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

C. Heurteaux, C. Laigle, N. Blondeau, G. Jarretou, M. Lazdunski

► To cite this version:

C. Heurteaux, C. Laigle, N. Blondeau, G. Jarretou, M. Lazdunski. ALPHA-LINOLENIC ACID AND RILUZOLE TREATMENT CONFER CEREBRAL PROTECTION AND IMPROVE SURVIVAL AFTER FOCAL BRAIN ISCHEMIA. *Neuroscience*, 2006, 137 (1), pp.241-251. 10.1016/j.neuroscience.2005.08.083 . hal-03357424

HAL Id: hal-03357424

<https://hal.science/hal-03357424>

Submitted on 28 Sep 2021

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

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

C. HEURTEAUX,* C. LAIGLE, N. BLONDEAU,
G. JARRETOU AND M. LAZDUNSKI

*Institut de Pharmacologie Moléculaire et Cellulaire, UMR 6097, CNRS
Université de Nice Sophia Antipolis, Institut Paul Hamel, 660 Route
des Lucioles, Sophia-Antipolis, 06560 Valbonne, France*

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 acid 500 or riluzole 4 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. © 2005 Published by Elsevier Ltd on behalf of IBRO.

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

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 (pO₂), partial pressure of carbon dioxide (pCO₂) 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, Crapeyronne, 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% para-formaldehyde 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 mm³ 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.

Fig. 1. Flowchart illustrating the different paradigms. Timing of MCAO/reperfusion, ALA and RLZ treatment at different doses, neurological score, mortality rate, TTC-staining, Bax expression, TUNEL staining and cell counting. (A) Single dose paradigm (RLZ4 or ALA500 or PAL500). (B) Three-day therapy paradigm (RLZ2 or ALA250). (C) Three-week therapy paradigm (RLZ2 or ALA250). (D) Combined therapy paradigm (ALA250+RLZ2).

In addition Cresyl Violet, a dye that stains the Nissl bodies in the stellate somas of viable neurons was used to confirm the evolution and the extent of infarct volume and analyze cell survival in ischemic mice. Coronal frozen sections of brain (10 μ m-thick) were added to a solution of 1% Cresyl Violet in 0.25% acetic acid for 3 min, rinsed, dehydrated and mounted with Entellan. Sections were analyzed under light microscopy. Cell counting using standard methods (Blondeau et al., 2002) were performed in a blind fashion in striatal and cortical penumbra at 30 days to determine the level of cerebral damage. Staining coronal brain sections with TTC and Cresyl Violet after 24 h and 30 days of recovery, respectively has been shown to provide a reliable estimate of the infarct margins and the boundary between penumbra and core, which was estimated by the difference between the size of both infarcts studied at 24 and 3 weeks post-ischemia. Histologically, the boundaries between core and penumbra can be well evaluated. Unlike the core region where many neurons are dead, the penumbra region appears with intact neurons and cells presenting pyknosis.

TUNEL staining

Terminal deoxynucleotidyl transferase (TdT)-mediated 2'-deoxyuridine 5'-triphosphate-biotin nick-end labeling (TUNEL) staining was performed by using the *In Situ* Cell Death Detection Kit (Roche, Basel, Switzerland), which allows to demonstrate apoptotic cells in ischemic brain tissue as described previously (Blondeau et al., 2002). Coronal 10 μ m frozen sections were rehydrated in ethanol (95, 70 and 50%) followed by PBS and bathed in 0.3% hydrogen peroxide/methanol to inactivate endogenous peroxidase. Sections were then permeabilized in 0.3% Tween/PBS and washed twice in PBS. Labeling of 3'-OH terminal DNA fragments was then performed at 37 °C for 2 h by using the TUNEL reaction mixture according to the manufacturer's protocol. Positive control was obtained by pre-incubation of a section with DNase I (20 μ g/ml) for 15 min at 37 °C before incubation with biotinylated dUTP. Quantitative (number of TUNEL-positive cells/0.5 mm²) analyses were performed in the ipsilateral cortical penumbra. TUNEL-pos-

itive cells displayed a brown staining within the nucleus in the apoptotic cells.

Western blot analysis

Whole-cell protein extraction was performed using standard procedures. Samples were obtained from the MCA territory cortex on the ischemic sides and from nonischemic controls. ($n=3$ per group). Fresh brain tissue was cut into pieces after 24 h of reperfusion and homogenized in four volumes of cold lysis buffer (20 nmol/l Tris pH: 7.5, 137 mmol/l NaCl, 2 mmol/l EDTA, 1% Triton X-100, 10% glycerol, and protease inhibitor cocktail) on ice. The homogenates were centrifuged at $12,000\times g$ for 30 min at 4 °C. The supernatant was stored at -70°C until further use. Protein concentrations were measured using conventional Bradford's method. Fifty microgram proteins from each experimental group were applied to 10% SDS PAGE gels and electrophoresed for 1 h at 100 mA. Proteins were transferred onto nitrocellulose membrane (Hybond) in blotting buffer (156 mmol/l Tris, 1 mol/l glycine, PBS) for 90 min at 80 mA and blocked with 5% skim milk (Regilaite) in PBS for 2 h at room temperature. The blotted membrane was incubated with the rabbit polyclonal Bax antibody (Santa Cruz, SC493, diluted 1/1000) for 2 h at room temperature. Western blots were incubated with horseradish peroxidase-conjugated anti-rabbit IgG (Jackson ImmunoResearch, diluted 1/15,000) for 1 h at room temperature and revealed using enhanced chemiluminescence Western blotting detection reagents (Pierce). To control for sample loading, stripped membranes were rehybridized with a α -tubulin antibody as internal control (data not shown). Films with specific bands were scanned and quantified using an imaging densitometer.

Immunohistochemistry

Frozen brain sections 25 μm thick were immersed in 0.3% H_2O_2 /methanol for 10 min, permeabilized in 0.1% Triton/PBS for 10 min and blocked with 3% goat serum/PBS for 2 h at room temperature followed by a single rinse in PBS. Sections were incubated then with the primary antibody overnight. The antiserum used was the rabbit polyclonal Bax antibody (Santa Cruz, SC493, diluted 1/300). After the primary incubation and three rinses in PBS, sections were then incubated in biotinylated horse anti-rabbit IgG (Jackson ImmunoResearch, diluted 1/15,000) for 2 h at room temperature. Bax expression was visualized by 3,3'-diaminobenzidine (DAB) staining using the VectaStain ABC kit (Biovalley). All sections were washed in PBS and then in distilled water, and mounted with Entellan.

Statistical analysis

Data were expressed as mean $\pm$ S.D. of n observations, where n represents the number of animals or samples. Comparison between multiple groups was evaluated by one-way analysis of variance (ANOVA) followed by a PLSD (protected least significant difference) Fisher's test. When measures were repeated (physiological variables) data were analyzed by a two-factorial ANOVA for repeated measures. Significance was assumed at a value of $P<0.05$.

RESULTS

A single dose of ALA500 or RLZ4 induced cerebral protection at 24 h and 30 days after MCAO

To determine whether acute administration of ALA or RLZ protects against ischemic stroke, mice were subjected to transient (1 h) MCAO and injected intravenously with a

Table 1. Physiological parameters 30 minutes after injection of drugs ($n=5$ per group) and neurological deficit score after 1-hour MCAO in drug- and vehicle-treated mice (24 hours following reperfusion)

	VEH	LIN	RLZ	PALM
MABP (mm Hg)	90 $\pm$ 2	92 $\pm$ 3	94 $\pm$ 6	92 $\pm$ 4
paCO ₂ (mm Hg)	39 $\pm$ 6	44 $\pm$ 3	46 $\pm$ 2	41 $\pm$ 3
paO ₂ (mm Hg)	444 $\pm$ 41	420 $\pm$ 9	462 $\pm$ 29	452 $\pm$ 27
pH (arterial)	7.30 $\pm$ 0.02	7.35 $\pm$ 0.01	7.31 $\pm$ 0.02	7.33 $\pm$ 0.03
RT (°C)	37.4 $\pm$ 0.3	37.2 $\pm$ 0.4	37.2 $\pm$ 0.4	37.4 $\pm$ 0.3
Neurological score	2.48 $\pm$ 0.11	1.79 $\pm$ 0.14*	1.78 $\pm$ 0.13*	2.59 $\pm$ 0.15

* $P<0.05$ vs. vehicle-treated ischemic mice.

single dose of ALA500 or RLZ4 at different times after reperfusion. This paradigm corresponds to Fig. 1A. Administration of RLZ or fatty acids (ALA or PAL) did not affect MABP, arterial pO₂ or pCO₂ and arterial blood pH (Table 1). The intraluminal suture method resulted in an infarct in the right MCA perfused region. Representative photographs of brain slices stained with TTC at 24 h after MCAO are shown in Fig. 2A. As indicated by the white area, cerebral infarcts in ALA500- and RLZ4-treated mice were reduced. The infarct spread into the dorsomedial cortex in the region of the anterior cerebral artery/MCA and the caudate-putamen was particularly inhibited by both treatments. Quantitative assessment of infarct volume 24 h after MCAO revealed the therapeutic window of ALA500 or RLZ4 treatment (Fig. 2B). Administration of ALA500 or RLZ4 up to 6 h after reperfusion drastically decreased cerebral infarction. Maximal ALA-induced protection was achieved with a dose of 500 nmol/kg injected 2 h post-ischemia with a 86% reduction as compared with vehicle-group ($P<0.001$), an effect that was lost when treatment was initiated 12 h after reperfusion. Administration of RLZ up to 3 h after reperfusion at a dose of 4 mg/kg reduced the stroke volume by approximately 75% ($P<0.001$) as compared with control mice, with no significant difference between reperfusion times. In contrast, PALM500, a saturated fatty acid, had no beneficial effect on the infarct size when injected 2 h after reperfusion, a time corresponding to the best cerebral protection obtained with ALA, a polyunsaturated fatty acid (Fig. 2B).

Based on these results, we chose as reference treatment the dose of RLZ4 or ALA500, injected 1 or 2 h after reperfusion, respectively. In this context, neurological deficits 24 h after ischemia were also significantly improved by ALA500 or RLZ4 (Table 1).

The protection induced by ALA500- or RLZ4-injection was also associated with a reduction in cytopathological features of cell injury, including cell shrinkage (cresyl violet staining, not shown), DNA-fragmentation (Fig. 3A), and Bax expression (Fig. 3B–D). TUNEL method revealed massive DNA fragmentation by 24 h in the ipsilateral cortex of vehicle-treated mice, which underwent 1 h-MCAO as compared with sham-operated animals. Fig. 3A shows the number of TUNEL-positive cells, characterized by shrunken cytoplasm and dark nuclei in the ipsilateral cortex of different groups. ALA500- or RLZ4-injection signifi-

Fig. 2. Effects of a single injection of ALA, RLZ or PALM on infarct size in brain sections after 1-hour reversible MCAO. (A) Representative samples of TTC-stained from mice killed 24 h after ischemia. One-hour MCAO/reperfusion animals treated with vehicle or PALM showed severe infarctions. The white areas represent the infarct regions in these sections. In contrast, ALA500 RLZ4 treatment had a significant reduction in infarction. (B) Therapeutic window of ALA500 and RLZ4 on infarct volume 24 h after MCAO. ALA, RLZ or PALM was i.v. injected ($n=15$ per experimental group). Data are expressed as means $\pm$ S.E.M. * $P<0.05$; ** $P<0.001$ versus vehicle-treated ischemic mice.

cantly reduced the percentage of positive cells with a better protection with RLZ (13.9% in RLZ- and 36.5% in ALA-groups vs 49% in vehicle-group, $P<0.001$). Expression of the protein Bax, known to have a role in promoting apoptosis pathways, is regarded as a diagnostic marker of neurons undergoing cell death (Tsujimoto and Shimizu, 2000). The induction of the Bax protein in the cortical penumbra of vehicle- and treated-mice has been assessed using Western blotting (Fig. 3B and 3C) and immunohistochemistry (Fig. 3D) at 3 h after reperfusion following MCAO. The Western blotting analysis, using the polyclonal anti-Bax antibody revealed a single major band at 21 kDa, the size predicted for the Bax- α protein (Fig. 3B). Fig. 3C summarizes the quantification of Bax expression in ipsilateral cortical extracts from the complete set of mice. Bax induction remained undetectable in protein extracts from cortex

of sham-operated groups. One hour MCAO induced strong Bax expression in cortical extracts of vehicle-treated mice 3 h after reperfusion. In contrast, injection of a dose of ALA500 or RLZ4 after MCAO led to a significant decrease in Bax expression in the cortical extracts of treated mice. While Bax expression increased 5.9-fold in vehicle-treated mice, injection of RLZ4 or ALA500 significantly prevented this increase. Immunostaining on brain sections (Fig. 3D) confirmed the findings obtained with Western blot analysis. Weak Bax immunoreactivity decorated the cytoplasm and cell processes of neurons in the cortex of sham-operated mice, but increased Bax immunoreactivity occurred in the cytoplasm of penumbra neurons of the ipsilateral cortex at 3 h following reperfusion. Post-treatment with ALA500 or RLZ4 strongly reduced Bax immunoreactivity in penumbra cortical neurons of ischemic brains (Fig. 3D).

Fig. 3. Decrease of TUNEL-positive cells and Bax expression following the acute injection of ALA500 or RLZ4 after MCAO. (A) Histograms representing percentage of TUNEL-positive cells/total in the ipsilateral penumbral cortex (per 0.5 mm²) 24 h after reperfusion ($n=10$ per group, 10 slides per mouse). * $P<0.05$, ** $P<0.001$ versus vehicle (VEH)-treated ischemic mice. (B) Western blotting analysis of Bax protein induction in cortical extracts isolated from sham-operated (Sham), VEH-injected brains or brains treated with a dose of RLZ4, ALA500 or PAL500 (PALM). RLZ4, ALA500 or PALM500 was i.v. injected 1 (for RLZ4) or 2 h (for ALA500 or PALM500) after reperfusion. Brains were removed from mice 3 h after reperfusion. (C) Quantification of Bax expression obtained in Western blotting. The specific bands were quantified using an imaging system as described in Experimental Procedures. Values are expressed as a percentage of sham-operated mice. Data are expressed as mean $\pm$ S.E.M. Data are representative of three separate experiments ($n=4$ per experimental group). * $P<0.05$, ** $P<0.001$ versus sham-operated mice. (D) Decreased expression of Bax protein in cortical penumbra of RLZ4- or ALA500-treated ischemic mice as compared with VEH- or PALM-injected ischemic mice at 3 h after reperfusion following MCAO. Data are representative of three separate experiments ($n=5$ per experimental group). Scale bar = 100 μ m.

Importantly, the reduction of the infarct volume induced by ALA500- or RLZ4-injection, previously observed by TTC staining, was still occurring at 30 days after MCAO with Nissl staining. The lesion volume, revealed by the lack of Nissl staining, was drastically reduced in ALA500- or RLZ4-treated mice, as compared with vehicle-treated animals (data not shown). The infarct size reduction was associated to better cell preservation after ALA500 or RLZ4 administration that lasted for at least one month as determined by cell counting in well-known structures injured by MCAO (i.e. dorsomedial cortex and caudate-putamen) at 30 days postischemia (Fig. 4A). As expected, one hour MCAO produced severe cell loss. Compared with the sham-operated group, only 57.9 and 43% penumbra neurons of cerebral cortex and striatum were preserved, respectively. This cellular loss was almost completely inhibited by ALA500- or RLZ4-injection in both structures. Cortical cell survival was around 95% in ALA500- and RLZ4-treated groups ($P<0.001$). Administration of ALA500 or RLZ4 limited striatal cell destruction to 5.9 and 7.9% of neurons, respectively ($P<0.001$). In contrast, injection of the saturated fatty acid PALM500 failed to protect

the brain and no rescue of the cell number was observed in both structures (Fig. 4A).

Three-day and three-week therapies reduced mortality and increased cell survival following ischemia at 30 days after MCAO

As indicated in the paradigms of Fig. 1B and 1C, the efficiency of a single dose of RLZ4 or ALA500 injected 1 (RLZ4) or 2 h (ALA500) after reperfusion was compared with a dose of RLZ2 or ALA250 given 1–2, 48 and 72 h after reperfusion or 1–2 h after reperfusion following MCAO and once a week during the next two weeks of reperfusion. The protection was assessed by neuronal counting and mortality rate one month after MCAO. In terms of cell survival, the weekly injection of ALA250 or RLZ2 during three weeks also induced a potent protection (Fig. 4B and 4C). It limited the striatal cell destruction to 4.6 and 5.7% of neurons (Fig. 4B), and induced a cortical cell survival of 96.5 and 95.1%, respectively ($P<0.001$) (Fig. 4C). In terms of mortality (Fig. 5), the best protection against ischemia was obtained with a weekly treatment of ALA250 during three weeks (70% survival rate) followed by RLZ-treatment at a dose of 2 mg/kg with the

Fig. 4. Comparative long-term protective effects between single, three-day or three-week therapy and administration of a combined dose of ALA or RLZ on cell death one month after 1-hour reversible MCAO. Histograms represent the neuronal density per 0.5 mm² brain structure (cortex or caudate-putamen in (i) ipsi- and (c) contralateral sides) assessed in cresyl-violet-stained sections (cortex and caudate-putamen) one month after ischemia. (A) Single dose paradigm: RLZ4 or ALA500 was i.v. injected 1 or 2 h after reperfusion, respectively. (B, C) Three-day, three-week and combined therapies paradigms. Days 1–3: a dose of RLZ2 or ALA250 given 1–2, 48 and 72 h after reperfusion. Weeks 1–3: a dose of RLZ2 or ALA250 given 1–2 h after reperfusion and once each week during the next two weeks of reperfusion. Combined: a single dose of RLZ2+ALA250 injected once 2 h after reperfusion. Data are expressed as means±S.E.M. (*n*=15 per experimental group, 10 slides per animal). ** *P*<0.001 versus vehicle-treated (VEH) ischemic mice.

same periodicity (60% survival rate). Compared with the reference treatment (a single dose of ALA500 or RLZ4), the

three-day therapy did not significantly improve the survival rate of ischemic mice (Fig. 5).

Fig. 5. Comparative effects between three-day or three-week therapies and administration of a single or combined dose of ALA and RLZ on survival rate of mice one month after 1-hour reversible MCAO. Single dose: a dose of RLZ4 or ALA500 injected 1 or 2 h after reperfusion. Days 1–3: a dose of RLZ2 or ALA250 given 1–2, 48 and 72 h after reperfusion. Weeks 1–3: a dose of RLZ2 or ALA250 given 1–2 h after reperfusion and once each week during the next two weeks of reperfusion. Combined: a single dose of ALA250+RLZ4 injected once 2 h after reperfusion ($n=15$ per group).

Combined doses of RLZ2 and ALA250 induced the best brain resistance to ischemia

As indicated in paradigm D, we next tested the hypothesis that combined administration of a dose of RLZ2+ALA250 given 2 h after MCAO could improve the protection against the deleterious effects of ischemia. Representative photographs of brain slices stained with TTC at 24 h after MCAO

with the corresponding quantitative assessment of infarct volume are shown in Fig. 6. As indicated by the white area, the cerebral infarct in mice treated with a combined injection of RLZ2+ALA250 was significantly reduced. Cell counting in the peri-infarct zone 30 days after MCAO shows that a combined injection of RLZ2 and ALA250 induced a penumbra cortical and striatal cell survival of

Fig. 6. Effects of combined administration of ALA250 and RLZ2 on infarct size in brain sections after 1-hour MCAO. (A) Representative samples of TTC-stained from mice killed 24 h after ischemia. The white areas represent the infarct regions in these sections. (B) Histograms representing the corresponding infarction volumes 24 h after ischemia. A single dose of ALA250+RLZ2 was i.v. injected 2 h after reperfusion ($n=15$ per group). Data are expressed as means±S.E.M. ($n=15$ per experimental group). ** $P<0.001$ versus vehicle-treated (VEH) ischemic mice.

98.7 and 98%, respectively ($P < 0.001$) (Fig. 4B and 4C). One month after MCAO, the mortality rate was reduced to 20% (Fig. 5) compared with 75% in the sham-operated group.

DISCUSSION

The purpose of the current study was to investigate the protective effects of ALA and RLZ in a clinically relevant model associating transient focal ischemia and post-treatment. After 24 h of MCA occlusion, histological changes indicating ischemic brain damage have been observed in caudate putamen, pallidum, basolateral cortex, hippocampus and parts of thalamus, which is consistent with previous studies of intraluminal MCA occlusion (Hata et al., 2000; Ding-Zhou et al., 2002). We have tested a dose-range of both drugs (ALA500 or RLZ4) based on previously published data in other models of neurodegeneration (Pratt et al., 1992; Lang-Lazdunski et al., 1999; Lauritzen et al., 2000). In the first step of the study, we showed that an i.v. injection with a single dose of ALA500 or RLZ4 initiated after the onset of MCAO significantly reduced the infarct size and improved the neurological score, both examined at 24 h post-ischemia. ALA500 and RLZ4 were both efficient up to 6 h following reperfusion, i.e. within a therapeutic window, which is relevant clinically. Ischemic injury is associated with progressive DNA fragmentation in both striatal and cortical neurons within the infarct area including the core and penumbra. At 24 h post-reperfusion, TUNEL labeling appeared intense and abundant in these damaged structures. Ischemia-induced DNA fragmentation contains not only internucleosomal fragmentation but also random breakdown products often associated with necrosis. The TUNEL procedure labels all types of DNA fragments. It cannot discriminate accurately between apoptotic and necrotic cells (Charriaut-Marlangue et al., 1996; Unal-Cevik et al., 2004), and therefore is mostly considered as a marker of damaged cells. In that context, we observed that a single dose of ALA500 or RLZ4 injected 1–2 h following MCAO reduced dramatically the number of TUNEL-positive cells in cortex after 24 h reperfusion. Programmed cell death and apoptosis are regulated by members of the Bcl-2 family, including Bcl-2, which promotes cell survival and Bax, which promotes cell death. Several studies *in vivo* have demonstrated modifications in the expression levels of members of the Bcl-2 family. Bax upregulation has been found following ischemia (Krajewski et al., 1995; Ferrer et al., 2003) in association with Bcl-2 reduction in the infarct core (Matsushita et al., 1998). Bax is a potent proapoptotic molecule, which triggers activation of terminal caspases and subsequently promotes neuronal cell death and has been described to increased in the penumbra 3 h following the reperfusion (Ferrer et al., 2003). Using Western blotting and immunohistochemistry, this work confirms the upregulation of the Bax protein in the penumbra cortical neurons of ischemic vehicle-treated mice at 3 h following reperfusion and demonstrated that a single dose of ALA500 or RLZ4 strongly reduced Bax expression. Taken together with the fact that

penumbra is the privileged site for apoptosis, both reduction of TUNEL labeling and Bax reactivity suggest that the neuroprotection induced by ALA500 and RLZ4 involves a decrease of apoptotic pathways occurring in this area. Nevertheless, we cannot exclude a direct effect of these drugs on apoptosis.

In the second step, to design the best type of treatment we compared neuronal protection, but also long term survival induced by a single dose of ALA500 or RLZ4 to three day or three week therapies with ALA250 or RLZ2 or a combination therapy of ALA250+RLZ2 injected 2 h after reperfusion. The results show that the greatest differences between paradigms appeared in the survival rate of the mice. The model of focal ischemia used in this study is very severe, since more than 75% of the ischemic mice treated with vehicle died within the month of reperfusion. Interestingly, three-week therapies with ALA250 or RLZ2 improved the survival rate (at one month) as compared with a single dose of ALA500 or RLZ4. The best protection in terms of survival rate (83.6%) was obtained with the cocktail therapy of ALA250+RLZ2 injected 2 h after reperfusion, suggesting a combined effect of both drugs. When we compare the different paradigms and analyze the results obtained in terms of brain protection and mice survival, it seems that salvaging cortical and caudate neurons did not necessarily improve survival. This point can be explained by the development of the ischemic edema (vasogenic and cytotoxic), that we have not measured in this study. As mentioned in the Experimental Procedures, neurological deficit was assessed at 24 h post-ischemia. Mice with a neurological deficit above grade 4 (decreased level of consciousness), were excluded from the study to exclude ischemic edema, one of the causes of clinical deterioration within the first 24 h. The clinical manifestations of ischemic brain swelling generally appear between 2 and 5 days after stroke and this propensity of ischemic brain tissue to develop edema and swelling remains the major cause of death within a week. Then, cresyl violet staining, used to count viable neurons and analyze cell survival in ischemic mice, was performed at three weeks on the animals that had survived, probably overcoming the deleterious effects of edema. In this context, we cannot exclude that the single combined therapy (ALA250+RLZ2) as well as the three daily injections (ALA250 or RLZ2) reduced the development of edema and consequently reduced brain and animal death.

Taken together, this study demonstrates that alpha-linolenic acid and riluzole induce a beneficial effect on the infarct size, neurological score, neuronal survival and mortality rate of ischemic mice in a clinically relevant model of stroke. The neuroprotective properties of RLZ and PUFAs have already been described in other models of neurodegeneration (Malgouris et al., 1989; Pratt et al., 1992; Barneoud et al., 1996; Voskuyl et al., 1998; Ettaiche et al., 1999; Lang-Lazdunski et al., 1999; Leaf et al., 1999; Lauritzen et al., 2000). The main interest of this work has been to compare the neuroprotective effects of RLZ or/and ALA, both at short (24 h) and long terms (one month). Most studies using the MCAO model describe the beneficial

effect of a drug by the reduction of the infarction and the improvement of the neurological score at 24 h. As for the rapid ischemic preconditioning protection, which disappears when the reperfusion is extended to 7 days (Perez-Pinzon et al., 1997), it is known that neuronal protection can only be temporary with some therapies (Dietrich et al., 1993). In our study, we analyzed not only the effectiveness of RLZ or/ and ALA on the infarct size and the neurological outcome at 24 h post-ischemia, but also the cell survival in the damaged cerebral structures and the death rate of mice one month after ischemia. Looking at cell survival shortly after stroke, it appears that the various paradigms (single dose of ALA or RLZ, 3 days or three weeks therapy, or combined treatment) equally induce a remarkable protection of the neurons. On the other hand, for animal survival, only the 3 week therapy or combined treatment provides a real improvement. This observation is interesting for later clinical applications, since it shows that one protocol effective in rescuing cellular populations does not necessarily improve survival.

It is clear that multiple and complex pathways involving excitotoxicity, oxidative and nitrosative stress, periinfarct depolarization, inflammation and apoptosis-like mechanisms cause cell death in stroke (Dirnagl et al., 1999; White et al., 2000). In this context, it is reasonable to develop combination therapies targeting several of deleterious mechanisms, simultaneously. In this work, we show that a combined injection of RLZ and ALA not only induced effective neuronal survival but more importantly an improvement of the animals' survival. Several research groups have analyzed the different targets of RLZ and ALA. Both molecules are known to reduce hyperexcitability and excitatory neurotransmission (Cheramy et al., 1992; Martin et al., 1993; Doble, 1996; Lauritzen et al., 2000). They both partially inhibit voltage-sensitive Na⁺ channels and voltage-sensitive Ca⁺⁺ channels (Benoit and Escande, 1991; Hebert et al., 1994; Huang et al., 1997). More importantly both activate the background TREK-1, TREK-2 and TRAAK potassium channels (Lesage and Lazdunski, 1999, 2000; Duprat et al., 2000; Patel and Honore, 2001). On the other hand recent work has shown enhanced expression of the lipid-sensitive 2P-domain K⁺ channels (the TREK/TRAAK channel family) in cortex and hippocampus after MCAO and suggested a correlation between TREK channels and acute cerebral ischemia (Li et al., 2005). This idea was reinforced by recent data demonstrating that TREK-1 channels were required for the protective properties of polyunsaturated fatty acids against global ischemia and kainate-induced seizures (Heurteaux et al., 2004).

Our data on the neuroprotective effects of alpha-linolenic acid and riluzole in a model of focal ischemia are supportive of the idea that a combined therapy with these molecules may have important clinical implications for a successful therapy of stroke in the future. They also strengthen the general opinion that polyunsaturated fatty acids have beneficial effects in human health.

Acknowledgments—This work was supported by the Centre National de la Recherche Scientifique (CNRS) and the Paul Hamel

Foundation. We are grateful to the Fondation de la Recherche Médicale for a fellowship to C. Laigle, to Professor M. Plotkine for training with the focal ischemia model, and to Dr. Lochlan Rash for careful reading of the manuscript.

REFERENCES

- Barneoud P, Mazadier M, Miquet JM, Parmentier S, Dubedat P, Doble A, Boireau A (1996) Neuroprotective effects of riluzole on a model of Parkinson's disease in the rat. *Neuroscience* 74:971–983.
- Bederson JB, Pitts LH, Tsuji M, Nishimura MC, Davis RL, Bartkowski H (1986) Rat middle cerebral artery occlusion: evaluation of the model and development of a neurologic examination. *Stroke* 17:472–476.
- Benoit E, Escande D (1991) Riluzole specifically blocks inactivated Na channels in myelinated nerve fibre. *Pflugers Arch* 419:603–609.
- Bensimon G, Lacomblez L, Meininger V (1994) A controlled trial of riluzole in amyotrophic lateral sclerosis. ALS/Riluzole Study Group. *N Engl J Med* 330:585–591.
- Blondeau N, Lauritzen I, Widmann C, Lazdunski M, Heurteaux C (2002) A potent protective role of lysophospholipids against global cerebral ischemia and glutamate excitotoxicity in neuronal cultures. *J Cereb Blood Flow Metab* 22:821–834.
- Blondeau N, Widmann C, Lazdunski M, Heurteaux C (2001) Polyunsaturated fatty acids induce ischemic and epileptic tolerance. *Neuroscience* 109:231–241.
- Charriaut-Marlangue C, Margail I, Represa A, Popovici T, Plotkine M, Ben-Ari Y (1996) Apoptosis and necrosis after reversible focal ischemia: an in situ DNA fragmentation analysis. *J Cereb Blood Flow Metab* 16:186–194.
- Cheramy A, Barbeiro L, Godeheu G, Glowinski J (1992) Riluzole inhibits the release of glutamate in the caudate nucleus of the cat *in vivo*. *Neurosci Lett* 147:209–212.
- Dietrich WD, Busto R, Alonso O, Globus MY, Ginsberg MD (1993) Intrischemic but not postischemic brain hypothermia protects chronically following global forebrain ischemia in rats. *J Cereb Blood Flow Metab* 13:541–549.
- Ding-Zhou L, Marchand-Verrecchia C, Croci N, Plotkine M, Margail I (2002) L-NAME reduces infarction, neurological deficit and blood-brain barrier disruption following cerebral ischemia in mice. *Eur J Pharmacol* 457:137–146.
- Dirnagl U, Iadecola C, Moskowitz MA (1999) Pathobiology of ischaemic stroke: an integrated view. *Trends Neurosci* 22:391–397.
- Doble A (1996) The pharmacology and mechanism of action of riluzole. *Neurology* 47:S233–S241.
- Duprat F, Lesage F, Patel AJ, Fink M, Romey G, Lazdunski M (2000) The neuroprotective agent riluzole activates the two P-domain K⁺ channels TREK-1 and TRAAK. *Mol Pharmacol* 57:906–912.
- Ettaiche M, Fillacier K, Widmann C, Heurteaux C, Lazdunski M (1999) Riluzole improves functional recovery following ischemia in the rat retina. *Invest Ophthalmol Vis Sci* 40:729–736.
- Ferrer I, Friguls B, Dalfo E, Justicia C, Planas AM (2003) Caspase-dependent and caspase-independent signalling of apoptosis in the penumbra following middle cerebral artery occlusion in the adult rat. *Neuropathol Appl Neurobiol* 29:472–481.
- Ginsberg MD, Busto R (1989) Rodent models of cerebral ischemia. *Stroke* 20:1627–1642.
- Golanov EV, Reis DJ (1995) Contribution of cerebral edema to the neuronal salvage elicited by stimulation of cerebellar fastigial nucleus after occlusion of the middle cerebral artery in rat. *J Cereb Blood Flow Metab* 15:172–174.
- Hankey GJ, Warlow CP (1999) Treatment and secondary prevention of stroke: evidence, costs, and effects on individuals and populations. *Lancet* 354:1457–1463.
- Hata R, Maeda K, Hermann D, Mies G, Hossmann KA (2000) Evolution of brain infarction after transient focal cerebral ischemia in mice. *J Cereb Blood Flow Metab* 20:937–946.

- Hebert T, Drapeau P, Pradier L, Dunn RJ (1994) Block of the rat brain IIA sodium channel alpha subunit by the neuroprotective drug riluzole. *Mol Pharmacol* 45:1055–1060.
- Heurteaux C, Guy N, Laigle C, Blondeau N, Duprat F, Mazzuca M, Lang-Lazdunski L, Widmann C, Zanzouri M, Romey G, Lazdunski M (2004) TREK-1, a K(+) channel involved in neuroprotection and general anesthesia. *EMBO J* 23:2684–2695.
- Huang C-H, Song J-H, Nagata K, Yeh JZ, Narahashi T (1997) Effects of the neuroprotective agent riluzole on the high voltage-activated calcium channels of rat dorsal root ganglion neurons. *J Pharmacol Exp Ther* 282:1280–1290.
- Krajewski S, Mai JK, Krajewska M, Sikorska M, Mossakowski MJ, Reed JC (1995) Upregulation of bax protein levels in neurons following cerebral ischemia. *J Neurosci* 15:6364–6376.
- Lang-Lazdunski L, Blondeau N, Jarretou G, Lazdunski M, Heurteaux C (2003) Linolenic acid prevents neuronal cell death and paraplegia after transient spinal cord ischemia in rats. *J Vasc Surg* 38:564–575.
- Lang-Lazdunski L, Vaillant N, Widmann C, Heurteaux C, Lazdunski M (1999) Riluzole improves neurological function following severe spinal cord ischemia. *J Thorac Cardiovasc Surg* 117:881–889.
- Lauritzen I, Blondeau N, Heurteaux C, Widmann C, Romey G, Lazdunski M (2000) Polyunsaturated fatty acids are potent neuroprotectors. *EMBO J* 19:1784–1793.
- Leaf A, Kang JX, Xiao Y-F, Billman GE, Voskuyl RA (1999) The antiarrhythmic and anticonvulsant effects of dietary N-3 fatty acids. *J Membr Biol* 172:1–11.
- Lesage F, Lazdunski M (1999) Potassium channels with two P-domains. In: Potassium ion channels. Molecular structure, function, and diseases: Current topics in membranes, Vol. 46 (Kurachi Y et al., eds), pp 199–222. San Diego, CA: Academic Press.
- Lesage F, Lazdunski M (2000) Molecular and functional properties of two pore domain potassium channels. *Am J Physiol* 279:793–801.
- Li ZB, Zhang HX, Li LL, Wang XL (2005) Enhanced expressions of arachidonic acid-sensitive tandem-pore domain potassium channels in rat experimental acute cerebral ischemia. *Biochem Biophys Res Commun* 327:1163–1169.
- Longa EZ, Weinstein PR, Carlson S, Cummins R (1989) Reversible middle cerebral artery occlusion without craniectomy in rats. *Stroke* 20:84–91.
- Malgouris C, Bardot F, Daniel M, Pellis F, Rataud J, Uzan A, Blanchard J-C, Laduron PM (1989) Riluzole, a novel antilutamate prevents memory loss and hippocampal neuronal damage in ischemic gerbils. *J Neurosci* 9:3720–3727.
- Martin D, Thompson MA, Nadler JV (1993) The neuroprotective agent riluzole inhibits release of glutamate and aspartate from slices of hippocampal area. *Eur J Pharmacol* 250:473–476.
- Matsushita K, Matsuyama T, Kitagawa K, Matsumoto M, Yanagihara T, Sugita M (1998) Alterations of Bcl-2 family proteins precede cytoskeletal proteolysis in the penumbra, but not in infarct centres following focal cerebral ischemia in mice. *Neuroscience* 83: 439–448.
- Patel AJ, Honore E (2001) Properties and modulation of mammalian 2P domain K⁺ channels. *Trends Neurosci* 24:339–346.
- Perez-Pinzon MA, Xu GP, Mumford PL, Dietrich WD, Rosenthal M, Sick TJ (1997) Rapid ischemic preconditioning protects rats from cerebral anoxia/ischemia. *Adv Exp Med Biol* 428:155–161.
- Pratt J, Rataud J, Bardot F, Roux M, Blanchard JC, Laduron PM, Stutzmann JM (1992) Neuroprotective actions of Riluzole in rodent models of global and focal cerebral ischemia. *Neurosci Lett* 140:225–230.
- Sharp FR, Lu A, Tang Y, Millhorn DE (2000) Multiple molecular penumbras after focal cerebral ischemia. *J Cereb Blood Flow Metab* 20:1011–1032.
- Tsujimoto Y, Shimizu S (2000) Bcl-2 family: life-or-death switch. *FEBS Lett* 466:6–10.
- Unal-Cevik I, Kilinc M, Can A, Gursay-Ozdemir Y, Dalkara T (2004) Apoptotic and necrotic death mechanisms are concomitantly activated in the same cell after cerebral ischemia. *Stroke* 35:2189–2194. Epub 2004 Jul 2115.
- Voskuyl RA, Vreugdenhil M, Kang JX, Leaf A (1998) Anticonvulsant effect of polyunsaturated fatty acids in rats, using the cortical stimulation model. *Eur J Pharm* 341:145–152.
- White BC, Sullivan JM, DeGracia DJ, O'Neil BJ, Neumar RW, Grossman LI, Rafols JA, Krause GS (2000) Brain ischemia and reperfusion: molecular mechanisms of neuronal injury. *J Neurol Sci* 179:1–33.