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ALPHA-LINOLENIC ACID AND RILUZOLE TREATMENT CONFER CEREBRAL PROTECTION AND IMPROVE SURVIVAL AFTER FOCAL BRAIN ISCHEMIA

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Abstract—We investigated here the effects of alpha-linolenic acid and riluzole, both activators of the 2P-domain K^+ channel family TREK/TRAAK, in a model of focal ischemia clinically relevant to stroke, not only assessing neuronal protection, but also long term survival. Moreover, all the drug treatments were initiated post-ischemia.

Mice were subjected to transient middle cerebral artery occlusion (1 h) and reperfusion according to the intraluminal filament model. Drugs were injected into the jugular vein according to three protocols: (i) a single dose of 4 mg/kg riluzole or 500 nmol/kg alpha-linolenic acid at different reperfusion time; (ii) a three-day therapy (a single dose of 2 mg/kg riluzole and 250 nmol/kg alpha-linolenic acid given 1–2, 48 and 72 h after reperfusion); (iii) a three-week therapy (a single dose of 2 mg/kg riluzole and 250 nmol/kg alpha-linolenic acid given once a week during three weeks after reperfusion. A combined treatment with 2 mg/kg riluzole+250 nmol/kg alpha-linolenic acid injected 2 h after reperfusion was also tested.

A single dose of riluzole (4 mg/kg) or alpha-linolenic acid (500 nmol/kg) injected up to 3 h after reperfusion reduced drastically the stroke volume by 75% and 86%, respectively. Neurological deficits 24 h after ischemia were significantly improved by alpha-linolenic acid500 or riluzole4 with a neurological score of 1.8 as compared with 2.5 observed in vehicle-treated mice. Alpha-linolenic acid- and riluzole treatment were associated with a reduction in cytopathological features of cell injury, including DNA fragmentation and Bax expression in the cortex and the caudate putamen. With regard to the survival rate at 30 days, the best protections were obtained with the alpha-linolenic acid-injection in the three-week therapy as well as with a single dose of the combined treatment (2 mg/kg riluzole+250 nmol/kg alpha-linolenic acid). Palmitic acid, a saturated fatty acid that does not activate the 2P-domain K-channel TREK/TRAAK family, did not provide any neuroprotection.

Taken together, these data suggest that the TREK/TRAAK K-channel family may be a promising target for neuroprotection, and that riluzole and alpha-linolenic acid could be of therapeutic value against focal ischemia/reperfusion injury to the brain.

Key words: neuroprotection, polyunsaturated fatty acids, riluzole, focal ischemia, long-term survival, 2P-domain K^+ channels.

Stroke remains the third leading cause of death and the most common cause of adult disability in industrialized countries. Until now, the only treatment for acute ischemic stroke that has a proven efficacy is i.v. thrombolysis within 3 h of onset. However, only a small percentage of the patients are eligible for this therapy. Anticoagulant therapy is also performed for the prevention of stroke in patients with high risk factors and for halting the evolution of a thrombotic stroke (Hankey and Warlow, 1999). It remains therefore of foremost importance to develop efficacious neuroprotective agents.

The neuroprotective effects of riluzole (RLZ, 2-amino-6-trifluoromethoxy benzothiazole) and alpha-linolenic acid (ALA) have been well established in different models of neurodegeneration. RLZ currently in clinical use among patients with amyotrophic lateral sclerosis (Bensimon et al., 1994) induces major protective effects against brain, spinal cord and retina injuries (Malgouris et al., 1989; Pratt et al., 1992; Martin et al., 1993; Barneoud et al., 1996; Ettaiche et al., 1999; Lang-Lazdunski et al., 1999).

Both *in vitro* and *in vivo* studies have shown alpha-linolenic acid-benefit for neurons in hyperexcitability-induced neuronal death such as epileptic seizures (Voskuyl et al., 1998; Leaf et al., 1999; Lauritzen et al., 2000; Blondeau et al., 2001). It also prevents neuronal cell death and paraplegia after transient spinal cord ischemia (Lang-Lazdunski et al., 2003).

The middle cerebral artery occlusion (MCAO) using the intraluminal suture technique has been shown to reliably and rapidly induce neurological deficits and hemispheric infarcts restricted to the territory of the MCA 24 h after the onset of ischemia (Ginsberg and Busto, 1989; Sharp et al., 2000). Using this model described as the closest to human stroke and monitoring the long-term recovery, the aim of the present work is to demonstrate the relevance of the protective effect of alpha-linolenic acid and RLZ against stroke for future clinical study. Both drug treatments were initiated post-ischemia, to determine their therapeutic windows. Three-day and three-week therapies effects were

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Abbreviations: ALA, alpha-linolenic acid; ALA250, 250 nmol/kg α-linolenic acid; ALA500, 500 nmol/kg α-linolenic acid; ANOVA, analysis of variance; MABP, mean arterial blood pressure; MCA, middle cerebral artery; MCAO, middle cerebral artery occlusion; PALM, palmitic acid; PAL500, 500 nmol/kg palmitic acid; pCO_2, partial pressure of carbon dioxide; pO_2, partial pressure of oxygen; RLZ, riluzole; RLZ2, 2 mg/kg riluzole; RLZ4, 4 mg/kg riluzole; TRAAK, TWIK-1 related arachidonic acid-stimulated potassium channel; TREK, TWIK-1-related potassium channel; TTC, 2,3,5-triphenyltetrazolium chloride; TUNEL, terminal deoxynucleotidyl transferase (TdT)-mediated 2′-deoxyuridine 5′-triphosphate-biotin nick-end labeling; TWIK-1, tandem of pore domains in a weak inward rectifying potassium channel.

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then analyzed to characterize a potential improvement in the survival of animals and their cerebral protection. The drug combination was also tested to evaluate whether the association of these drug actions could enhance the protective effects. To test the link between alpha-linolenic acid, riluzole and the 2P-domain K^+ channel TREK/TRAAK family, palmitic acid (PALM), a saturated fatty acid, which does not activate these channels (Lesage and Lazdunski, 1999, 2000; Patel and Honore, 2001) was injected as negative control and his effects analyzed. Moreover, we paid a particular attention to the comparison of the short-term and the long-term effects of these agents administered in single or combined doses and in repeated single-drug treatment over 3 days and 3 weeks.

EXPERIMENTAL PROCEDURES

Animals

All experiments were conducted according to the NIH guide and the Society of Neurosciences guidelines for the Care and Use of Laboratory Animals (NIH publications No. 80-23, 1985). All efforts were made to minimize the number of animals used and their suffering. Adult male C57/Bl6 mice, weighing 22–26 g were used in this study. The animals housed under controlled laboratory conditions with a 12-h light/dark cycle, a temperature of 21±2 °C, and a humidity of 60–70% for at least one week prior to drug treatment or surgery. The mice had free access to standard rodent diet and tap water.

Physiological parameters

General anesthesia was induced with 3% isoflurane and maintained with 1% isoflurane by means of an open facemask for each mouse. Mice were allowed to breathe spontaneously. A subset of animals (n=5 per group) was monitored for physiological parameters including mean arterial blood pressure (MABP), rectal temperature, arterial blood gases and pH before, during and after ischemia. The right femoral artery was catheterized with PE-10 polyethylene tubing and connected to a blood pressure transducer (Harvard Apparatus, Les Ulis, France) for continuous monitoring of MABP (mm Hg). A heparinized blood sample (75 μl) was then obtained from the catheterized femoral artery and blood partial pressure of oxygen (pO_2), partial pressure of carbon dioxide (pCO_2) and pH were measured using an Acid-Base Laboratory system (ABL 555, Radiometer-Copenhagen, Neuilly/Plaisance, France). Core temperature was monitored continuously with a thermometer (3-mm probe diameter, Harvard Apparatus), inserted into the rectum and maintained at physiological temperatures using a thermostatically controlled heating blanket (Harvard Apparatus).

Induction of transient focal cerebral ischemia

Focal ischemia was induced by occlusion of the left middle cerebral artery (MCA) using an intraluminal filament technique (Ding-Zhou et al., 2002) based on modifications of the original rat model (Longa et al., 1989). After a midline neck incision was made, the left common and external carotid arteries were isolated and ligated with a 4-0 silk suture (Ethicon, Johnson and Johnson Intl, Brussels, Belgium). A temporary Yasargil aneurysm clip (BMH31, Aesculap, Tuttlingen, Germany) was temporarily placed on the internal carotid artery. A 6-0 nylon monofilament (Ethicon), blunted at tip with an open flame, was introduced through a small incision into the common carotid artery and 13 mm distal to the carotid bifurcation for occlusion of the origin of the MCA. Animals were kept at 37 °C for one hour, after which time the thread was carefully withdrawn to allow reperfusion of the MCA territory. To control the MCAO severity regional CBF (rCBF) was determined by laser-Doppler flowmetry (Perimed, Craponne, France) using a flexible 0.5-mm fiber optic extension to the master probe fixed on the intact skull over the ischemic cortex. Sham-operation was performed inserting the thread into the common carotid artery without advancing it to occlude the MCA. The animals were allowed to regain full consciousness on a heating pad before returning to the cage.

Drug treatments

The experimental protocol is shown in Fig. 1. It includes one study at short term, where the animals were analyzed at 24 h post-ischemia (Fig. 1A) and three studies at long term (Fig. 1B, C, D) where the mice were evaluated one month following MCA occlusion. In study A, mice received a single dose of 4 mg/kg riluzole (RLZ4) or 500 nmol/kg alpha-linolenic acid (ALA500), or 500 nmol/kg palmitic acid (PAL500) at different times after MCAO to determine the therapeutic window. The doses used were selected based on our previous studies in different animal models (Lang-Lazdunski et al., 1999; Lauritzen et al., 2000; Blondeau et al., 2001) and from pilot studies on mice searching for the best protection (data not shown). Single doses of RLZ and ALA were tested at different time points to establish therapeutic windows. It is based on these results that we chose as reference treatment a fixed dose for a determined time point of injection after reperfusion.

Next, the efficiency of a single dose of RLZ4 or ALA500 injected one (RLZ4) or two (ALA500) hours after reperfusion was compared with a dose of 2 mg/kg riluzole (RLZ2) or 250 nmol/kg alpha-linolenic acid (ALA250) given 1–2, 48 and 72 h after reperfusion (Fig. 1B) or 1–2 h after reperfusion following MCAO and once a week during the next 2 weeks (Fig. 1C). In study C, mice were treated with a combined treatment of RLZ2+ALA250 injected 2 h after reperfusion following MCAO (Fig. 1D). RLZ (RBI, USA), ALA and PALM (Biomol, USA) were injected as a bolus directly in the jugular vein. Sham-operated animals and mice injected with vehicle were used as controls.

Neurological deficits

Neurological deficits of mice were assessed 24 h post-ischemia in a blinded fashion according to a scoring scale in a postural reflex test developed by Bederson et al. (1986): Grade 0: no visible deficits; Grade 1: forelimb flexion; Grade 2: unidirectional circling when the animal is pulled by the tail; Grade 3: circling and rolling movement; Grade 4: decreased level of consciousness. Mice with a neurological deficit above grade 3 were excluded from the study.

Determination of infarct volume

To assess the infarct volume in the short-term study (single injection of ALA, RLZ or PALM), mice were killed at 24 h after reperfusion. Their brains were removed and sectioned into six 1 mm-thick coronal slices using a tissue chopper (Phymep, France). Coronal brain slices were immediately immersed into 2% 2,3,5-triphenyltetrazolium chloride (TTC, Sigma, France) for 20 min at room temperature in the dark followed by fixation in a 4% paraformaldehyde solution overnight prior to analysis as described previously (Ding-Zhou et al., 2002). The striatal and cortical areas of infarction, outlined in light were measured on each section using a computer image analysis system and corrected for brain edema according to Golanov and Reis (1995). Infarct volume, expressed in mm^3 was calculated by a linear integration of the corrected lesions areas. The same TTC staining at 24 h post-ischemia was also applied to assess the first effect on infarct size of the combined administration used in the long-term study.