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Levosimendan exerts anticonvulsant properties against PTZ-induced seizures in mice through activation of nNOS/NO pathway: Role for K_{ATP} channel

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ABSTRACT

Aims: Although approving new anticonvulsants was a major breakthrough in the field of epilepsy control, so far we have met limited success in almost one third of patients suffering from epilepsy and a definite and reliable method is yet to be found. Levosimendan demonstrated neuroprotective effects and reduced mortality in conditions in which seizure can be an etiology of death; however, the underlying neuroprotective mechanisms of levosimendan still eludes us. In the light of evidence suggesting levosimendan can be a K_{ATP} channel opener and nitrergic pathway activator, levosimendan may exert antiseizure effects through K_{ATP} channels and nitrergic pathway.

Main methods: In this study, the effects of levosimendan on seizure susceptibility was studied by PTZ-induced seizures model in mice.

Key findings: Administration of a single effective dose of levosimendan significantly increased seizures threshold and the nitrite level in the hippocampus and temporal cortex. Pretreatment with noneffective doses of glibenclamide (a K_{ATP} channel blocker) and L-NAME (a non-selective NOS inhibitor) neutralize the anticonvulsant and nitrite elevating effects of levosimendan. While 7-NI (a neural NOS inhibitor) blocked the anticonvulsant effect of levosimendan, Aminoguanidine (an inducible NOS inhibitor) failed to affect the anticonvulsant effects of levosimendan. Cromakalim (a K_{ATP} channel opener) or L-arginine (an NO precursor) augmented the anticonvulsant effects of a subeffective dose of levosimendan. Moreover, co-administration of noneffective doses of Glibenclamide and L-NAME demonstrated a synergistic effect in blocking the anticonvulsant effects of levosimendan.

Significance: Levosimendan has anticonvulsant effects possibly via K_{ATP}/nNOS/NO pathway activation in the hippocampus and temporal cortex.

1. Introduction

With a prevalence of 1–2%, epilepsies undoubtedly impose a major burden upon patients and societies [1]. Despite major breakthroughs in the field of epilepsy research, anti-seizure medications do not provide sufficient seizure control in almost one-third of patients suffering from epilepsy [2,3]. Thus, investigation on new evidence-based therapeutical strategies is mandatory, and neuropharmacology can be a promising field for this purpose.

Levosimendan is a novel pyridazinone-dinitrile derivate, well-known for positive inotropic effects through enhancing the sensitivity between Ca^{+2} and myofilaments and inhibiting the phosphodiesterase III activity [4]. Some studies suggest possible impact of calcium sensitizers including levosimendan on CNS, demonstrated as central symptoms such as headaches, vertigo, flushing, and nausea [5]. Recent studies demonstrated that levosimendan can improve the survival rates in calcium channel blockers (CCBs) toxicity, which partly might be through seizure reduction as a common consequence of CCB toxicity [6–8].

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Several lines of evidence confirmed that levosimendan, in addition to its inotropic effects, exerts cardio-protection, vasodilation, and antioxidant activity through modulation of mitochondrial adenosine triphosphate-sensitive potassium (K_{ATP}) channels and nitric oxide (NO) production [9–12].

K_{ATP} channels are non-voltage dependent, inward-rectifier potassium channels, regulated by intracellular ATP/ADP ratio as a marker of the cellular energy supply. Besides, various pharmacologic agents (e.g., cabergoline and glibenclamide) can modulate the activity of these channels without affecting cellular ATP concentration [13–15]. Recent studies have found that the K_{ATP} channels have antiepileptic effects and help to control neuronal excitability and seizure propagation [16–20].

NO is a gaseous neuronal messenger and neurotransmitter modulator in the brain, synthesized by NO synthase (NOS) either constitutive (eNOS, and nNOS) or inducible isoforms (iNOS) from L-arginine [21]. There are several studies reporting a controversial anticonvulsant or pro-convulsant but, at the same time, a fundamental role for the nitrergic system in the modulation of seizures [22–26].

The pivotal role of K_{ATP} channels and NO in both mechanism of action of levosimendan and modulation of seizure thresholds raise the possibility that levosimendan may affect the seizure susceptibility through K_{ATP} and NO-dependent mechanisms. To this end, this study aims to shed light on the effects of levosimendan on PTZ-induced seizure threshold in mice as a model of generalized clonic seizures (GCS) and then ascertain the nature of this effect by delineating the molecular pathways involved in the exertion of this effect.

2. Methods and materials

2.1. Subjects

Swiss male mice at 12–16 weeks were used throughout this study. The mice were housed at a constant temperature (23 °C) and relative humidity (60%) in groups of 6–8 with free access to food and water and a fixed 12 h light/dark cycle. Procedures involving mice and their care were conducted in conformity with the institutional guidelines that are in compliance with the national and international laws and policies. Each mouse was used only once, and each treatment group comprised of 6–8 animals. Additionally, all efforts were made to minimize animal suffering and to use only the minimal number of animals required to produce reliable scientific data.

2.2. Pharmacological treatments

The following drugs were used in the study: levosimendan [a calcium sensitizer (Sigma, St Louis, MO, USA)], pentylenetetrazole (PTZ) [$GABA_A$ receptor antagonist; (Sigma, UK)], L-arginine (L-Arg) [NO precursor; (Sigma, St Louis, MO, USA)], N(G)-nitro-L-arginine methyl ester (L-NAME) [a non-selective NOS inhibitor; (Sigma, St Louis, MO, USA)], 7-nitroindazole (7-NI) [a selective nNOS inhibitor; (Sigma, St Louis, MO, USA)], aminoguanidine (AG) [an iNOS inhibitor; (Sigma, St Louis, MO, USA)], Cromakalim [a K_{ATP} channel opener; (Sigma, St Louis, MO, USA)], Glibenclamide [a K_{ATP} channel blocker; (Sigma, St Louis, MO, USA)].

All drugs were freshly prepared prior to use, and injection volume (10 $mL \cdot kg^{-1}$) was kept constant for in vivo experiments. Levosimendan, L-NAME, 7- NI, AG, L-ARG, Cromakalim and glibenclamide were administered intraperitoneally (i.p.). 7-NI was suspended in a 1% aqueous solution of Tween 80 and all other drugs were dissolved in normal saline. To assess clonic seizure, PTZ was administered intravenously in the tail vein (0.5%, i.v.). The dosage selections, route of drug administration, and injection time of different compounds were based on our previously published data, preliminary experiments and pharmacokinetic considerations [20,22,24]. Control mice were injected with the corresponding volume of vehicles before PTZ-induced seizures. Pharmacological experiments were carried between 9.00 am and 2.00 pm.

2.3. Study design

309 mice were randomized into two categories. The first category, consisting of 35 groups, was used in seizure paradigm to assess the PTZ-induced seizure threshold. Ten groups of mice were randomly assigned to produce scientific and reliable data regarding levosimendan effects on PTZ-induced seizures dose response and time course. In order to delineate the role of K_{ATP} channel and nitrergic system on the anticonvulsant properties of levosimendan mice were randomly assigned to the following groups: 1) glibenclamide (0.5 $mg \cdot kg^{-1}$; i.p.); 2) glibenclamide (1.0 $mg \cdot kg^{-1}$; i.p.); 3) glibenclamide (0.5 $mg \cdot kg^{-1}$; i.p.) + levosimendan (2.0 $mg \cdot kg^{-1}$; i.p.); 4) glibenclamide (1.0 $mg \cdot kg^{-1}$; i.p.) + levosimendan (2.0 $mg \cdot kg^{-1}$; i.p.); 5) Cromakalim (0.5 $mg \cdot kg^{-1}$; i.p.); 6) Cromakalim (1.0 $mg \cdot kg^{-1}$; i.p.); 7) Cromakalim (0.5 $mg \cdot kg^{-1}$; i.p.) + levosimendan (0.2 $mg \cdot kg^{-1}$; i.p.); 8) Cromakalim (1.0 $mg \cdot kg^{-1}$; i.p.) + levosimendan (0.2 $mg \cdot kg^{-1}$; i.p.); 9) L-NAME (1.0 $mg \cdot kg^{-1}$; i.p.); 10) L-NAME (5.0 $mg \cdot kg^{-1}$; i.p.); 11) L-NAME (1.0 $mg \cdot kg^{-1}$; i.p.) + levosimendan (2.0 $mg \cdot kg^{-1}$; i.p.); 12) L-NAME (5.0 $mg \cdot kg^{-1}$; i.p.) + levosimendan (2.0 $mg \cdot kg^{-1}$; i.p.); 13) L-ARG (30.0 $mg \cdot kg^{-1}$; i.p.); 14) L-ARG (60.0 $mg \cdot kg^{-1}$; i.p.) 15) L-ARG (30.0 $mg \cdot kg^{-1}$; i.p.) + levosimendan (0.2 $mg \cdot kg^{-1}$; i.p.); 16) L-ARG (60.0 $mg \cdot kg^{-1}$; i.p.) + levosimendan (0.2 $mg \cdot kg^{-1}$; i.p.); 17) 7-NI (15.0 $mg \cdot kg^{-1}$; i.p.); 18) 7-NI (30.0 $mg \cdot kg^{-1}$; i.p.); 19) 7-NI (15.0 $mg \cdot kg^{-1}$; i.p.) + levosimendan (2.0 $mg \cdot kg^{-1}$; i.p.); 20) 7-NI (30.0 $mg \cdot kg^{-1}$; i.p.) + levosimendan (2.0 $mg \cdot kg^{-1}$; i.p.); 21) AG (30.0 $mg \cdot kg^{-1}$; i.p.); 22) AG (100.0 $mg \cdot kg^{-1}$; i.p.); 23) AG (30.0 $mg \cdot kg^{-1}$; i.p.) + levosimendan (2.0 $mg \cdot kg^{-1}$; i.p.); 24) AG (100.0 $mg \cdot kg^{-1}$; i.p.) + levosimendan (2.0 $mg \cdot kg^{-1}$; i.p.); 25) L-NAME (1.0 $mg \cdot kg^{-1}$; i.p.) + glibenclamide (0.5 $mg \cdot kg^{-1}$; i.p.) + levosimendan (2.0 $mg \cdot kg^{-1}$; i.p.) (Table 1).

The second, consisting of 6 groups, was sacrificed to harvest brain tissues for nitrite assays. To study the effects of levosimendan on NO content in brain tissue mice were randomly assigned to the following groups: 1) saline; 2) levosimendan (2.0 $mg \cdot kg^{-1}$; i.p.); 3) L-NAME 5.0 $mg \cdot kg^{-1}$; i.p.); 4) glibenclamide (1.0 $mg \cdot kg^{-1}$; i.p.); 5) L-NAME (5.0 $mg \cdot kg^{-1}$; i.p.) + levosimendan (2.0 $mg \cdot kg^{-1}$; i.p.); 6) glibenclamide (1.0 $mg \cdot kg^{-1}$; i.p.) + levosimendan (2.0 $mg \cdot kg^{-1}$; i.p.).

2.4. Seizure paradigm

In order to analyze the seizure susceptibility of mouse, we recruited PTZ-induced seizure threshold as a model of GCS. The seizure paradigm test was performed by researchers blinded to experimental conditions using standardized test according to a protocol used previously in our laboratory [22,27]. Following i.v. PTZ administration, eventually a sequence of seizure signs beginning with twitch and progressing to clonus and tonic limb extension is observed. In the present study, forelimb clonus followed by full clonus of the body was used as the endpoint. In preliminary experiments, forelimb clonus was found to be more sensitive to levosimendan than other seizure phases. A 30-gauge, 3/4 in. butterfly needle was inserted into the tail vein of unrestrained freely moving animals, and the needle was secured with a piece of adhesive tape. The needle was connected by a polyethylene tube to a 1 mL syringe mounted on an infusion pump (NE 1000, New Era Pump System, Inc.). PTZ solution (5 $mg \cdot mL^{-1}$) was infused at a constant rate of 1 $mL \cdot min^{-1}$. Infusion was halted at 1 min or when forelimb clonus followed by full clonus of the body was observed, whichever occurred first. The animals were anesthetized by increasing carbon dioxide concentrations and killed by cervical displacement. The PTZ-induced seizure threshold ($mg \cdot kg^{-1}$) was determined according to the following formula: [infusion duration (min) $*$ infusion rate ($mL \cdot min^{-1}$) $*$ PTZ concentration ($mg \cdot mL^{-1}$) $*$ 1000] / [weight of mouse (g)].