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Bepridil reducing levothyroxine-induced enhancement of mitochondria Ca^{2+} Mg^{2+}-ATPase activity in rat cerebrum

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KEY WORDS calcium channel blockers; bepridil; propranolol; levothyroxine; cerebrum; mitochondria; Ca^{2+} Mg^{2+}-ATPase

AIM: To study if bepridil (Bep) could affect the enhancement of activity of cerebral mitochondria Ca^{2+} Mg^{2+}-ATPase caused by levothyroxine (Lev) in relation to ischemic overload calcium cerebrum injury. **METHODS**: The experimental hyperthyroidism model with ischemic cerebrum was developed in rats by ig Lev 1 mg·kg^{-1}·d^{-1} for 7 d. Ca^{2+} Mg^{2+}-ATPase activity and its kinetic parameters were assayed. **RESULTS**: The activity, V_{max} and K_m of cerebral mitochondria Ca^{2+} Mg^{2+}-ATPase in control rats were 3.1 ±0.8, 5.1±2.3 mmol·P_i·h^{-1}/g protein and 0.81 ±0.08 mmol·L^{-1} (ATP) respectively, whereas those of hyperthyroid rats were significantly altered to 4.6±0.5, 8.5±1.9 mmol·P_i·h^{-1}/g protein and 0.49±0.11 mmol·L^{-1} (ATP) respectively. After treated with Bep 10 or 20 mg·kg^{-1}·d^{-1} ig for 3 d, allabove 3 parameters of the enzyme were very significantly reduced *vs* those of either control or hyperthyroid. **CONCLUSION**: Bep, via decreasing Ca^{2+} Mg^{2+}-ATPase activity and increasing the affinity of Ca^{2+} Mg^{2+}-ATPase to ATP, could prevent rat cerebrum from ATP depletion and ischemic overload calcium injury.

The cerebrum and its mitochondria are very sensitive to ischemia[1]. A high dose of levothyroxine (Lev) can cut off oxidative phosphorylation coupling and stimulate metabolism leading tissues to ATP depletion and ischemia[2]. Calcium channel blockers, eg, verapamil, had generally been applied for anti-ischemic cerebral injury in clinical settings[3]. In the present paper, the effects of Bepridil (Bep)[4], a novel calcium channel blocker, on elevated mitochondril Ca^{2+} Mg^{2+}-ATPase activity caused by Lev in rat cerebrum were studied. Propranolol (Pro), an anti-ischemia and anti-hyperthyroidism drug, was chosen as positive control drug.

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MATERIALS AND METHODS

Establishment and treatment of hyperthyroidism model SD rats, $n = 50$, weighing 190 - 230 g, were medicated with Lev 1 mg·kg^{-1}·d^{-1} ig for 7 d. From d 8 to d 10, rats were treated by ig normal saline (NS), Pro 10 mg·kg^{-1}·d^{-1}, Bep 10 or 20 mg·kg^{-1}·d^{-1} for 3 d were administered respectively, meanwhile rats were stopped medicating Lev.

Preparation of mitochondria On d 11, Rats were killed by neck concussion and jugular incision, the cerebrum were taken and washed with cold saline, then, cerebrum was immediately placed in liquid nitrogen. The cerebrum, being cut into small pieces, were homogenized in 10 vol of 10 mmol·L^{-1} imidazole buffer containing sucrose 0.25 mol·L^{-1}, the homogenate was centrifuged at 750 × *g* for 20 min, the supernatant was recentrifuged at 9000 × *g* for 20 min. The pellet, containing mitochondria, was resuspended in homogenizing medium at an appropriate protein concentration and used for the study. All procedures were performed at 4 ℃.

Ca^{2+} Mg^{2+}-ATPase assay[5] The incubation media contained edetic acid 0.1, $MgCl_2$ 5, $CaCl_2$ 0.3, ATP 1.0, ouabain 1, imidazole 30 mmol·L^{-1} and about 100 μg *mitochondria protein at pH 7.4*. The *final incubation volume* was 1.0 mL. The reaction was initiated by addition of ATP, and incubation was carried out by shaking at 37 ℃ for 10 min. Reaction was terminated by the addition of 0.5 mL 15 % trichloroacetic acid. Liberated inorganic phosphate (P_i) was determined by spectrophotometric method at 660 nm. One minute after addition of P_i reagent (ascorbic acid 10 % : H_2SO_4 3 mol·L^{-1} : ammonium molybdate 2.5 % : H_2O, 1:1:1:2, vol/vol), 24 % sodium citrate were added to complex P_i liberated by nonenzymetic molybdate catalytic ATP hydrolysis. Ca^{2+} Mg^{2+}-ATPase activity was expressed as mmol of P_i liberated per g mitochondria protein per hour (mmol·P_i·h^{-1}/g protein).

Measurement of Ca^{2+} Mg^{2+}-ATPase apparent K_m (ATP) and V_{max} Under the condition of different ATP concentrations (0.25, 0.5, 0.75, and 1.0 mmol·L^{-1}), measuring Ca^{2+} Mg^{2+}-ATPase activity respectively. Then, according to Lineweaver-Burk double-reciprocal plot method,

the apparent K_m and V_{max} of Ca^{2+} Mg^{2+}-ATPase were calculated by linear regression[6]. Prorein was determined by the procedure of Bradford[7], using bovin serum albumin as a standard.

Chemicals Pro (910516, Wuxi Fourth Pharmaceutical Factory). Bep (901101, Changzhou Fourth Pharmaceutical Factory). Lev (sodium salt, Sigma). ATP (grade II Sigma). Ouabain (extra pure, Merck). Imidazle (289 829 589, 99.0 % purity Fluka). Other chemicals were AR

Statistical analysis Data were expressed as $\bar{x} \pm s$ and compared with t test.

RESULTS

Bep diminished the elevated Ca^{2+} Mg^{2+}-ATPase V_{max} and activity caused by Lev The cerebral mitochondria Ca^{2+} Mg^{2+}-ATPase activity and V_{max} of untreated rats (normal saline group, NS) were increased by 50 % and 67 % respectively compared with those of control rats. Medication of Bep 10 or 20 $mg \cdot kg^{-1} \cdot d^{-1}$ ig for 3 d, the enzyme activity was reduced by 63.3 % and 88.5 % respectively compared with that of NS, and even lowered by 45 % and 83 % compared with that of control. Similarly, V_{max} was very significantly reduced compared with either NS or control.

Bep enhanced the affinity of mitochondria Ca^{2+} Mg^{2+}-ATPase toward ATP The cerebral mitochondria Ca^{2+} Mg^{2+}-ATPase K_m(for ATP) in NS group rats was lowered by 25.9 % *vs* that of control. After treated by Bep 10 or 20 $mg \cdot kg^{-1} \cdot d^{-1}$ ig for 3 d, the affinity of the enzyme toward ATP was enhanced *vs* NS and control, ie, K_m value was further diminished (Tab 1).

Tab 1. Effects of propranolol (Pro), bepridil (Bep) on activity, V_{max} and K_m of cerebral mitochondria Ca^{2+} Mg^{2+}-ATPase in experimental hyperthyroidism rats induced by levothyroxine (Lev). $n = 8 - 9$, $\bar{x} \pm s$. $^cP < 0.01$ *vs* control; $^fP < 0.01$ *vs* NS.

Groups/ $mg \cdot kg^{-1} \cdot d^{-1}$	Ca^{2+} Mg^{2+}-ATPase activity/ $mmol \cdot P_i \cdot h^{-1}$/g protein	V_{max}	K_m/ $mmol \cdot L^{-1}$, ATP
Control	3.1±1.0	5.1±2.3	0.81±0.08
NS	4.6±0.5^c	8.5±1.9^c	0.49±0.11^c
Pro 10	1.3±0.3^{cf}	1.7±0.5^{cf}	0.192±0.022^{cf}
Bep 10	1.69±0.22^{cf}	2.01±0.20^{cf}	0.18±0.04^{cf}
Bep 20	0.53±0.09^{cf}	0.58±0.06^{cf}	0.118±0.021^{cf}

Pro, as similar as Bep, could intervene all of 3 parameters of the enzyme mentioned above. Thus, all 3 parameters of the enzyme in Bep- and Pro-treated rats were very significantly reduced compared with those of either NS or control.

DISCUSSION

A high dose of Lev interrupts the oxidative phosphorylation coupling bringing the cerebrum to ATP depletion and ischemia[2]. Also, Lev can increase the expression and biosynthesis of mRNA and protein including cardiac, not cerebrum, Ca^{2+} Mg^{2+}-ATPase[8-11]. Here, we observed that Lev, as similar as in cardiac, markedly increased the activity and V_{max} of cerebral Ca^{2+} Mg^{2+}-ATPase in rats. This fact was agreement with reports[8-11] and suggested that Lev also increased the cerebral Ca^{2+} Mg^{2+}-ATPase biosynthesis. In addition to above pathway, Ca^{2+} Mg^{2+}-ATPase could be stimulated by high Ca^{2+} level and Ca^{2+}-stimulated protein kinase C (PKC)[12]. A high calcium ion level in Lev caused ischemic tissues[2,13], which could stimulate PKC and "bomb" injury mitochondria[1,14]. The high activity Ca^{2+} Mg^{2+}-ATPase of mitochondria would hydrolyze a great number of ATP for lowering cytosol calcium ion level, and then further enhance ATP depletion and ischemic cerebral damage[1,14].

β-Blocker Pro can prevent cerebrum from ischemia, and decrease cardiac protein synthesis[15]. In our study, Pro was chosen as positive control drug.

Bep, a calcium channel blocker by decreasing intracellular Ca^{2+} level, lowered cerebral mitochondria Ca^{2+} Mg^{2+}-ATPase activity, and Bep, as same as Pro, markedly diminished the V_{max} of mitochondria Ca^{2+} Mg^{2+}-ATPase in hyperthyroid cerebrum. By diminishing the activity and V_{max} of mitochondria Ca^{2+} Mg^{2+}-ATPase and cytosol calcium level, Bep could greatly decrease cerebral ATP depletion and overload calcium injury. K_m reduction of the enzyme by Bep showed that Bep could increase the sensitivity of Ca^{2+}-pump for ischemic overload calcium and calcium level fluctuation. Thus, Bep enhanced the sensitivity of Ca^{2+}-pump for maintenance intracellular calcium

homeostasis. There existed a dose-efficacy relationship between Bep two doses. It should be further done that Bep affects the cerebral ATP content and malondialdehyde (MDA) content.

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苄普地尔降低左甲状腺素诱发升高的大鼠脑线粒体钙$^{2+}$镁$^{2+}$-ATP酶活力

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关键词 钙通道阻滞剂；苄普地尔；普萘洛尔；左甲状腺素；大脑；线粒体；钙$^{2+}$镁$^{2+}$-ATP酶

目的：研究苄普地尔是否能影响左甲状腺素诱发升高的大鼠脑线粒体钙$^{2+}$镁$^{2+}$-ATP酶活力及其与缺血性超负荷钙脑损伤的关系 **方法**：ig左甲状腺素1 mg·kg^{-1}·d^{-1} 7 d建立具缺血大脑的实验性甲亢大鼠模型，钙$^{2+}$镁$^{2+}$-ATP酶活力测定和酶动力学分析. **结果**：正常大鼠脑线粒体钙$^{2+}$镁$^{2+}$-ATP酶活力，V_{max}和K_m分别为3.1±0.8，5.1±2.3 mmol·P_i·h^{-1}/g蛋白质和0.81±0.08 mmol·L^{-1} ATP，甲亢大鼠中该酶相应值分别为4.6±0.5，8.5±1.9 mmol·P_i·h^{-1}/g蛋白质和0.49±0.11 mmol·L^{-1} ATP. 经苄普地尔10或20 mg·kg^{-1}·d^{-1} ig 3 d，该酶上述三项参数均显著低于甲亢或正常大鼠. **结论**：苄普地尔通过降低钙$^{2+}$镁$^{2+}$-ATP酶活力和提高该酶对ATP的亲和力，防止大脑ATP耗竭和缺血性超负荷钙损伤.

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