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Increased P-glycoprotein expression and decreased phenobarbital distribution in the brain of pentylenetetrazole-kindled rats

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Abstract

The purposes of this study were to investigate whether P-glycoprotein (P-GP) is overexpressed in the brain of pentylenetetrazole (PTZ)-kindled rats, and to investigate the effects of P-GP up-regulation on the distribution of phenobarbital (PB) in brain and its antiepileptic effects. Kindled rats were developed using a subconvulsive dose of PTZ (30 mg/kg, once every 2 days, i.p.) for 24 days. P-GP expression and function were measured by Western blot analysis and rhodamine 123 (Rho 123) distribution in brain. Kindled rats received 10 mg/kg of PB alone or co-administration of cyclosporine A (CsA, 5 mg/kg). At 60 min after administration of PB, concentrations of PB in brain and plasma were measured and the tissue-to-plasma concentration ratios of PB were calculated. Anticonvulsive effects of PB (13.2 mg/kg) alone or co-administration of CsA on the kindled rats were observed. The results showed that kindling resulted in 1.7-fold increase of P-GP level in brain, accompanied by significant decrease of tissue-to-plasma concentration ratios of Rho 123 and PB in hippocampus and cortex without affecting their concentrations in plasma. Co-administration of CsA reversed the decrease of PB concentration in brain without affecting PB level in plasma and significantly potentiated the anticonvulsive effects of PB. The present study demonstrated that chronic PTZ-kindling might increase P-GP expression and function in brain of rat, resulting in decrease of Rho 123 and PB levels in brain tissues. Co-administration of CsA increased PB levels in brain and enhanced anticonvulsive effects of PB by inhibiting P-GP function.

Keywords: P-glycoprotein; Epilepsy; Blood–brain barrier; Antiepileptic drugs; Kindling; Phenobarbital

1. Introduction

About 30% of patients with epilepsy do not respond to clinically established antiepileptic drugs (AEDs), although their plasma concentrations are within the ''therapeutic range''. Patients resisting to a major AED are also refractory to other AEDs even though the action mechanisms of these AEDs are different (Löscher and Potschka, 2002). The underlying causes of refractory epilepsy are still not fully understood. A plausible hypothesis is that the permeability of AEDs across the blood–brain barrier (BBB) may be altered in patients with medically intractable epilepsy (Löscher and Potschka, 2002). This hypothesis is supported by the overexpression of the multidrug resistance transporter P-glycoprotein (P-GP) in the brain microvessels endothelial cells of patients with pharmacoresistant epilepsy (Dombrowski et al., 2001; Sisodiya et al., 2002; Rogawski, 2002). In brain microvessels endothelial cells, P-GP functions as an outwardly directed efflux transporter that limits the access of drugs into the brain parenchyma. Since some studies have demonstrated that some major AEDs are substrates of P-GP, overexpression of P-GP in endothelial cells will decrease the access of AEDs to their targets in the brain (Löscher and Potschka, 2002).

An answerable question is whether the overexpression of P-GP in epileptogenic brain tissue of patients with pharmacoresistant epilepsy is a consequence of epilepsy, uncontrolled seizures, and chronic treatment with AEDs, or combinations of these factors. Animal models of epilepsy may help to

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resolve this issue, and a number of recent studies have demonstrated that experimentally induced seizures may result in overexpression of P-GP at brain (Volk et al., 2004a,b, 2005; Brandt et al., 2006).

Phenobarbital (PB) is recommended by the World Health Organization as first-line for partial and tonic–clonic seizures in the developing world, and remains a popular choice in many developed countries (Brodie and Kwan, 2004). Limited information showed that the transport of PB at BBB was mediated by P-GP (Potschka et al., 2002).

The purposes of this study were, *firstly*, to investigate whether overexpression of P-GP occurs in brain of rats kindled by pentylenetetrazole (PTZ), similarly to kainite or pilocarpine; *secondly*, to clarify whether up-regulation of P-GP expression affects function of P-GP using rhodamine 123 (Rho 123) as the model drug; *thirdly*, to investigate whether up-regulation of P-GP expression and function affects distribution of PB in brain; and *finally*, to examine whether co-administration of modulator cyclosporine A (CsA) increases distribution of PB in brain of kindled rats and potentiates antiepileptic effects of PB on kindled rats by PTZ.

2. Methods

2.1. Chemicals

PTZ was purchased from Alfa Aesar (Ward Hill, MA, USA), and rhodamine 123 (Rho 123) was purchased from Sigma Co. (St. Louis, MO, USA). PTZ and Rho 123 were dissolved in 0.01 M phosphate-buffered saline (PBS, pH 7.4) before use. Sodium phenobarbital (PB) and CsA were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China) and Novartis Pharma (Beijing, China), respectively.

2.2. Animals

Adult male Sprague–Dawley rats (190–210 g) were purchased from Sippr–BK Lab Animal Co. (Shanghai, China). The rats were housed under controlled environmental conditions (temperature 22–23 °C and humidity 50–60%) and kept under a 12-h light/dark cycle with free access to food and water. All animal experiments were performed under a license granted by Jiangsu Science and Technology Office (China), with approval from Animal Ethics Committee of China Pharmaceutical University, and in compliance with Cruelty to Animals Act, 1876. Every effort was made to minimize stress to the animals.

2.3. Kindled model of epilepsy

Kindling was induced according to the method described previously (Suzuki et al., 2001). A subconvulsive dose of PTZ (30 mg/kg) was intraperitoneally (i.p.) given to rats at 9:00 am every 2 days up for 12 injections. The convulsive activity was monitored for 30 min following dose of PTZ. The intensity of seizure response was scored according to the following scale (Sarro and Naccari, 1999): *0*, no response; *1*, mouth and facial jerks; *2*, nodding or myoclonic body jerks; *3*, forelimb clonus; *4*, rearing, falling down, hindlimb clonus and forelimb tonus; and *5*, tonic extension of hindlimb, status epileptic and/or death. The maximum response was recorded for each animal. Incidence of seizure and latency of seizure were also recorded. Only animals showing at least five consecutive stage 2 seizures or three consecutive stage 4 seizures were considered to be kindled rats and included in the study. One separate group of rats received only physiological saline throughout the study and was served as control group.

2.4. Western blot analysis

Half an hour after the final PTZ or saline injection, the rats were sacrificed under light ether anesthesia and brains were quickly removed. The cerebral cortex weighing 300 mg was homogenized in ice-cold cell lysis containing 10 mM Tris–HCl, 1 mM $MgCl_2$, 1 mM EGTA, 1 mM mercaptoethanol, 1% glycerin and protease inhibitor cocktail (1 mM dithiothreol, 2 mM phenylmethylsulfluoride) (Sigma Chemical Co., Ltd., St. Louis, MO, USA), and the homogenate was centrifuged at 13,000 rpm for 10 min at 4 °C. The supernatant was obtained as membrane fractions for Western blot analysis. Protein concentration in the solution was measured with a Bio-Rad Protein Assay (Bio-Rad Laboratories, CA, USA) using bovine serum albumin (Sigma) as a standard. An aliquot of tissue sample was diluted with a volume of 4× sodium dodecyl sulfate (SDS) sample buffer containing 0.1 M Tris–HCl, 4% SDS, 200 mM DDT, 20% glycerin, and 0.2% bromphenol blue. The protein (20 μg) was separated by electrophoresis on 8% SDS–polyacrylamide gel and electrophoretically transferred to a nitrocellulose membrane. The membrane was blocked in PBS containing 0.1% Tween-20 and 5% dried skim milk at room temperature for 1 h and washed three times for 15 min in PBS containing 0.1% Tween-20. The membrane was incubated with the primary monoclonal antibody C219 (Calbiochem-Novabiochem, Seattle, WA, USA), diluted 200-folds in PBS containing 0.1% Tween-20, overnight at 37 °C. After washing the membrane with PBS containing 0.1% Tween-20, it was incubated with the secondary polyclonal antibody IRDye 800 conjugated affinity purified anti-mouse IgG (Rockland Inc., Rockland, Ontario, Canada) diluted 12,000-folds in PBS containing 0.1% Tween-20, at room temperature for 1 h. Detection was performed by the Odyssey Infrared Imaging System (LI-COR Inc., USA). All blots were stripped and reprobed with polyclonal anti-β-actin antibody (Boshide Biotech Co., Wuhan, China) to ascertain equal loading of protein.

2.5. Distribution of Rho 123 and PB in brain

To elucidate the effect of kindling on P-GP function at BBB, Rho 123 (0.2 mg/kg), a typical substrate of P-GP, was given intravenously (i.v.) to kindled rats and control rats. At 60 min after the injection of Rho 123, the rats were sacrificed under light ether anesthesia, and blood and brain samples were immediately collected for analysis.

To study the effect of kindling on PB brain distribution, a single dose of PB (10 mg/kg, i.v.) was given to kindled rats and control rats. At 60 min after the injection of PB, the rats were sacrificed under light ether anesthesia, and blood and brain samples were immediately collected for analysis.

To examine the effect of P-GP modulator CsA on the brain distribution of PB, 10 mg/kg of PB (i.v.) was given to kindled rats at 5 min following intravenous injection of 5 mg/kg CsA. At 60 min after the injection of PB, the rats were sacrificed under light ether anesthesia, and blood and brain samples were immediately collected for analysis.

Blood samples were immediately centrifuged to yield plasma. Hippocampus and cerebral cortex were obtained and weighed. All plasma and brain samples were stored at −80 °C until analysis. The tissue-to-plasma concentration ratios of Rho 123 and PB were calculated.

2.6. Effect of repeated dose of PB or PB + CsA on PTZ-kindled seizure in rats

To study the treatment of PB or PB + CsA for seizure induced by PTZ-kindling, the kindled rats were randomly grouped into three groups (four in each group) and received following treatments at 9:00 am once every day up to 4 days, respectively. Group 1 was served as model rats, only received physiological saline. Group 2 received PB (13.2 mg/kg, i.v.) and group 3 received CsA (5 mg/kg, i.v.) followed by PB (13.2 mg/kg, i.v.) 5 min later. At 30 min after receiving PB (group 2 and group 3) and physiological saline (group 1), each rat received PTZ (30 mg/kg, i.p.). Seizure stage, latency and times of seizure were recorded according to the scale described above. Age-matched rats were selected as control and only received PB (13.2 mg/kg, i.v.) once every day for 4 days.

On day 4, the rats were sacrificed under light ether anesthesia at 60 min after the last injection of PB. Plasma and brain samples were collected and