characteristics of [3H]GABA uptake by TR-iBRB2 cells under hypertonic conditions
(A) Time-course of [3H]GABA uptake by TR-iBRB2 cells under hypertonic (closed circle) and isotonic conditions (open circle). The [3H]GABA uptake (0.20 μCi, 11 nM) was performed at 37°C after 24 h-preincubation of hypertonic culture medium or isotonic culture medium. Each point represents the mean ± S.E.M. (n = 4). (B) Na⁺- and Cl⁻-dependence of [3H]GABA uptake by TR-iBRB2 cells under hypertonic (closed bar) and isotonic conditions (open bar). [3H]GABA uptake (0.20 μCi, 11 nM) was performed in the presence (control) or absence of Na⁺ and Cl⁻ at 37°C for 5 min after 24 h-preincubation of hypertonic culture medium or isotonic culture medium. Each column represents the mean ± S.E.M. (n = 4). *p < 0.01, #p < 0.01 significantly different from each control. (C) Comparison of [3H]GABA, [3H]taurine, and [3H]L-leucine uptake by TR-iBRB2 cells under hypertonic conditions. [3H]GABA (0.20 μCi, 11 nM), [3H]taurine (0.10 μCi, 16 nM) and [3H]L-leucine (0.10 μCi, 8 nM) uptake at 37°C for 5 min after 24 h-preincubation of hypertonic culture medium (closed bar) or isotonic culture medium (open bar). Each column represents the mean ± S.E.M. (n = 4). *p < 0.01 significantly different from isotonic conditions. (D) Concentration-dependence of GABA uptake by TR-iBRB2 cells. The [3H]GABA uptake (0.20 μCi) was performed at 37°C, for 5 min, over the GABA concentration range 10 μM to 10 mM after 24 h-preincubation of hypertonic culture medium (closed circle) or isotonic culture medium (open circle). Each point represents the mean ± S.E.M. (n = 4). Data were subjected to Michaelis-Menten (main) and Eadie-Scatchard analyses (inset).
BA uptake than that of taurine, β-alanine, GABA or nipecotic acid. The inhibitory effect of TR-iBRB2 cells on [3H]GABA uptake under hypertonic conditions was similar to that under isotonic conditions (Table 1). These results support the hypothesis that an unknown GABA transport process and TauT-mediated GABA transport are enhanced by hypertonicity.

**Discussion**

In the present study, we report GABA uptake in retinal capillary endothelial cells under hypertonic conditions.

[3H]GABA uptake by TR-iBRB2 cells under hypertonic conditions was significantly greater than that under isotonic conditions (Figs. 1 and 2A). This suggests that retinal capillary endothelial cells maintain their cell volume at least partly by GABA influx when the retinal osmolarity is increased. Generally, in response to hypertonic stress, cells initially shrink and the volume recovers following the uptake of inorganic and organic osmolytes, such as small amino acids and methylamines. Of these amino acids, although taurine is mostly used as an organic osmolyte in the cells, GABA plays a role as an osmolyte in the retina and brain. Hyperglycemic syndromes increase the serum glucose level and concomitantly serum osmolarity. Severe hyperglycemia causes hypertonic states in patients with more than 350 mOsm/kg serum. Therefore, retinal capillary endothelial cells are exposed to hypertonic serum in hyperglycemic patients. We previously found that TauT transports GABA as a substrate and TauT is expressed in retinal capillary endothelial cells. It is conceivable that the low-affinity GABA transport system, TauT, rather than high-affinity GABA transporters, such as GAT-1 (Slc6a1), GAT-2 (Slc6a13), GAT-3 (Slc6a11), and betaine/GABA transporter (BGT-1/Slc6a12), make a great contribution to the removal of GABA from interstitial fluid when GABA is released from the intracellular space and is present in relatively high concentrations under pathologic conditions.

Thus, it appears that TauT-mediated GABA transport is enhanced in TR-iBRB2 cells exposed to hypertonic culture medium. Under hypertonic conditions, [3H]GABA uptake by TR-iBRB2 cells was Na\(^{+}\), Cl\(^{-}\), and concentration-dependent with the K_m of 0.324 and 5.48 mM (Figs. 2B and 2D) and there was greater inhibition of TauT substrates, such as taurine and β-alanine, than GATs substrates, such as GABA, nipecotic acid, and betaine (Table 1). K_m is similar that obtained from GABA uptake by TauT expressing HeLa cells (1.5 mM). V_max of GABA uptake by TR-iBRB2 cells under hypertonic conditions was 2.23-fold greater than that under isotonic conditions. These transport characteristics are consistent with the hypothesis that TauT is involved in GABA transport enhancement under hypertonic conditions. This supports the evidence that TauT mRNA expression and [3H]taurine uptake by TR-iBRB2 cells exposed to 24 h-hypertonic culture medium increased by 170% and 216% (Fig. 2C), respectively, in comparison with isotonic culture medium. On the other hand, [3H]-leucine uptake by TR-iBRB2 cells remained unchanged under both hypertonic and isotonic conditions. It appears that hypertonicity specifically enhances TauT-mediated GABA transport as well as taurine transport although there is no effect on LAT1 (Scl7a5)-mediated L-leucine transport in TR-iBRB2 cells. These results suggest that TR-iBRB2 cells sustain a taurine-response element (TonEBP) pathway, which regulates osmoosensitive transcription of the TauT gene.

The high-affinity GABA transport process is up-regulated under hypertonic conditions (Fig. 2D). K_m is comparable with that obtained for GABA uptake by rat osteoblasts expressing BGT-1 (K_m = 789 μM). [3H]GABA uptake by TR-iBRB2 cells under hypertonic conditions was inhibited by 22% in the presence of 2 mM betaine. Although these results suggest that BGT-1 is responsible for the high-affinity GABA transport process under hypertonic conditions, RT-PCR analysis using specific primers for rat BGT-1 demonstrated that BGT-1 mRNA was not present in TR-iBRB2 cells under both isotonic and hypertonic conditions (data not shown). Moreover, in the presence of 2 mM L-leucine, L-α-alanine, creatine, and probenecid, inhibitory effect of TR-iBRB2 cells on [3H]GABA uptake under hypertonic conditions increased compared with that under isotonic conditions. It is conceivable that an unknown GABA transport process is involved in GABA uptake under hypertonic conditions. Further study is needed to identify this process which is induced under hypertonic conditions.
In conclusion, hypertonicity enhances GABA uptake by TR-iBRB2 cells via activation of TauT and unknown GABA transport process. It appears that TauT in retinal capillary endothelial cells is regulated by retinal conditions such as tonicity and is involved in GABA uptake as well as taurine. These findings significantly contribute to our understanding of the protective role of TauT-mediated GABA in the barrier function at the inner BRB under hypertonic conditions.

Acknowledgments: This study was supported, in part, by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (JSPS).

References