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nNOS and p-ERK involvement in the neuroprotection exerted by remote postconditioning in rats subjected to transient middle cerebral artery occlusion

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ABSTRACT

It has recently been hypothesized that a sub-lethal ischemic insult induced in one organ is able to protect from a harmful ischemia occurring in a different organ. The objective of this study is to identify new putative mechanisms of neuroprotection elicited by remote ischemic femoral postconditioning. A 50% reduction in the infarct volume was observed when 100 min of middle cerebral artery occlusion was followed, 10 min later, by the remote postconditioning stimulus represented by 20 min of femoral artery occlusion. The use of in vivo silencing strategy allowed to demonstrate that NO production through nNOS mediates part of the neuroprotection. Indeed, whereas CNS nNOS expression was up-regulated by remote postconditioning, the pharmacological inhibition of nNOS or its silencing-mediated knocking-down partially prevented this neuroprotective effect. This nNOS overexpression seemed to be p-ERK dependent. In fact, p-ERK expression increased in brain cortex after remote postconditioning, and its pharmacological inhibition prevented both nNOS overexpression and remote postconditioning-mediated neuroprotection. Interestingly, neuroprotection induced by remote postconditioning was partially prevented when ganglion transmission was pharmacologically interrupted by hexamethonium, thus showing that neural factors are involved in this phenomenon.
Collectively, the present study demonstrates that p-ERK and nNOS take part to the complex cascade of events triggered by ischemic remote postconditioning.

Introduction

Two endogenous neuroprotective mechanisms have recently been characterized: ischemic preconditioning and ischemic postconditioning (Dirnagl and Meisel, 2008). These two neuroprotective strategies refer to natural adaptive responses used by the brain and other organs to protect themselves from a potentially lethal ischemic insult. However, ischemic preconditioning, a neuroprotective strategy induced by a subliminal ischemic episode applied before a longer harmful ischemia (Gidday, 2006), though scientifically fascinating is hardly practicable in clinical medicine owing to the unpredictable nature of ischemic events. Therefore, a post ischemic neuroprotective approach, named ischemic postconditioning, appears to be much more attractive as it can be more easily translatable into an innovative clinical therapeutic strategy. Indeed, recent studies in vivo have demonstrated that a sub-lethal cerebral ischemia, induced by a short period of tMCAO, following a prolonged harmful brain ischemic episode, reduces the volume of the ischemic lesion (Pignataro et al., 2008, 2009; Zhao, 2007, 2009; Zhao et al., 2006, 2012). Although this type of local postconditioning may ultimately prove to be successful, it does present some major drawbacks, for re-occlusion of a cerebral artery could lead to worse clinical outcomes, thus rendering this strategy not applicable and safe to treat cerebral ischemia in humans.

In the last years, several groups of investigators have directed considerable attention toward evidence indicating that a short term occlusion of a peripheral artery results in a marked protection of a distant organ undergoing an ischemic insult (Andreka et al., 2007; Ates et al., 2002; Gho et al., 1996; Kin et al., 2005; Konstantinov et al., 2004; Moses et al., 2005; Singh and Chopra, 2004; Takaoka et al., 1999).

Differently from remote preconditioning, it has been recently demonstrated that a short occlusion of a distant artery is able to protect the brain from a previous harmful ischemic insult, a phenomenon called remote postconditioning (Gao et al., 2008; Geng et al., 2012; Ren et al., 2008, 2009; Zhao et al., 2012).

It is possible to hypothesize that to achieve such a long-distance protection diffusible factors, such as nitric oxide, and rapid activated pro-survival factors, such as p-ERK and p-AKT, are recruited. Therefore, the objective of this paper is to characterize the remote femoral postconditioning neuroprotective effect on focal brain ischemia and to investigate the role of nitric oxide and prosurvival kinases such as p-ERK and p-AKT in mediating such a neuroprotective effect.

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Methods

Experimental groups

382 male Sprague–Dawley rats (Charles River) weighing 250 to 300 g were housed under diurnal lighting conditions (12 h darkness/light). Experiments were performed according to the international guidelines for animal research. The experimental protocol was approved by the Animal Care Committee of the "Federico II" University of Naples.

Chemicals

N(G)-nitro-L-arginine methyl ester (L-NAME; 10 mg/kg intraperitoneal; Sigma-Aldrich Ltd), aminoguanidine (AG; 400 mg/kg, intraperitoneal), N(5)-(1-Iminoethyl)-L-ornithine HCl (L-NIO; 2 mg/kg, intraperitoneal; Cayman Chemical), and hexamethonium (400 mg/kg, intraperitoneal), were dissolved in distilled water.

7-Nitroindazole (7-NI; 35 mg/kg, intraperitoneal) was dissolved in dimethyl sulfoxide (DMSO) solution. U0126 (10 μM, 5 uL, icv, Sigma-Aldrich Ltd) was dissolved in aCSF. All drug solutions were freshly prepared before use and administered at the indicated dosage 30 min after cerebral ischemia induction.

Small interference double-stranded RNA oligonucleotides (siRNA) against nNOS (GenBank accession number NM_001204214.1) were from Qiagen.

Transient middle cerebral artery occlusion model

Transient focal ischemia was induced by suture occlusion of the middle cerebral artery (MCA) in male rats anesthetized using 2% sevofluorane, 60% N_2O, and 38% O_2 (Pignataro et al., 2008). Under an operating stereomicroscope (Nikon SMZ800), the right carotid bifurcation was carefully exposed, and the external carotid artery (ECA) was coagulated distal to the bifurcation. A silicon-coated nylon filament (Doccol Co) was inserted through the ECA stump and gently advanced (19 mm) into the right internal carotid artery until it blocked the origin of the MCA. The surgical wound was closed and the filament was left in place. After 100-min MCA occlusion, the filament was gently withdrawn to restore blood flow. In order to expose the animals to the same concentration of gaseous anesthesia, all animals were kept under anesthesia for 3 h. Achievement of ischemia was confirmed by monitoring regional cerebral blood flow in the area of the right MCA. Cerebral blood flow was monitored through a disposable microtip fiber optic probe (diameter 0.5 mm) connected through a master probe to a laser Doppler computerized main unit (PF5001; Perimed) and analyzed using PSW Perisoft 2.5 (Kawano et al., 2006). Once a stable CBF signal was obtained, the MCA was occluded. CBF monitoring was continued up to 30 min after the end of the surgical procedure, when the occurred reperfusion was verified. To verify the effect of remote postconditioning on CBF, in a group of animals CBF was monitored from the onset of remote postconditioning procedure up to 3 h after No changes in CBF values were found after remote postconditioning (CBF after FAO 123 ± 11.8; CBF in sham-operated animals 118 ± 17.3; CBF 1 h after tMCAO 131 ± 8.0; CBF 1 h after tMCAO and FAO 129 ± 24.2). Animals that did not show a CBF reduction of at least 70% were excluded from the experimental group (34 out of 382, 8.9%), as were animals that died after ischemia induction (19 out of 348, 5.4%).

Intracerebroventricular (icv) administration

In rats positioned on a stereotaxic frame, a 23-g stainless steel guide cannula (Small Parts) was implanted into the right lateral ventricle using the stereotaxic coordinates of 0.4 mm caudal to bregma, 2 mm lateral and 2 mm below the dura[20]. The cannula was fixed to the skull using dental acrylic glue and small screws.

U0126 (5 μL, 10 μM) was icv administered at 30 min before tMCAO.

siRNA control or siRNA against nNOS (1 μL, 5 μM) were icv administered at 24, 18 and 6 h before tMCAO. In particular, we selected the best siRNA sequence able to block selectively nNOS expression out of three commercially available siRNA for nNOS. All siRNA were continuously infused (1 μL/h for 48 h) icv through an Alzet osmotic minipump implanted in a subcutaneous pouch and connected to the lateral ventricles through a plastic tube and a cannula stereotaxically implanted.

Limb remote postconditioning

Remote ischemic postconditioning (Rem PostC) was induced by subjecting ischemic animals to brief cycles of femoral artery occlusion (FAO). Briefly, femoral artery was identified, isolated and occluded with two microserrafine clips (FST) to stop the blood flow. The achievement of femoral artery blockade was verified by measuring blood flow.

To identify the experimental protocol able to induce neuroprotection, rats subjected to 100′ tMCAO were then exposed to several cycles and different time intervals of reperfusion and occlusion of the femoral artery (Figs. 1A, C, and 2A, C). Sham operated animals were subjected to the same surgical procedure except for the occlusion of femoral or middle cerebral artery that did not take place. To avoid possible confounding effects due the use of gaseous anesthetic, all animals included in the study were subjected to the same amount of anesthesia.

Immunohistochemical analysis revealed that in all experimental groups, FAO induced no damage to the region supplied by the femoral artery (data not shown).

In some of the animals subjected to tMCAO and FAO, a catheter was inserted into the femoral artery to measure arterial blood gasses with a blood gas analyzer before and after experimental procedures (Rapid Laboratory 860, Chiron Diagnostic). No differences between the examined experimental groups were detected in PaO_2, $PaCO_2$, and pH mean values (data not shown).

Evaluation of the infarct volume and of neurologic deficit scores

Animals were killed with isoflurane 24 h or 7 days after ischemia. Brains were quickly removed, sectioned coronally at 1 mm intervals, and stained by immersion in the vital dye (2%) 2,3,5-triphenyltetrazolium hydrochloride (TTC). The infarct volume was calculated by summing the infarction areas of all sections and by multiplying the total by slice thickness. To avoid that edema could affect the infarct volume value, it has been chosen to express the infarct volume as percentage of the infarct, calculated by dividing the infarct volume by the total ipsilateral hemispheric volume (Pignataro et al., 2004a, 2004b).

Neurological scores were evaluated 24 h or 7 days after reperfusion according to 2 scales: a general neurologic scale and a focal neurologic scale. In the general score, the following 6 general deficits were measured: (1) hair conditions, (2) position of ears, (3) eye conditions, (4) posture, (5) spontaneous activity, and (6) epileptic behavior. For each of the 6 general deficits measured, animals received a score ranging between 0 and 12 depending on the severity of signs. The scores of investigated items were then summed to provide a total general score.

In the focal score, the following 7 areas were assessed: (1) body symmetry, (2) gait, (3) climbing, (4) circling behavior, (5) front limb symmetry, (6) compulsory circling, and (7) whisker response. For each of these items, animals were rated between 0 and 4 depending on severity. The 7 items were then summed to give a total focal score.

Infarct volumes and neurological scores were evaluated in a blinded manner by individuals who did not perform the surgical procedures.