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Combination treatment with progesterone and vitamin D hormone is more effective than monotherapy in ischemic stroke: The role of BDNF/TrkB/Erk1/2 signaling in neuroprotection

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ABSTRACT

We investigated whether combinatorial post-injury treatment with progesterone (P4) and vitamin D hormone (VDH) would reduce ischemic injury more effectively than P4 alone in an oxygen glucose deprivation (OGD) model in primary cortical neurons and in a transient middle cerebral artery occlusion (tMCAO) model in rats. In the OGD model, P4 and VDH each showed neuroprotection individually, but combination of the "best" doses did not show substantial efficacy; instead, the lower dose of VDH in combination with P4 was the most effective. In the tMCAO model, P4 and VDH were given alone or in combination at different times post-occlusion for 7 days. *In vivo* data confirmed the *in vitro* findings and showed better infarct reduction at day 7 and functional outcomes (at 3, 5 and 7 days post-occlusion) after combinatorial treatment than when either agent was given alone. VDH, but not P4, upregulated heme oxygenase-1, suggesting a pathway for the neuroprotective effects of VDH differing from that of P4. The combination of P4 and VDH activated brain-derived neurotrophic factor and its specific receptor, tyrosine kinase receptor-B. Under specific conditions VDH potentiates P4's neuroprotective efficacy and should be considered as a potential partner of P4 in a low-cost, safe and effective combinatorial treatment for stroke.

1. Introduction

Despite extensive effort and costs, the results of well over one hundred industry-sponsored clinical trials for the treatment of stroke by medication have been disappointing, with genetically engineered tissue plasminogen activators (tPAs) still the only agents approved by the FDA. Unfortunately, tPA has a high risk-to-benefit ratio (causing intracranial hemorrhage in some patients) and is used in fewer than 5% of stroke victims. Several years ago an NIH-sponsored consensus meeting on neuroprotective treatments for brain injury specifically recommended directing therapeutic strategies toward combinations of neuroprotective agents acting on different pathways (Margulies and Hicks, 2009). The consensus report singled out the neurosteroid hormone progesterone (P4) as one of the pleiotropic agents particularly well-suited for studies of combination therapies for brain injury.

P4 treatment for traumatic brain injury (TBI), and more recently for stroke (Sayeed and Stein, 2009), is under investigation. In addition to leading to improved functional/behavioral outcomes in several injury models, P4 treatment reduces inflammatory cytokines, brain tissue necrosis, apoptosis, and cerebral edema (Stein, 2008). P4 is in national and international Phase III clinical trials for moderate to severe TBI (Stein, 2011). We and others have recently demonstrated substantial neuroprotection by P4 in several different stroke models (Liu et al., 2012; Dang et al., 2011; Gibson et al., 2011, 2005; Gibson and Murphy, 2004; Ishrat et al., 2009; Sayeed and Stein, 2009; Sayeed et al., 2007, 2006).

Accumulating pre-clinical evidence of the complex systemic pathophysiology of stroke suggests that it is unrealistic to confine research to neuroprotective agents targeted primarily to a single, or a small number of, injury mechanisms. Drugs like P4 with pleiotropic consequences are more likely to provide effective neuroprotection. There are at least two good reasons to consider 1, 25-dihydroxyvitamin D_3 hormone (VDH) as a potential combinatorial treatment with P4. First, it is well known that VDH insufficiency is common in acute stroke patients, and low levels of VDH are independently predictive for fatal strokes (Pilz et al., 2008). Second, both P4 and VDH are natural hormones known to have neuroprotective properties (Cekic et al., 2009). While both P4 and VDH can have neuroprotective effects, the latter exerts some of its

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actions on different signaling pathways from those of P4 (Cekic et al., 2009). Therapies for stroke that combine P4 with other agents that, like VDH, act on both similar and different injury pathways, may be able to improve stroke outcomes when given in the acute stage of injury. There is evidence to support this notion. We know that combinatorial treatment with P4 and VDH enhances the neuroprotective efficacy of P4 against excitotoxic cell death *in vitro* (Atif et al., 2009) and improves functional outcomes when given after TBI (Cekic et al., 2011).

P4 and VDH have high safety profiles, act on different injury and pathological mechanisms, and are clinically relevant, easy to administer and inexpensive, making them good choices for a new form of stroke therapy. Here, we investigated whether combinatorial treatment with P4 and VDH would produce better outcomes than P4 alone in reducing ischemic neuronal death *in vitro* and in reducing cerebral ischemia-induced brain infarction and restoring functional outcomes *in vivo*. We also explored some of their mechanisms of action by examining the effects of P4 and VDH on (1) growth factor signaling; (2) inflammatory markers (interleukin-6 (IL-6)) and nuclear factor kappa B (NFκB); (3) apoptosis markers (cleaved caspase-3 and BCl-2); and (4) an oxidative injury marker (heme oxygenase-1 (HO-1)). For growth factor signaling, we examined the expression of brain-derived neurotrophic factor (BDNF) and its specific receptor tropomysin-related kinase B (TrkB), along with their downstream signaling mediated by extracellular, signal-regulated kinase1/2 (Erk1/2) and phosphoinositide 3-kinase/Protein kinase B (PI3K/Akt).

2. Materials and methods

2.1. Neuronal culture

NeuroPure™ E18 primary rat cortical cells were commercially procured (N200200, Genlantis, San Diego, CA) as micro-surgically dissected regions from day 18 embryonic Sprague-Dawley rat brains. Enzymatic pre-treatment of the cells was followed by mechanical dissociation by incubation in sterile NeuroPapain™ enzyme solution at 30 °C for 30 min. The cells were then centrifuged and transferred to fresh plating medium and dissociated into isolated neurons using a P-1000 pipettor with a sterile 1-ml plastic tip (0.8–1.0-mm diameter opening). The cells were again centrifuged and seeded in multi-well plates pre-coated with poly-D-lysine (0.15 ml/cm^2, 50 μg/ml) and maintained at 37 °C in a humidified 5% CO_2 atmosphere. All experiments were performed on the neuronal cells after 9–10 days in culture.

2.2. Oxygen glucose deprivation (OGD) and drug treatment

OGD was carried out in 8-day *in vitro* (DIV) cultures, as required for the primary neurons to express all their cell surface receptors. The medium was replaced with pre-warmed Dulbecco's Modified Eagle's Medium (DMEM) without glucose. Cell cultures were then transferred into an anaerobic chamber equilibrated with 95% N_2 and 5% CO_2. The chamber was kept in a 37 °C incubator. Sham OGD cultures were maintained in a normal oxygenated DMEM containing 25 mM glucose. After 2 h, cultures were placed back into the normoxic incubator with normal culture medium. For concurrent treatment, P4 (0.1, 1, 5, 10, 20, 40, 80 μM) and VDH (1, 20, 50, 75, 100, 500 nM and 1, 5 μM) were presented either individually or in different combinations (PROG: 10, 20 μM + VDH: 1, 20, 50 and 100 nM) in the culture medium during OGD and reoxygenation. For the controls, only vehicle was added to the culture medium.

2.3. MTT reduction assay

Neuronal death was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The reaction is based on the cleavage of the tetrazolium ring of the pale yellow MTT into dark blue formazan crystals by mitochondrial dehydrogenase enzyme in viable cells. These blue formazan crystals accumulate within the cells due to their impermeability to the cell membrane, and are then solubilized by adding dimethyl sufoxide (DMSO; 50 μl). The intensity of blue-colored formazan solution is directly proportional to the number of surviving cells. Concentrations were determined by photometric analysis. Ten μl of MTT was added per well and incubated at 37 °C for 4 h until purple precipitate was visible. DMSO was added to solubilize the crystals and the absorbance was read at 570 nm.

2.4. Propidium iodide (PI) staining

PI-staining of neuronal cell cultures was performed as described previously (Endres et al., 2004). Cortical neurons were incubated for 1 min with 0.02 mg/mL PI (stock solution 1 mg/mL, 1:50) in medium with gentle shaking and rinsed once with phosphate-buffered saline. Conditioned medium was reapplied and phase contrast and fluorescent pictures were taken immediately with an inverse fluorescence microscope attached to a digital camera.

2.5. Animals and treatment regimen

Thirty adult male Sprague-Dawley rats (300–325 gm; Charles River Laboratories, Wilmington, MA) were used according to procedures approved by the Institutional Animal Care and Use Committee, Emory University, Atlanta, GA, USA (protocol 151-2005). The rats were quarantined for 7 days before the experiment and housed in individual cages in a room maintained at 21–25 °C, 45–50% humidity, a 12-h light/dark cycle and free access to pellet chow and water. Rats were randomized to the treatment conditions and the identity of the groups was coded to avoid experimenter bias while testing. There were 5 groups (n = 6/group). One group served as sham-operated, vehicle-treated controls (SHAM). Animals from the other groups were given middle cerebral artery occlusions (MCAO) followed by treatment with either vehicle (VEH), P4 (8 mg/kg), VDH (1 μg/kg body weight/day), or a combination of P4 (8 mg/kg) + VDH (1 μg/kg body weight/day) for 6 days. P4 (P-0130; Sigma-Aldrich Co., St. Louis, MO) was dissolved in 22.5% 2-hydroxypropyl-cyclodextrin (HBC). VDH stock was prepared in absolute ethanol and further diluted in 22.5% HBC. P4 was given intraperitoneally (i.p.) 5 min prior to reperfusion followed by subcutaneous (s.c.) injections at 6 h post-occlusion and then on days 1, 2, 3, 4, 5 and 6 post-occlusion. The i.p. injection of P4 provides rapid absorption, peak blood P4 levels, and quick access to the brain. Since P4 has a very short half-life, and washes out in a short period, remaining injections were s.c. for slow absorption/release to maintain high blood P4 levels for a longer period. The P4 8-mg/kg dose and routes of administration were determined from previous studies showing that this amount provided the maximal protective effects for stroke (Sayeed et al., 2007, 2006). One i.p. injection of VDH was given 5 min prior to reperfusion followed by daily s.c. injections for 6 days either alone or in combination with P4. On day 7, animals were killed and their brains removed after transcardial perfusion.

All efforts were made to minimize animal suffering and we conducted several experiments *in vitro* to reduce the number of animals. For each outcome measure, we calculated the starting sample sizes and power needed to reject the null hypothesis with a P-value of 0.05. The number of rats per group at these criteria was determined to be 6 to reject the null hypothesis (H_0) at the 0.05 level at a power of 0.8.

2.6. Transient middle cerebral artery occlusion (tMCAO)

Focal cerebral ischemia was induced by the occlusion of the right middle cerebral artery as previously described (Longa et al., 1989). A midline incision was made on the ventral surface of the neck, and the right common carotid arteries were isolated and ligated with 6.0 silk suture. The internal carotid artery and the pterygopalatine artery were temporarily occluded using a microvascular clip. A 4-0 Doccol™ filament (Doccol Corporation, Redlands, CA) was introduced into the internal carotid artery through the incision in the external carotid artery. The filament was advanced approximately 20 mm distal to the carotid bifurcation. Relative cerebral blood flow was monitored by laser Doppler (LD) for the entire 90 min of occlusion. Drug treatment was randomly assigned 5 min before onset of reperfusion. After 90 min of MCAO, the occluding filament was withdrawn back into the common carotid artery to allow reperfusion. Relative cerebral blood flow was monitored for 5 min before the wound was sutured and rats permitted to recover from anesthesia. Rats subjected to MCAO with less than 40% of baseline LD flowmetry were randomly assigned to receive drug treatments.

2.7. Analysis of infarct volume

On day 7, animals were deeply anesthetized using isoflurane. After transcardial perfusion with 10% buffered formalin, brains were extracted and fixed in gradient sucrose solution and cut into 20-μm sections for histological analysis. Brain sections were stained in 0.1% cresyl violet solution for 10 min at 45 °C, and then rinsed in distilled water. Stained sections were fixed by serial dehydration in alcohol and xylene and mounted with xylene-based cytoseal. Fixed sections were coded for hiding group identity and then scanned. Infarct volume was calculated using NIH Image-J software.

2.8. Behavioral testing

2.8.1. Motor coordination–accelerating rotarod

Motor impairment was assessed with the accelerating rotarod (Ishrat et al., 2009). Rats were given 3 training sessions 5 min apart before surgery. The animals were habituated to the stationary rod, and then exposed to the rotating rod.