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Molecular mechanisms underlying the neuroprotective role of atrial natriuretic peptide in experimental acute ischemic stroke

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ABSTRACT

Along with its role in regulating blood pressure and fluid homeostasis, the natriuretic peptide system could be also part of an endogenous protective mechanism against brain damage. We aimed to assess the possibility that exogenous atrial natriuretic peptide (ANP) could protect against acute ischemic stroke, as well as the molecular mechanisms involved. Three groups of rats subjected to transient middle cerebral artery occlusion (tMCAO, intraluminal filament technique, 60 min) received intracerebroventricular vehicle, low-dose ANP (0.5 nmol) or high-dose ANP (2.5 nmol), at 30 min reperfusion. Neurofunctional condition, and brain infarct and edema volumes were measured at 24 h after tMCAO. Apoptotic cell death and expression of natriuretic peptide receptors (NPR-A and NPR-C), K^+ channels (K_{ATP}, K_V and BK_{Ca}), and PI3K/Akt and MAPK/ERK1/2 signaling pathways were analyzed. Significant improvement in neurofunctional status, associated to reduction in infarct and edema volumes, was shown in the high-dose ANP group. As to the molecular mechanisms analyzed, high-dose ANP: 1) reduced caspase-3-mediated apoptosis; 2) did not modify the expression of NPR-A and NPR-C, which had been downregulated by the ischemic insult; 3) induced a significant reversion of ischemia-downregulated K_{ATP} channel expression; and 4) induced a significant reversion of ischemia-upregulated pERK2/ERK2 expression ratio. In conclusion, ANP exerts a significant protective role in terms of both improvement of neurofunctional status and reduction in infarct volume. Modulation of ANP on some molecular mechanisms involved in ischemia-induced apoptotic cell death (K_{ATP} channels and MAPK/ERK1/2 signaling pathway) could account, at least in part, for its beneficial effect. Therefore, ANP should be considered as a potential adjunctive neuroprotective agent improving stroke outcome after successful reperfusion interventions.

1. Introduction

Stroke still remains as a leading cause of death, permanent disability and dementia worldwide. If the current trends continue, by 2030 there will be almost 12 million stroke deaths, 70 million stroke survivors, and more than 200 million disability-adjusted life-years lost globally (Feigin et al., 2014). Major advances in the treatment of acute ischemic stroke (AIS) came from vascular approaches to dissolve (thrombolysis with "recombinant tissue-type plasminogen activator", rt-PA) or remove (endovascular thrombectomy) the occluding clot responsible of the ischemia and subsequent infarction in the relevant part of the brain. However, only a minority of patients benefit from such treatments (Hankey, 2017). Although reperfusion therapies are therefore restricted, they are more clinically beneficial than neuroprotective strategies, which have failed in the translation from bench to bedside (Chamorro et al., 2016). Although the track report of monotherapeutic approaches in stroke is very poor and could at some point to be abandoned, neuroprotectants should be revisited in the era of recanalisation with thrombectomy since it would be more beneficial when carried out along with delivery of neuroprotectants (Babadjouni et al., 2017; Corbett et al., 2015). In this context, the natriuretic peptide (NP) system represents a serious choice.

NPs are important endocrine, autocrine and paracrine mediators which play a major role in the regulation of blood pressure and fluid

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homeostasis through their natriuretic, diuretic and vasorelaxant activities, counterbalancing the renin-angiotensin-aldosterone and neurohormonal systems. Three distinct but structurally related endogenous NPs have been identified: atrial natriuretic peptide (ANP), B-type natriuretic peptide (BNP) and C-type natriuretic peptide (CNP). ANP and BNP are secreted in the heart in response to volume expansion and pressure overload, and CNP is mainly released in endothelium in response to vasoactive agents or to proinflammatory cytokines. NPs interact with three different types of receptors: 1) NP receptor A (NPR-A), which binds both ANP and BNP with the greatest affinity for ANP; 2) NP receptor B (NPR-B), which preferably binds CNP; and NP receptor C (NPR-C), a clearance receptor which binds with similar affinity all three NPs (Kerkelä et al., 2015; Potter et al., 2006).

The three NPs are also produced in the CNS, and both their distribution and their NPRs have been described. This has two important implications: first, in addition to their peripheral actions, NPs could also contribute to the central control of cardiovascular function; and second, the NP system may be also involved in a variety of aspects of the physiology and pathophysiology of the CNS, e. g. neural development, synaptic transmission (Cao and Yang, 2008), behavior (Hodes and Lichtstein, 2014), cerebral hemodynamics (Guo et al., 2014), fluid homeostasis, neuro-inflammation, anxiety and memory (Mahinrad et al., 2016). Of particular interest is the neuroprotective role of NPs shown in several experimental paradigms of neuronal damage, leading to the premise that NPs could be part of an endogenous protective mechanism against brain damage (Hodes and Lichtstein, 2014). Focusing on ANP in the experimental setting, its ability to regulate fluid and electrolyte balance has been shown to reduce brain edema and subsequent intracranial pressure rise after focal (Naruse et al., 1991) or global (Akdemir et al., 1997; Nakao et al., 1990) cerebral ischemia, as well as after intracerebral hemorrhage (Rosenberg and Estrada, 1995). By contrast, some studies from the clinical realm have given conflicting results lending support either to a beneficial (Nogami et al., 2001; Pereira et al., 2015) or a detrimental (Berntsson et al., 2014; Katan et al., 2010) role of ANP.

To our knowledge, the possibility that exogenous ANP could improve neurofunctional status and reduce infarct volume, the two main endpoints in AIS neuroprotection, has not yet been investigated. We aimed to assess this possibility as well as the mechanisms which could be involved by using a rat model of AIS with reperfusion, hence mimicking endovascular thrombectomy along with adjunctive neuroprotection. Specifically we investigated: 1) brain damage as assessed by neurofunctional status, and infarct and edema volumes; 2) apoptotic cell death; 3) NPR-A and NPR-C expression; 4) K^+ channels (K_{ATP}, K_V and K_{Ca}) expression, as they could mediate ANP-induced responses (Jan and Jan, 1997); and 5) activation of the PI3K/Akt and MAPK/ERK1/2 signaling pathways, as previous reports have shown that ANP receptors could signal through these pathways (Pandey, 2014; Prins et al., 1996). ANP was delivered directly into the CNS (intracerebroventricular) to bypass the blood-brain barrier thus being distributed throughout the brain (Cohen-Pfeffer et al., 2017; Levin et al., 1987).

2. Materials and methods

2.1. Animals and ethical issues

Fifty-eight male Wistar rats (300–350 g, Charles River, Barcelona, Spain) were housed under standard conditions. Experiments were conducted in compliance with the legislation on protection of animals used for scientific purposes in Spain (RD 53/2013) and the EU (Directive 2010/63/EU). Protocols were approved by the Animal Experimentation Ethics Committee from IIS La Fe.

2.2. Transient focal cerebral ischemia

Animals were anesthetized by intraperitoneal injection of 5 mg/kg diazepam, 100 mg/kg ketamine and 0.3 mg/kg atropine. Anesthesia was maintained with 0.5–1% sevoflurane in 70% N_2O plus 30% O_2. Transient right middle cerebral artery occlusion (tMCAO) was performed by following the intraluminal suture procedure as originally described (Longa et al., 1989) and adapted to our experimental setup (Burguete et al., 2006). This includes continuous monitorization of cerebrocortical laser-Doppler flow (cortical perfusion, CP), arterial blood pressure (ABP) and core temperature (T), and discontinuous measurement of pH, PaO_2, $PaCO_2$ and glucose at pre-ischemia (basal), ischemia and reperfusion stages. MCAO was maintained for 60 min, after which reperfusion was monitored for 30 min. Twenty four hours after the ischemic insult the animals were subjected to neurofunctional evaluation and euthanized by intracardiac injection of KCl (200 mg/kg) under anesthesia to obtain the brain according to specific requirements for each determination.

2.3. Experimental groups, inclusion/exclusion criteria and treatments

Animals were randomly assigned to three experimental (tMCAO) groups: vehicle-, low-dose ANP- (0.5 nmol) and high-dose ANP- (2.5 nmol) treated rats. Twenty-four rats were excluded from the study according to the following criteria: 1) CP did not drop after filament insertion (no ischemia), n = 4; 2) CP did not recover after filament withdrawal (no reperfusion), n = 5; 3) subarachnoid hemorrhage during filament insertion/withdrawal, n = 4; 4) no brain infarction in spite of a right ischemia-reperfusion pattern, n = 1; and 5) death before 24 h, n = 10. Therefore, the three groups had the following "n": vehicle- (n = 11), low-dose ANP- (n = 10) and high-dose ANP- (n = 13) treated rats. Allocation was concealed since the experimenter carrying out the surgical procedure had no knowledge of the experimental group to which an animal belonged.

Human ANP (Sigma-Aldrich, Madrid, Spain) was dissolved in 0.5% aqueous acetic acid and diluted in 5% bovine serum albumin in phosphate-buffered saline solution. To avoid low penetration of ANP across the blood brain barrier (Levin et al., 1987), treatments were applied intracerebroventricularly (ICV, bregma −1 mm anteroposterior, 1.5 mm right mediolateral and 4.5 mm dorsoventral) by slow injection through a brain infusion kit (Alzet, Durect Corp., Cupertino, CA, USA). Injection was carried out 30 min after filament withdrawal, i. e. at 30 min reperfusion what equals to 90 min from the onset of ischemia. Doses were selected on the basis of the effect of ICV ANP on ischemic brain edema in rats (Akdemir et al., 1997; Nakao et al., 1990), and the neuroprotective effect of intravitreal ANP against NMDA-induced neurotoxicity in the rat retina (Kuribayashi et al., 2006).

2.4. Neurofunctional evaluation, and infarct and edema volumes measurement

Neurofunctional condition based on four tests was examined just before euthanization (Burguete et al., 2006): (a) spontaneous activity (moving / exploring = 0, moving without exploration = 1, no moving or only when pulled by the tail = 2); (b) circling to the left (none = 0, when elevated by the tail and pushed or pulled = 1, spontaneously = 2, circling without displacement (spinning top) = 3); (c) parachute reflex: protective abduction of forelimbs (symmetrical = 0, asymmetrical = 1, contralateral forelimb retracted = 2); and (d) resistance to left forepaw stretching (stretching not allowed = 0, stretching allowed = 1, no resistance = 2). The total score could range from 0 (no neurological deficits) to 9 (highest neurological deficits).

Brain infarct volume was determined by the 2,3,5-triphenyltetrazolium chloride (TTC) vital staining method (Bederson et al., 1986), followed by morphometric analysis (Burguete et al., 2006). Briefly, rats were euthanized and the brain was sliced in seven 2-mm thick coronal sections, which were immersed in a 2% solution of TTC in saline solution at 37 °C for 15 min, fixed in 10% phosphate-buffered formalin (pH 7.4) overnight, and digitally photographed for image analysis.