Neuroscience 158 (2009) 1390–1396

WHOLE-BODY PERIODIC ACCELERATION REDUCES BRAIN DAMAGE IN A FOCAL ISCHEMIA MODEL

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Abstract—Stroke is the second most common cause of death and major cause of disability worldwide. Actual treatment involves surgery and/or thrombolytic drugs, but there is an urgent need for new approaches. Periodic acceleration, a rocking headward to footward movement of the whole body, is a non-invasive method to induce pulsatile shear stress on the vascular endothelium eliciting an enhanced production and secretion of endothelium-derived products such as nitric oxide, prostacyclin, prostaglandin E2, tissue plasminogen activator (tPA), and adrenomedullin. All these products have been shown to protect the brain from ischemic injuries. A rat model of focal brain ischemia was treated with application of periodic acceleration for 3 h immediately after the onset of ischemia. Controls remained static for the same period of time. Brain damage was assessed by magnetic resonance imaging (MRI) and biochemical markers. A significant reduction in brain damage was observed, 7 days post-ischemia, in rocked rats when compared with the static controls, through MRI. Furthermore, rocked animals had significantly lower levels of Beclin 1 and fractin than their static counterparts, and some isoforms of nitric oxide synthase were regulated by periodic acceleration. Our results show that periodic acceleration may provide a novel, affordable, non-invasive therapeutic option for the treatment of stroke.

Key words: stroke, endothelium-derived products, apoptosis, brain damage, MRI.

Stroke is a major cause of death and long-term disability in industrialized countries. As an example, stroke accounted for more than 1 in every 15 deaths in the United States in 2003 (http://www.americanheart.org). More than 85% of all strokes are caused by cerebral ischemia (Zia et al., 2007), resulting in devastating neurological sequelae (Hermann et al., 2008) accompanied by severe morphological and molecular alterations (Oechmichen and Meissner, 2006). Treatment for stroke usually involves either decompressive surgery, cerebral angioplasty (Gupta and Jovin, 2007), or application of clot-dissolving drugs such as aspirin, tissue plasminogen activator (tPA) and its derivatives (Simpson et al., 2006), but there is an urgent need for new and complementary approaches since the number of cases is reaching epidemic proportions (Donnan et al., 2008).

Periodic acceleration (pGz) is a non-invasive method to induce pulsatile shear stress on the vascular endothelium by producing headward to footward movements of the body at frequencies of around 180 cycles per minute (cpm) and acceleration forces of approximately 0.4 Gz (Adams et al., 2001, 2003; Wu et al., 2006). pGz enhances production and secretion of endothelium-derived products such as nitric oxide (NO), prostacyclin, prostaglandin E2 (PGE2), tPA, and adrenomedullin (Adams et al., 2005; Martinez et al., 2008). All these substances have been shown to protect the brain from ischemic injuries, reducing the volume of the region that gets damaged following hypoxia and reperfusion (Miyashita et al., 2006; Liang et al., 2007; Martinez-Gonzalez and Badimon, 2007; Sallustio et al., 2007). On the other hand, potentially damaging molecules such as interleukin-4, interleukin-6, cortisol, or catecholamines do not get affected by pGz (Adams et al., 2005; Martinez et al., 2008). These molecules have been shown to worsen ischemia-induced brain damage (Cook et al., 2004; Anne et al., 2007; Rupp, 2007; Shlipak et al., 2008).

Therefore we hypothesized that pGz could constitute an alternative and/or adjuvant therapy for stroke. To investigate whether this was true, we used a model of focal brain ischemia in the basal ganglia that has shown great reproducibility in our hands (Martinez-Murillo et al., 2007), and brain injury was measured by a panel of methods. These included imaging of the infarct area by magnetic resonance imaging (MRI) and several biochemical markers. Among all possible biochemical markers of brain injury we have chosen two molecules that provide complementary information on the mechanism of cell damage produced by ischemia; Beclin 1 is a protein involved in the process of autophagy (Rami et al., 2008; Rami and Kogel, 2008), whereas fractin is a product of the apoptotic cascade (Haynes et al., 2008; Leonardo et al., 2008). Thus between MRI and these markers we cover three aspects of brain damage: vascular edema, autophagy, and apoptosis. In addition, we also studied the regulation of the nitrergic system since it is critical to understand the physiopathology of ischemia (Ginsberg, 2008). Our study shows that animals that had been subjected to pGz presented a lower infarct volume in their brains and had a lower expression of markers of brain damage than the static controls. In addition, the brain-damaging isoforms of nitric oxide synthase (NOS) were downregulated in pGz-treated rats.

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Abbreviations: cpm, cycles per minute; MRI, magnetic resonance imaging; NO, nitric oxide; NOS, nitric oxide synthase; pGz, periodic acceleration; tPA, tissue plasminogen activator.

0306-4522/09
doi:10.1016/j.neuroscience.2008.12.005

EXPERIMENTAL PROCEDURES

Animals

Male Wistar rats ($n=28$) provided by Harlan (Barcelona, Spain) weighing 250–350 g were housed under controlled light, temperature, and relative humidity. The animals had free access to food and water. All procedures were carried out in accordance with the European Communities Council Directive (86/609/EEC) on animal experiments, under a protocol approved by the Animal Welfare Committee of the Instituto Cajal. Special care was taken to minimize pain and discomfort in the experimental animals and to minimize their number.

Ischemia model and pGz treatment

Given our previous experience that has shown the high reproducibility of a focal brain ischemia model using endothelin-1, a potent vasoconstrictor, in the basal ganglia (Martinez-Murillo et al., 2007), unilateral ischemic lesions of the striatum were induced in the experimental animals. Briefly, rats were anesthetized with 2-chloro-2-(difluoromethoxy)-1,1,1-trifluoro-ethane (isoflurane, Baxter S.L., Valencia, Spain) with a vaporizer from MSS International Ltd. (Keighley, UK) and placed in a stereotaxic instrument (David Kopf Instruments, Tujunga, CA, USA). A small hole was drilled on the right side of the skull at 0.2 mm anterior to bregma and −3.0 mm lateral to mid-line. Dura was removed and 2 μl of endothelin-1 (0.2 μg/μl, Calbiochem, La Jolla, CA, USA) were injected at 5.6 mm deep at a rate of 1 μl/3 min. The scalp was sutured and the animals were immediately subjected to one of the following treatments: i) pGz on an orbital shaker (SBS Instruments, Rubí, Spain) at 180 cpm for 3 h under anesthesia (pGz group); or ii) remain under anesthesia for 3 h in a static position (static group).

Magnetic resonance

Infarct volume was analyzed by MRI in 10 rats 2 and 7 days after surgery, as previously described (Martinez-Murillo et al., 2007). Images were acquired in a Bruker Biospec 47/40 spectrometer, with a main field of 4.7 T and a 40 cm bore. This magnet is equipped with an unshielded gradient coil with a maximum strength of 372 mT/m. The RF coil, acting as a transmitter and receiver, was a 30-mm-diameter surface coil placed just on top of the head of the rat. Initially, localization fast spin echo images were acquired both in the coronal and in the sagittal directions, and coronal slices were selected for imaging. Diffusion-weighted images were acquired with a 30×30 mm field of view, a slice thickness of 1 mm, and a 256×64 acquisition matrix, using a slice-selective spin echo sequence, with one slice, two averages, and five different values of the diffusion gradient, TE=50 ms, TR=200 ms, T=20.56 ms, and δ=10 ms. Spatial resolution was therefore 1000 (slice thickness)×469×117 μm. Extent of vasogenic edema or infarction was calculated from T2WI 3D MR images using the ParaVision 3.0.1 (Bruker, Ettlingen, Germany) software. In T2WI MR images, vasogenic edema appeared hyperintense. Addition of all the hyperintensive areas in all serial slices yielded an estimation of the lesion volume. Results are expressed as mean±S.E.M. Data were analyzed by regression analysis based on the mixed models procedure of the Statistical Analysis System (Proc mixed; SAS for Personal Computers, version 8, SAS Institute, Cary, NC, USA). The main effects of the model were treatment and time with the treatment-by-time interaction further tested. Animal within treatment was used as the error term. P values lower than 0.05 were considered statistically significant.

Molecular biology

Other animals ($n=12$) were used to obtain RNA at 24 h and 5 days after endothelin-1 injection. These time points were chosen to investigate gene expression at an early (24 h) and a late (5 days) phase after ischemic onset, but always before statistically significant differences were observed by MRI at day 7. Following deep anesthesia, the striatum was rapidly dissected out and frozen in liquid N_2, taking care to separate the right side (ischemic) from the left side (non-ischemic). The RNA was extracted with Trizol (Invitrogen, Carlsbad, CA, USA) and reverse transcribed using SuperScript reverse transcriptase (Invitrogen). Real time PCR was performed using the Chromo4 (MJ Research, Hercules, CA, USA) thermocycler and software. Amplification was done in a final volume of 25 μl, containing 2 μl cDNA (diluted 1:10), 2 μl of primer mixture (at 10 nM), and 12.5 μl of 2× SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA). Cycling parameters were 45 cycles of 94 °C (denaturation), 60 °C (annealing), and 72 °C (extension). Values were determined by interpolation within a standard curve. At the end of the PCR, a melting curve was generated to ascertain amplicon quality. All gene expression values were normalized according to the β-tubulin concentration of each sample. Primers are shown in Table 1.

Western blotting

Fresh striatum tissues from rocked and static rats ($n=3$ for each group) were collected 5 days after ischemic onset. This time point was chosen because protein modulation usually takes longer time than RNA changes and because is still previous to the statistically significant changes observed by MRI at day 7. These tissues were homogenized in lysis buffer [50 mM Tris–HCl pH 7.5, 150 mM NaCl, 1% Triton X-100, 1% deoxycholate, 0.1% (w/v) NaN_3, 1 mM EGTA, 0.4 mM EDTA, 1 mM PMSF, 0.2 mM Na_3VO_4, and protease inhibitor cocktail] and sonicated. Lysates were clarified by centrifugation at 15,000×g for 10 min and protein concentration was estimated in the supernatant using a ND-1000 spectrophotometer (Nanodrop, Wilmington, DE, USA). Equal amounts of total protein (50 μg) were loaded into 4–12% NuPage Bis-Tris gels (Invitrogen), electrophoresed in MES buffer, and transferred to nitrocellulose membranes (Invitrogen). An antibody against fractin

Table 1. Primers used for real time RT-PCR

Target	GenBank accession number	Primer sequences	Annealing temp.
nNOS	NM_052799	5′-GAC-AAC-GTT-CCT-GTG-GTC-CT-3′ 5′-TCC-AGT-GTG-CTC-TTC-AGG-TG-3′	60 °C
eNOS	NM_021838	5′-AAG-TGG-GCA-GCA-TCA-CCT-AC-3′ 5′-GCT-TGA-CCC-AAT-AGC-TGC-TC-3′	60 °C
iNOS	NM_012611	5′-CAC-CTT-GGA-GTT-CAC-CCA-GT-3′ 5′-ACC-ACT-CGT-ACT-TGG-GAT-GC-3′	60 °C
Beclin1	NM_053739	5′-CGC-CTC-CTA-TTC-CAT-CAA-AA-3′ 5′-AAC-TGT-GAG-GAC-ACC-CAA-GC-3′	60 °C
β-Tubulin	BC133064	5′-GTC-CTG-GAC-GTG-GTA-AGG-AA-3′ 5′-TGC-GGT-CTG-GGT-ACT-CTT-CT-3′	60 °C