Experimental Neurology 230 (2011) 58–66

Contents lists available at ScienceDirect

Experimental Neurology

journal homepage: www.elsevier.com/locate/yexnr

Evaluation of cyclosporine A in a stroke model in the immature rat brain

Pierre-Louis Leger [a,b,c], Damien De Paulis [d], Sonia Branco [a,b], Philippe Bonnin [e], Elisabeth Couture-Lepetit [d], Olivier Baud [b], Sylvain Renolleau [a,b,c], Michel Ovize [d,f], Abdallah Gharib [d], Christiane Charriaut-Marlangue [a,b,*]

[a] UMR-CNRS 7102, Université P&M Curie-Paris6, 75005 Paris, France
[b] INSERM U676, Equipe AVENIR R05230HS, Hôpital R. Debré, 75019 Paris, France
[c] AP-HP, Service de Réanimation, Hôpital Armand Trousseau, UPMC-Paris6, 75012 Paris, France
[d] INSERM U886, Laboratoire de Physiologie Lyon-Nord, Université C. Bernard Lyon 1, France
[e] AP-HP, Hôpital Lariboisière, Physiologie-Explorations Fonctionnelles, Université-Denis, Diderot-Paris7; INSERM U965; 75010 Paris, France
[f] Hospices Civils de Lyon, Hôpital Louis Pradel, Service de cardiologie, F-69677, Bron, France

ARTICLE INFO

Article history:
Received 29 October 2009
Revised 27 May 2010
Accepted 8 June 2010
Available online 16 June 2010

Keywords:
Neonatal brain injury
Permeability transition pore
Mitochondria
Rat pups
Reperfusion
Brain damage
Calpain
Alpha-spectrin

ABSTRACT

The effects of ischemia–reperfusion on opening of the mitochondrial permeability transition pore (mPTP) and its blockade in the immature brain are not fully understood. Presently, we evaluated the effect of cyclosporine A (CsA) on cell death and mPTP opening in a model of transient focal ischemia induced by permanent left middle cerebral artery, and homolateral transient common carotid artery occlusion (50 min) in P7 rats. CsA (10 mg/kg) was administered 14 h before induction of ischemia and effects were analyzed at 30–40 min and 48 h after reperfusion. CsA administration reduced infarct size, DNA fragmentation and apoptotic bodies, and inflammatory responses in mild but not severe injury. CsA increased the Ca^{2+} load required to open the mPTP (78.4 ± 19.2 vs. 50.2 ± 19.9 nmol.mg^{-1} protein, $p<0.05$) in limiting the decoupling of the respiratory chain by unchanged state 3 but reduced state 4, and attenuated early calpain-mediated alpha-spectrin proteolysis. In conclusion, CsA mediates inhibition of mPTP opening and has a tendency to protect immature rat brain against mild ischemic injury. This article is part of a Special Issue entitled "Interaction between repair, disease, & inflammation."

Introduction

Perinatal hypoxia–ischemia is an important cause of neonatal brain injury and results in cerebral palsy, learning disabilities, visual field deficits, and epilepsy (Ferriero, 2004). However, recent data suggest a higher incidence of focal ischemia in neonates compared to the incidence of global cerebral ischemia arising from systemic asphyxia (Golomb et al., 2008; Lynch et al., 2002), while mechanisms of arterial ischemic injury without the confounding effect of hypoxia are not fully understood.

Mitochondria play essential roles in energy metabolism, generation of reactive oxygen species, and regulation of apoptosis in response to neuronal brain injury (Achanta et al., 2005). Mitochondrial biogenesis is a highly regulated process and occurs on a regular basis in healthy cells, where it is controlled by the nuclear genome. Damage to the bioenergetic integrity of mitochondria plays a critical role in adult ischemia (Murphy et al., 1999), and the formation and opening of the mitochondrial permeability transition pore (mPTP) is one of the core mediators of this process (Ankarcrona et al., 1995; Bernardi, 1996; Crompton, 1999). Although calcium (Ca^{2+}) accumulation plays a pivotal role in normal neuronal function, excessive mitochondrial Ca^{2+} accumulation causes a severe reduction in mitochondrial membrane potential, triggers the formation and opening of the mPTP and membrane permeability leading to the release of apoptogenic proteins like cytochrome *c*, apoptosis-inducing factor (AIF), endonuclease G, Smac/Diablo from the mitochondrial intermembrane space (Halestrap, 2006; Halestrap, 2009), mitochondrial oxidative damage, and eventual cell death. Pharmacological inhibition of the pore opening by cyclosporine A (CsA), a potent inhibitor of mPTP formation, provides the strongest evidence that mPTP occurs during reperfusion after adult cerebral ischemia. However, for several years studies indicate that the pattern of cell death in the immature brain differs from that seen in the adult central nervous system (Vannucci and Hagberg, 2004). The contribution of mPTP in the immature brain was evidenced by entrapment of deoxy-glucose (Puka-Sundvall et al., 2001) and loss of glutathione (Wallin et al., 2000). However, CsA treatment did not provide neuroprotection after hypoxia–ischemia in 7-day-old rats (P7), whatever the dose as well as the route of administration used (Puka-Sundvall et al., 2001).

* Corresponding author. INSERM U676, Equipe AVENIR R05230HS, Hopital Robert Debré. Bâtiment Ecran - 3ème étage, 48 Bd Sérurier, 75019 Paris, France. Fax: +33 1 40 03 19 95.

0014-4886/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.expneurol.2010.06.009

To address the role of the mPTP in our model of ischemia–reperfusion (elicited by middle cerebral artery occlusion and transient common carotid occlusion) in P7 rats, we evaluate the effect of CsA at mid-term (48 h after ischemia) on lesion volume, cell death and inflammation, and at very short term (30–40 min after ischemia) on Ca^{2+}-induced mPTP opening and Ca^{2+}-dependent calpain-mediated alpha-spectrin degradation. We demonstrated that mPTP opening occurred in our stroke model and CsA was able to reduce cell injury only in the case of mild compared to severe lesion.

Materials and methods

Perinatal ischemia

All animal experimentation was conducted in accordance with the French and European Community guidelines for the care and use of experimental animals. Ischemia was performed on Wistar 7 day-old rat pups (17–21 g; Janvier, Le Genest St-Isle, France) of both sexes, as previously described (Renolleau et al., 1998). Rat pups were anesthetized with an intraperitoneally (i.p.) injection of chloral hydrate (350 mg/kg). The anaesthetized rat was positioned on its back and a median incision was made in the neck to expose the left common carotid artery (CCA). The rat was then placed on its right side and an oblique skin incision was made between the ear and the eye. After excision of the temporal muscle, the cranial bone was removed from the frontal suture to a level below the zygomatic arch. Then the left middle cerebral artery (MCA), exposed just after its appearance over the rhinal fissure, was electrocoagulated (MCAo) at the inferior level of the cerebral vein. After this procedure, a vascular clip (18055-04, Fine Science Tools, Heidelberg, Germany) was placed to occlude the left common carotid artery (tCCAo). Rats were then placed in an incubator to avoid hypothermia. After 50 min, the clip was removed. Carotid blood flow restoration was verified with the aid of a microscope. Both neck and cranial skin incisions were then closed. During the surgical procedure, external body temperature was maintained at 36–36.5 °C. After recovery, pups were transferred to their mothers.

Therapeutic protocol

Animals ($n=123$, see supplemental Table 1) were randomly assigned to one of the ten following groups. Control ischemic (receiving saline buffer) animals were distributed in 4 groups (G1, G3, G5 and G9). Cyclosporine (CsA, Sandimmum, Novartis®, Basel, Switzerland) dissolved in saline was administered either intraperitoneally (i.p., at a dose of 20 mg/kg in 200 µl) at the reperfusion (group G2) or intravenously (i.v., at a dose of 10 mg/kg in 100 µl under 1% isoflurane anesthesia) 1-h (group G4) or 14 h (groups G6 and G10) before the ischemic onset. Sham animals received an equivalent volume of 0.9% saline or CsA (groups G7 and G8). Mortality rate did not differ between CsA-treated and vehicle-treated groups in any of the experiments (≤10%).

Arterial blood flow monitoring using ultrasound imaging

Rats subjected to ischemia ($n=11$) were analyzed using ultrasound measurements via an echocardiograph (Vivid 7, GE Medical Systems ultrasound®, Horten, Norway) equipped with a 12-MHz linear transducer (12 L) as previously reported (Bonnin et al., 2008; Villapol et al., 2009). Doppler spectral recordings in the right and left internal carotid arteries and basilar trunk (Hilger et al., 2002), were evaluated (1) before surgery (basal level), (2) during ischemia, and (3) 15 min after reperfusion. Data were then transferred to an ultrasound image workstation for subsequent analysis (PC EchoPAC, GE Medical Systems ultrasound®). The repeatability coefficient values for intra-observer repeatability were 1.5 cm s^{-1} for the peak systolic, 1.7 cm s^{-1} for the end-diastolic, and 1.7 cm s^{-1} for the mean BFV in ICA. Blood flow values were expressed as percentage of basal level for each artery in each animal.

Measurement of infarct volume

P7 Rat were euthanized at 48 h (vehicle- and CsA-treated) and the extent of the pale ischemic lesion on the cortical surface was visually graded from 1 to 3, where 1, 2 and 3 indicated small, medium and large infarctus, respectively (Joly et al., 2004). Brains were then fixed for 2 days in 4 % buffered paraformaldehyde and cryoprotected in 20 % sucrose for 3 days. Coronal brain sections (50 µm) were cut on a cryostat and collected on gelatin-coated slides. Sixteen sections from anterior striatum to posterior hippocampus (corresponding to plates 9 to 27 in Paxinos' rat brain atlas) were selected, taken at equally spaced 0.5-mm intervals. Infarct size was determined on cresyl violet-stained sections using an image analyzer (Image-Pro, Paris, France). Infarct volumes were expressed as the percentage of the ipsilateral hemisphere.

Assessment of cell damage

Coronal sections (20 µm, $n=6$ for each condition) were prepared as described above and processed for DNA strand breaks (TUNEL assay) using the *in situ* Cell Death Detection Kit (Fluorescein, Roche®, Meylan, France) according to the manufacturer's instructions. TUNEL-positive nuclei were scored under fluorescence microscopy. Apoptosis was determined by nuclear condensation and/or fragmentation into apoptotic bodies, whereas flocculent chromatin signed necrosis.

Immunohistochemistry and immunofluorescence

Coronal sections ($n=6$ for each condition) were first incubated for 30 min with 5 % normal horse serum in PBS with 0.5 % triton X-100 (PBS-TX-NHS), then overnight at 4 °C with appropriately diluted primary antibody in PBS-TX-NHS. The primary antibodies against myeloperoxidase (N5787, Sigma, Sigma-Aldrich, France; 1:200), to stain neutrophils, were visualized after incubation with the anti-rabbit biotinylated secondary antibody (Vectastain Lab., AbCys, Paris, France, 1:200) followed by the streptavidin–biotin–peroxidase complex (Elite ABC kit, Vectastain Lab.). Nonspecific peroxidase activity was abrogated by incubating the sections in 1% hydrogen peroxide in PBS 0.1 M at the appropriate stage. Antibodies against tomatolectin (Vector, Burlingame, CA) to stain resident, activated macrophage/microglia were revealed using streptavidin, anti-mouse IgG antibody coupled to the red fluoresent marker Cy3 (Jackson Immunores laboratories, Interchim, Asnieres, France). All these sections were used for cell counting.

Mast cell (MC) quantification

Sections (12 per brain) were stained with 0.01 % acidic (pH = 3.8) toluidine blue as previously described (Biran et al., 2008). Quantitative data were obtained by counting MCs both in the pia mater (3 sections from Bregma 4.5 to 3.0 mm, every 500 µm) and choroid fissure (3 sections from Bregma −1.80 to −3.30 mm, every 500 µm).

Preparation of isolated mitochondria

Vehicle- and CsA-treated P7 rats (groups G9 and G10, see supplemental Table 1) were killed by decapitation at 30–40 min after reperfusion ($n=2$ animals for each preparation of isolated mitochondria with a total of 6 to 16 animals). The brains were then rapidly dissected out on a cold plate. Cortical tissues (corresponding to the middle cerebral artery territory) were harvested from both lesioned and sham animals. Mitochondria were isolated in a 50 mM Tris (pH 7.4) buffer containing 70 mM sucrose, 210 mM mannitol,