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Dual action of NO synthases on blood flow and infarct volume consecutive to neonatal focal cerebral ischemia ☆

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ABSTRACT

Research into neonatal ischemic brain damage is impeded by the lack of a complete understanding of the initial hemodynamic mechanisms resulting in a lesion, particularly that of NO-mediated vascular mechanisms. In a neonatal stroke rat model, we recently show that collateral recruitment contributes to infarct size variability.

Non-specific and selective NO synthase (NOS) inhibition was evaluated on cerebral blood-flow changes and outcome in a P7 rat model of arterial occlusion (left middle cerebral artery electrocoagulation with 50 min occlusion of both common carotid arteries). Blood-flow changes were measured by using ultrasound imaging with sequential Doppler recordings in both internal carotid arteries and basilar trunk. Cortical perfusion was measured by using laser Doppler flowmetry. We showed that global NOS inhibition significantly reduced collateral support and cortical perfusion (collateral failure), and worsened the ischemic injury in both gender. Conversely, endothelial NOS inhibition increased blood-flows and aggravated volume lesion in males, whereas in females blood-flows did not change and infarct lesion was significantly reduced. These changes were associated with decreased phosphorylation of neuronal NOS at Ser^{847} in males and increased phosphorylation in females at 24 h, respectively. Neuronal NOS inhibition also increased blood-flows in males but not in females, and did not significantly change infarct volumes compared to their respective PBS-treated controls.

In conclusion, both nNOS and eNOS appear to play a key role in modulating arterial blood flow during ischemia mainly in male pups with subsequent modifications in infarct lesion.

Introduction

Neonatal hypoxia–ischemia is a common cause of neonatal brain injury and results in cerebral palsy, learning disabilities and epilepsy (Ferriero, 2004). In addition to global cerebral ischemia arising from systemic asphyxia, recent data suggest a higher incidence of focal ischemia–reperfusion leading to stroke in near-term neonates (Golomb, et al., 2008).

We recently demonstrated that the establishment or not of collateral recruitment, as revealed by changes in blood velocities in the basilar trunk, determined the extension of the lesion in immature animals (Bonnin, et al., 2011). Among vasoactive molecules able to modulate collateral recruitment, nitric oxide (NO) is of special interest. NO is a small, highly diffusible and reactive molecule produced by the NO synthases (NOS) and released from endothelial cells (Moncada, et al., 1991) and perivascular nitrergic neurons (Toda and Okamura, 2003). It contributes to vasodilation, increased local blood flow, and decreased vascular resistance in cerebral circulation (Pinard, et al., 2000; Toda, et al., 2009). Studies of NOS inhibitors in ischemic models in adult rat have given contradictory results for effects on lesion size and cerebral blood flow (CBF), with many demonstrating beneficial effects, whereas others report contradictory findings (Willmot, et al., 2005). Information on NO and NOS in perinatal hypoxia and/or ischemia is limited and, in particular, no direct evidence for its generation according to CBF changes during injury has been provided.

We here investigated the regulation of arterial recruitment by NO during ischemia in the P7 rat, and explored the role of selective NOS inhibitors on infarct volume.

☆ The authors declare no conflict of interest.

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Methods

Neonatal ischemia–reperfusion

All experiments complied with ethical guidelines of Robert Debré Hospital Research Council Review Board (A75-19-01), INSERM and the ARRIVE guidelines (http://www.nc3rs.org/ARRIVE). Ischemia was induced in Wistar P7 rat pups (17–21 g; Janvier, Le Genest St-Isle, France; both sexes) (Bonnin, et al., 2011). Briefly, thermoregulated (37.0 ± 0.5 °C) and anesthetized pups [1% isoflurane in O_2/N_2O (1:3)] were exposed to left middle cerebral artery electrocoagulation (MCAo) combined with a transient (50 min) and concomitant occlusion of both common carotid arteries (CCA). Rat pups were sacrificed at 48 h. Two investigators, who were blind to the treatment group, determined the size of the lesion in each animal (n = 62).

Drug treatment

Animals were randomly assigned to NOS inhibitors or vehicle groups. NG-nitro-L-arginine methyl ester (L-NAME, Calbiochem, Merck Biochemicals, 20 mg/kg), a non-specific NOS inhibitor, (4S)-N(A-amino-5[aminoethyl]aminopentyl)-N'-nitroguanidine TFA (Calbiochem, 4 mg/kg) a nNOS inhibitor displaying a >2500-fold selectivity over eNOS, and N^5-(1-Iminoethyl)-L-ornithine Dihydrochloride (L-NIO, Calbiochem, 10 mg/kg) a selective eNOS antagonist (Chaitoff, et al., 2008; Yemisci, et al., 2009) were injected intraperitoneally (i.p.) one hour before ischemia. The doses (according to manufacturer's instructions) were chosen to ensure that the inhibitory effect of the inhibitors on vascular- and/or neuron-derived NO synthesis did not induce a high mortality rate after ischemia. Vehicle group received PBS i.p.

Ultrasound imaging

Thermoregulated rats were subjected to ultrasound measurements under 0.5% isoflurane anesthesia using an echocardiograph (Vivid 7, GE Medical Systems ultrasound®, Horten, Norway) equipped with a 12-MHz linear transducer (12 L) (Bonnin, et al., 2011). Time-average mean blood-flow velocities (mBFVs) were measured in both intra cranial internal carotid arteries (ICAs) and basilar trunk (BT) before surgery, during ischemia (at 40 min) and at 15 min after removal of the CCA occlusion (supplemental Fig. 1). Heart rates were measured and reflected changes in cardiac output, as ventricular volume is quite invariable in newborns.

Cortical regional CBF (rCBF) monitoring

In each animal (thermoregulated and anesthetized) the calvarium was exposed by incision, and left cortical rCBF measurements were made by laser Doppler flowmetry (Moor Instruments Ltd, Axminster, UK). Relative changes in rCBF were recorded in 3 regions of interest (supplemental Fig. 2) over a period of 5 min in basal, after MCAo, at the end of ischemia, and at 30 min after reperfusion and averaged; rCBF measurements were normalized to baseline in each animal.

Immunoblotting and densitometry

Animals (female and male, n = 20) were sacrificed 2 and 24 h after injury and the brains were rapidly dissected out on a cold plate. Cytosolic proteins were extracted as previously described (Villapol, et al., 2009). Equal amounts of protein (40 μg) were resolved by SDS-PAGE electrophoresis and immunoblotted using antibodies to phospho-Ser^{847} nNOS (ab16650, AbCam, Paris, France), nNOS (sc-648, Santa Cruz, Heidelberg, Germany) and β-actin (clone AC-15, Sigma Aldrich). Blots were scanned and analyzed using ImageJ (NIH, Bethesda, MD, USA).

Immunohistochemistry

Sections from L-NIO treated males and females subjected to ischemia and sacrificed at 48 h were processed as previously described (Villapol, et al., 2009) and incubated with a primary antibody against nitrotyrosine (NT, AB5411, 1:100, Millipore, St-Quentin-en-Yvelines, France). Sections were then incubated with a biotinylated anti-rabbit secondary antibody and immunolabeling was visualized using the streptavidin–biotin–peroxidase method. NT-positive cells were counted (in a blind manner) in three to four coronal sections using a ×20 objective.

Statistical analysis

All results are expressed as mean ± SD. One- or two-way ANOVA and post hoc Bonferroni test or/and paired or unpaired Student's t-tests were used to analyze mBFV differences between groups. The nonparametric Mann–Whitney *U* test was used to compare infarct volumes between two groups. Linear regression analysis was done using MedCalc Software (Mariakerke, Belgium).

Results

During ischemia, whereas both common carotid arteries were occluded, only the basilar trunk (BT) supplied the circle of Willis through posterior communicating arteries towards cerebral arteries (supplemental Fig. 1). Collateral recruitment was evidenced by an association of increased blood flows in the BT with subsequent small infarct volumes, and collateral failure with larger infarct volumes (Bonnin, et al., 2011).

Inhibition of nitric oxide (NO) reduces collateral recruitment during ischemia

Administration of the non-specific NOS inhibitor L-NAME, at the dose of 20 mg/kg, induced a significant reduction in mean blood-flow velocities (mBFVs) in both the internal carotid arteries (ICAs) and in the BT ($p<0.01$, Figs. 1A and B–D), without any change in heart rates (352 ± 42 vs 377 ± 28 bpm, PBS vs L-NAME). In addition, cortical regional CBF (rCBF) was reduced by 21 ± 4% in L-NAME-treated animals (n = 8) compared to PBS-treated animals (n = 8, $p<0.05$). During ischemia, mBFVs in the BT remained reduced in L-NAME-treated animals (5.0 ± 1.3 $cm.s^{-1}$) whereas they increased in PBS-treated animals to 8.1 ± 2.3 $cm.s^{-1}$ ($p<0.001$, Fig. 1D). Cortical rCBF during ischemia was decreased in L-NAME- to 20.5 ± 3.1% vs 29.1 ± 5.9% in PBS-treated rats ($p<0.05$) with no difference between males and females in both groups. At 15 min after reperfusion, mBFVs in the BT remained significantly reduced in L-NAME-treated animals while they returned to basal values in PBS-treated animals (Fig. 1D). In both ICAs, mBFVs were still reduced (no difference between PBS- and L-NAME-treated animals) and recovered 24 h after, as previously reported (Villapol et al., 2009). Cortical rCBF at 30 min of the reperfusion returned to the level obtained after MCAo (before CCA occlusion) in PBS- (46.5 ± 8.4%) but not in L-NAME-treated animals (34.3 ± 8.7%, $p<0.05$), with no difference between males and females. Mean infarct volume at 48 h was significantly increased in the L-NAME-treated animals (Fig. 2A) compared to PBS-treated animals (23.7 ± 7.1%. vs 13.1 ± 6.7%, $p<0.01$). L-NAME induced similar significant increase in lesion size both in males and females (Fig. 2B). A strong negative linear regression between mBFVs in BT and lesion volume was found in PBS- (slope: −0.161 ± 0.033, r = 0.88, $p<0.001$, Fig. 1E). L-NAME-treated animals exhibited either unchanged or reduced mBFVs meaning collateral failure and large infarct volumes.