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Estradiol inhibits the activity of proton-coupled amino acid transporter PAT1 expressed in Xenopus oocytes

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ABSTRACT

Estrogen has great potential as a therapeutic agent in focal ischemic brain injury. Amino acids as energy resources and neurotransmitters in the central nervous system are crucial for proper neuronal function and excitability. The proton-coupled amino acid transporter PAT1 has clear potential in drug absorption. In this paper, human brain PAT1 was cloned and expressed in Xenopus oocytes. The effects of estradiol on the activity of PAT1 were investigated. Glycine-induced membrane currents mediated by PAT1 were measured using the two-electrode voltage clamp technique. The amplitude of the glycine-elicited current was decreased progressively with increasing concentrations of β-estradiol. A concentration-dependent outwards current of PAT1 was also detected by the presence of β-estradiol. We conclude that estrogen attenuates the activity of PAT1 by directly closing PAT1 channel. Our results may provide an additional mechanism for estrogen on neurotransmission and neuronal metabolism during ischemic injury.

1. Introduction

Estrogen is a neuroactive hormone and has potential as a therapeutic agent in focal ischemic brain injury. In laboratory animals, multiple reports indicate that estrogen reduces oxidative stress and inflammation stress, attenuates excitotoxicity and decreases apoptosis on ischemic injury (Scott et al., 2012; Rao et al., 2011; Li et al., 2011). It enhances synaptic plasticity and improves cognitive performance (Brown et al., 2009; Suzuki et al., 2009). Epidemiological studies also indicate the decreased stroke incidence in women on hormone replacement therapy (Strom et al., 2011; Lobo, 2009). However, estrogen on seizures is still controversial. Several animal studies showed increased ischemic damage by evaluating the slow-release estrogen pellets (Theodorsson and Theodorsson, 2005; Harukuni et al., 2001). Estrogen enhances neuronal excitability and increases the oxidative stress as mechanisms of neurodamage (Scharfman and MacLusky, 2006; Bingham et al., 2005; Gordon et al., 2005). A systematic review concludes that physiological estrogen concentrations are neuroprotective while supraphysiological concentrations augment the damage from cerebral ischemia (Strom et al., 2011).

Amino acids are essentially required to maintain the neuronal function and acts as neutral excitatory or inhibitory neurotransmitter in the central nervous system. The proton-coupled amino acid transporter PAT1 is a pH-dependent, Na^+ – independent amino acid transporter and is functionally expressed in the intestine, liver and spleen (Anderson et al., 2009; Kennedy et al., 2005; Bermingham and Pennington, 2004; Boll et al., 2002). In central nervous system, PAT1 is immunolabeled particularly in regions rich in glutamatergic and GABAergic neurons (Agulhon et al., 2003; Chen et al., 2003). PAT1 transports a broad spectrum of substrates including small unbranched amino acids such as glycine, osmolytes such as taurine, D-amino acids such as D-serine and D-cycloserine, γ-aminobutyric acid (GABA) and its analogs (Thwaites and Anderson; 2007; Metzner et al., 2006). As a high-capacity transporter, it has clear potential in drug absorption and plays pharmacological importance in affecting diseases (Frølund et al., 2010; Metzner et al., 2009; Anderson et al., 2004). It may serve for lysosomal export of amino acids derived from protein breakdown (Rubio-Aliaga et al., 2004; Agulhon et al., 2003). And PAT1 transporter may contribute to specialized cellular functions such as neuronal metabolism, neurotransmission, and control of brain amino acid homeostasis. Moreover, based on its pH-dependent manner, PAT1 channel transports amino acids at a low pH environment. It may serve as a main amino acid transporter which is still kept in an active state in the acidification brain subjected to ischemia stress.

The effect of estrogen on regulating the function of PAT1 transporter is still not clear. In the current research, we cloned human brain PAT1 and employed Xenopus oocyte expression system to express the PAT1 transporter. Voltage clamp recording

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was used to monitor PAT1 activity. The current study is designed to evaluate the effects of estrogen on the function of human brain PAT1 and clarify its electrophysiological property.

2. Materials and methods

2.1. Animals and chemicals

Mature female Xenopus laevis frogs were fed with regular frog brittle twice weekly. The animal study protocol was approved by the Institutional Animal Care and Use Committee. α-estradiol and β-estradiol were purchased from Tocris Bioscience (San Diego, CA, USA). All Reagents including Glycine, γ-aminobutyric acid and taurine were purchased from Sigma-Aldrich (St. Louis, MO,USA).

2.2. Cloning of human brain PAT1

Human brain total RNA was purchased from Ambion biotechnology (Austin, TX, USA). Brain cDNA was synthesized by using super script III reverse transcript kit (invitrogen). Human PAT1 gene was cloned by polymerase chain reaction using cloning primers. The sequences of PAT1 cloning primers were as follows: PAT1-upper, 5'–ATGTCCACGCAGAGACTTC-3'; PAT1-lower, 5'–CTATATGAAGGCACAGGTGG-3'. PCR reaction was performed with phusion polymerase (NEB) on eppendorf PCR system as follows: 2 min at 98 °C, followed by 30 cycles of 30 s at 98 °C, 30 s at 55 °C and 1 min at 72 °C. 1.4-kb PCR product was purified by gel purification, and 10 ng PCR product was used as template for the second round of PCR amplification with PAT1 subcloning primers. The sequences of PAT1 subcloning primers were as follows: PAT1-FWD, 5′–GCTCAACTTTGGCCATGTCCACGCAGAGACTTC-3′; PAT1-REV, 5′–TTCTTGAGGCTGGTTTATATGAAGGCACAGGTGG-3′. The primers for amplifying pGEMHE vector were as following: Vec-start-reverse, 5′–CATGGCCAAAGTTGAGCGTTTATTCTG-3′; Vec-end-forward, 5′–TAAACCAGCCTCAAGAACACC-3′. PCR conditions were 98 °C for 30 s, 55 °C for 30 s, and 72 °C for 2 min (18 cycles). The PAT1 were sub-cloned into the pGEMHE vector by fastcloning methods. The final plasmid was named as pGEM-PAT1. PAT1 sequence was further confirmed by DNA sequencing.

2.3. PAT1 cRNA synthesis

Linearized pGEM-PAT1 plasmid was transcribed with T7 RNA polymerase with the Ambion mMESSAGE mMACHINE kit. Template DNA was removed with RNase-free DNase I, and the RNA was precipitated with sodium acetate and ethanol. PAT1 cRNA was resuspended in DEPC-treated water and further examined on agarose gels with ethidium bromide.

2.4. Xenopus laevis oocytes expressing PAT1

Stage VI oocytes from female Xenopus laevis were harvested and incubated at 16 °C before injection. Individual oocytes were injected with 40 ng/40 nL of PAT1 cRNA using an automated microinjector (Nanoject; Drummond Scientific Co., Broomall, PA). Oocytes were further incubated for 2–4 days at 18 °C in sterile oocyte incubation solution to make the PAT1 was expressed before the electrophysiologic recording. Oocyte incubation solution was composed of 88 mM NaCl, 1 mM KCl, 0.82 mM $MgCl_2$, 0.41 mM $CaCl_2$, 0.33 mM $Ca(NO_3)_2$, 2.4 mM $NaHCO_3$, and 10 mM MES/Tris at pH 6.5.

2.5. Electrophysiologic recording

To characterize the response current in oocytes expressing PAT1, the two-electrode voltage clamp technique was applied. Oocytes were placed in an open chamber with continuous perfusion of oocyte Ringer solution (containing (in mM): 93 NaCl, 2.5 KCl, 1 $CaCl_2$, 1 $MgCl_2$ and 5 Hepes, pH 6.5) or oocyte Ringer solution containing drugs. The chamber was grounded through an agar KCl bridge. Two microelectrodes were inserted into individual oocyte and voltage clamping of each oocyte was performed using a GeneClamp 500B amplifier (Axon Instruments, Union City, CA, USA) at a holding potential of −70 mV. The current signal was filtered at 20 Hz with low-pass Bessel filter in the GeneClamp 500 B and digitized at 50 Hz.

2.6. Statistical analyses

Values are expressed as mean ± S.E.M. Statistical analysis was examined using Student t test or ANOVA. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Expression of human brain PAT1 in Xenopus oocytes

To confirm the successful expression of PAT1 in oocytes, the function of PAT1 was monitored by the two-electrode voltage clamp. 50 mM glycine, taurine and GABA were evaluated as PAT1 inducer. As shown in Fig. 1a, when subjected to components perfusion, an inward current was detected by voltage clamp which indicated that the PAT1 channel opened and amino acids were transferred into the oocytes. Furthermore, dose-dependent activation of glycine, taurine and GABA was checked with the final concentrations (0 mM, 1 mM, 3.16 mM, 10 mM, 31.6 mM, 100 mM, 316 mM and 1 M). The I–V curves of glycine on PAT1 are

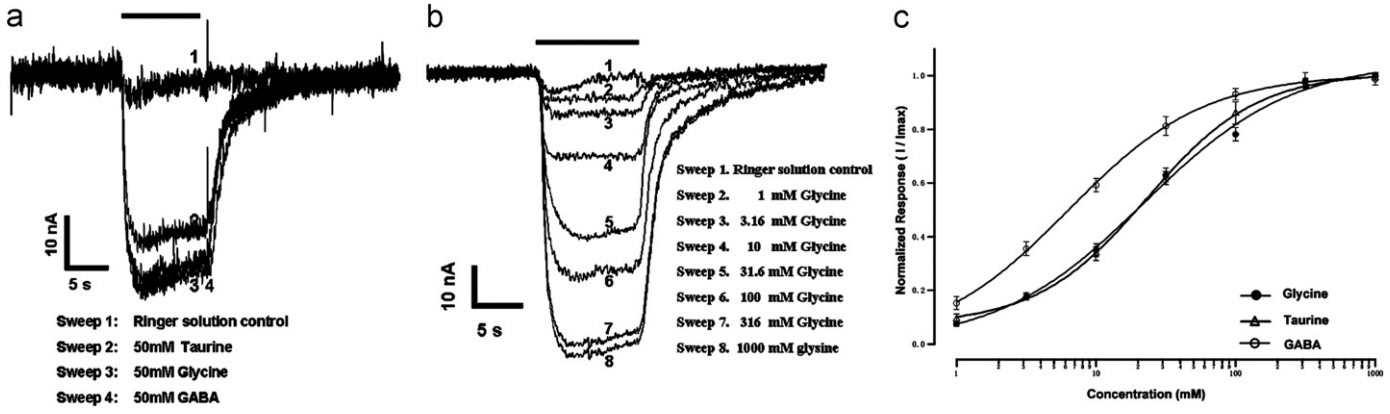

Fig. 1. Successful expression of human brain PAT1 in Xenopus oocytes. (a) Examples of the activation currents by 50 mM taurine, glycine and GABA on PAT1 expressed in oocytes, (b) dose–response curves of glycine concentrations (1 mM–1000 mM) on PAT1 channel and (c) normalized taurine, glysine and GABA dose response on PAT1.