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Progesterone and allopregnanolone attenuate blood–brain barrier dysfunction following permanent focal ischemia by regulating the expression of matrix metalloproteinases

Tauheed Ishrat, Iqbal Sayeed, Fahim Atif, Fang Hua, Donald G. Stein *

Department of Emergency Medicine, Emory University School of Medicine, Atlanta, GA 30322, USA

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ABSTRACT

Blood–brain barrier (BBB) breakdown after stroke is linked to the up-regulation of metalloproteinases (MMPs) and inflammation. This study examines the effects of progesterone (PROG) and its neuroactive metabolite allopregnanolone (ALLO) on BBB integrity following permanent middle cerebral artery occlusion (pMCAO). Rats underwent pMCAO by electro-coagulation and received intraperitoneal injections of PROG (8 mg/kg), ALLO (8 mg/kg) or vehicle at 1 h post-occlusion and then subcutaneous injections (8 mg/kg) at 6, 24, and 48 h. MMP activation and expression were analyzed by Western blot, immunohistochemistry and gelatin zymography 72 h post-pMCAO. Occludin1, claudin5, tumor necrosis factor-alpha (TNF-α) and Interleukin-6 (IL-6) were analyzed at 72 h post-pMCAO with Western blots. BBB permeability was measured by Evans blue extravasation and infarct size was evaluated by cresyl violet at 72 h after pMCAO. Ischemic injury significantly ($p<0.05$) increased the expression of MMP-9, MMP-2, TNF-α and IL-6, and reduced the levels of occludin1 and claudin5. These changes were followed by increased infarct size (% contralateral hemisphere) and Evans blue extravasation into the brain indicating compromise of the BBB. PROG and ALLO attenuated BBB disruption and infarct size following pMCAO by reducing MMPs and the inflammatory response and by preventing the degradation of occludin1 and claudin5. We conclude that PROG and ALLO can help to protect BBB disruption following pMCAO.

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Introduction

Ischemic stroke is most often caused by cerebral artery occlusion, and is characterized by a pathologic cascade including loss of cerebral blood flow, breakdown of the blood–brain barrier (BBB), and edema (Dirnagl et al., 1999). Rapid protection against BBB disruption is a critical capacity for any therapeutic intervention to minimize neuronal injury following ischemic stroke or other forms of traumatic brain injury (TBI). The BBB is composed of cerebral microvessel endothelial cells containing tight junction proteins such as occludin1 and claudin5, among others. These proteins appear to protect the brain against harmful substances entering from the bloodstream (Ballabh et al., 2004).

BBB breakdown has been linked to an increase in the expression of various cytokines and chemokines (Ballabh et al., 2004). In particular, tumor necrosis factor alpha (TNF-α) and Interleukin-6 (IL-6) have been shown to potentiate the neuroinflammatory response after ischemic injury (Meistrell et al., 1997; Nilupul Perera et al., 2006). These inflammatory factors stimulate the expression of matrix metalloproteinases (MMPs), a gene family of extracellular matrix enzymes which degrade junction proteins and change the permeability of the BBB (Rosenberg and Mun-Bryce, 2004). Elevated expression of MMP-9 and MMP-2 following cerebral ischemia leads to increased infarct size, BBB leakage and hemorrhagic activity (Machado et al., 2006; Rosenberg and Yang, 2007). In contrast, inhibition of MMP-9 is associated with attenuation of infarct size and reduced risk of hemorrhagic complications (Sumii and Lo, 2002). Increased plasma MMP-9 levels correlate with hemorrhage, edema and poor neurological outcome after thrombolytic treatment with tissue plasminogen activator (tPA) for acute ischemic stroke (Montaner et al., 2003; Sumii and Lo, 2002).

Experimental findings have consistently demonstrated the neuroprotective effects of progesterone (PROG) in a variety of animal and human injury models (Gibson et al., 2009; Ishrat et al., 2009; Sayeed et al., 2006, 2009; Wright et al., 2007). PROG reduces inflammation, BBB permeability, and edema in stroke (Betz and Coester, 1990; Djebaili et al., 2005; Gibson et al., 2009) and TBI (Guo et al., 2006; O'Connor et al., 2005; Wright et al., 2001). It exerts some of its actions through the intracellular membrane-bound PROG receptor (PR) or through its metabolite, allopregnanolone (ALLO), which does not bind to the PR (Mahesh et al., 1996). ALLO, a 3α-5α reduced neuroactive metabolite, is produced by the neuron–glia functional unit by the 5α-

* Corresponding author. Department of Emergency Medicine, Brain Research Laboratory, 1365B Clifton Road NE, Emory University, Atlanta, GA 30322, USA. Fax: +1 404 727 2388.
E-mail address: donald.stein@emory.edu (D.G. Stein).

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reductase-3α-hydroxysteroid oxydoreductase (5αR-3α-HSD) pathway induced by endogenous or exogenous PROG (Corpechot et al., 1993). ALLO acts as an agonist on the γ-aminobutyric acid (GABA-A) receptor, exerting anxiolytic, sedative and antiepileptic effects (Wang et al., 2008), and enhancing the myelination/remyelination process in the central and peripheral nervous systems (Schumacher et al., 2003; Wang et al., 2008).

Our own studies demonstrate that at a similar dose, ALLO treatment affords better neuroprotection than PROG in several models of brain injury including stroke (Sayeed et al., 2006; Sayeed et al., 2009) and TBI (Djebaili et al., 2005). The protective actions of PROG and ALLO on the functional and structural integrity of the BBB following permanent stroke have not been studied in as much detail. Here we hypothesize that PROG and ALLO treatment given soon after ischemic stroke will preserve BBB integrity by inhibiting the expression of key inflammatory cytokines and MMPs. This hypothesis is based on our recent results showing that PROG reduces infarct volume and improves functional outcome 72 h after injury produced by direct permanent ligation of the middle cerebral artery (MCA) (Ishrat et al., 2009). Our findings support the efficacy of post-administration of PROG and ALLO on the repair of BBB damage induced by ischemic stroke.

Experimental procedures

Animals and treatment regimen

Sixty adult male Sprague–Dawley rats (300–340 g; Charles River Laboratories, Wilmington, MA) were used according to procedures approved by the Institutional Animal Care and Use Committee, Emory University, Atlanta, GA (protocol # 306-2008). Animals were separated into four groups ($n = 15$): sham-operated vehicle-treated control (S); permanent middle cerebral artery occlusion (pMCAO) + vehicle (L); pMCAO + PROG (8 mg/kg) (LP); and pMCAO + ALLO (8 mg/kg) (LA). PROG (P-0130; Sigma-Aldrich Co., St. Louis, MO) and ALLO (EMD Biosciences, La Jolla, CA) were dissolved in 22.5% 2-hydroxypropyl-β cyclodextrin and administered by intraperitoneal (IP) injection 1 h post-occlusion to ensure relatively rapid absorption following injury, and then subcutaneously (SC) at 6, 24, and 48 h post-occlusion. The PROG and ALLO doses used in this experiment were determined from previous studies showing that these amounts provided the maximal protective effects (Djebaili et al., 2005; Sayeed et al., 2006, 2009).

Induction of permanent MCA occlusion (pMCAO)

Prior to pMCAO, isoflurane anesthesia was induced by 5% and then maintained at 1.5–2% during surgery in 2:1 nitrous oxide and oxygen. The left MCA was exposed and electrocoagulated as described previously (Ishrat et al., 2009). The occlusion was made midway between the inferior cerebral vein and the olfactory tract. Sham-operated rats were subjected only to exposure of the MCA without coagulation. Temperature was monitored and maintained (37 ± 2 °C) during surgery by a homeothermic heating blanket system (Harvard Apparatus, Holliston, MA). Pulse oximetry (SurgiVet™ V3304; Waukesha, WI) was used to maintain heart rate at approximately 350 bpm, with blood oxygen saturation (SpO2) levels >95%. Anesthesia duration was the same for all groups (Table 1).

Measurement of BBB permeability

Cerebral ischemia opens the BBB, leading to vascular permeability, significant edema, and claudin5 expression, all of which peak at 72 h after injury (Gotoh et al., 1985; Hatashita and Hoff, 1990; Hornig et al., 1985; Jiang et al., 2009). For this reason, we waited 72 h to evaluate BBB permeability. Rats were injected with Evans blue (Sigma Aldrich; 2% in saline, 3 ml/kg) through the tail vein. Six hours later, rats were anesthetized with Nembutal (2 ml/kg, IP), and intracardially perfused with 200 ml isotonic saline to remove the intravascular dye. Brains were quickly removed and separated into ipsilateral and contralateral hemispheres, and then weighed. For quantitative measurements, brain samples were homogenized in *N*, *N*-dimethylformamide (1.0 ml, Sigma-Aldrich), incubated for 72 h at 37 °C and then centrifuged at 21,000 × *g* for 30 min. Supernatant Evans blue concentrations were determined by a conventional spectrophotometric method using standard curves of Evans blue in formamide. Extravasation was expressed as micrograms of Evans blue per gram of wet tissue weight.

Table 1
Physiological monitoring.

Group	SpO2	Heart beats	Temperature
Sham (S)	94.84 ± 1.86	353.42 ± 6.82	36.76 ± 1.44
	95.24 ± 1.89	350.78 ± 5.68	36.90 ± 2.35
Lesion (L)	96.24 ± 3.75	354.45 ± 4.46	36.72 ± 3.22
	95.54 ± 2.95	351.60 ± 5.80	35.89 ± 2.34
L + PROG	94.75 ± 2.26	353.16 ± 5.28	36.78 ± 3.41
	95.32 ± 3.18	354.25 ± 6.42	36.89 ± 2.84
L + ALLO	95.15 ± 1.89	356.16 ± 7.38	36.43 ± 3.16
	94.82 ± 2.48	355.25 ± 5.62	37.12 ± 2.53
10 min before occlusion; 90 min after occlusion			

Values are expressed as mean ± SD. The physiological parameters (blood SpO2, heart beats and temperature) were monitored at 10 min before and 90 min after pMCAO. There were no significant differences among the groups for these parameters.

Assessment of cerebral infarct size

Cerebral infarct size was evaluated according to previously applied methods (Hua et al., 2009). Seventy-two hours after reperfusion, rats were given an overdose of Nembutal (2 ml/kg, IP) and then transcardiacally perfused with cold saline followed by 4% paraformaldehyde in PBS (pH 7.4) via the ascending aorta. Brains were removed and post-fixed in 4% paraformaldehyde for 48 h and then stored at 4 °C in a solution of 30% sucrose–PBS for 2 days. The brains were embedded in OCT and sectioned coronally in 12-μm-thick slices starting from the frontal pole at an interval of 2 mm. The sections were stained with 1% cresyl violet (Nissl staining). The infarct areas, defined as areas showing reduced Nissl staining under light microscopy, were traced and quantified with an image-analysis system. Infarct volumes are expressed as a percentage of the contralateral side ± SEM.

Tissue collection

Rats were overdosed with Nembutal (2 ml/kg, IP) at 72 h after pMCAO, and then decapitated while anesthetized. For sampling peri-infarct (penumbra) cortical regions, using a brain matrix, the brains were rapidly dissected into 4.0 mm coronal sections (approximately 0.5 and −3.5 mm from bregma). Based on the TTC staining we identified the penumbra and core areas in the brain sections. The peri-infarct cortical regions were snap-frozen in liquid nitrogen and then stored at −80 °C until needed (Fig. 1).

Western blotting

Peri-infarct cortical tissue was processed for protein analysis. Tissues were homogenized in T-per (Pierce, Rockford, IL) containing protease inhibitor cocktail (P8340, Sigma). Homogenates were centrifuged for 20 min at 10,000 × *g*. A bicinchoninic acid protein assay (Pierce, 23225) was performed for protein equalization. Forty micrograms of total protein was separated at 200 V for 1 h on 8–14% SDS gel and transferred onto PVDF membrane at 100 V for 30 min.

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