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

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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 level 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.

Keywords

permanent middle cerebral artery occlusion; progesterone; allopregnanolone; blood-brain barrier; cytokines; inflammatory response; ischemia; metalloproteinases; tight junction proteins; tumor necrosis factor-alpha

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DISCLOSURE/CONFLICT OF INTEREST

DGS is entitled to royalty payment from BHR Pharmaceuticals related to research on progesterone and brain injury. His future financial interests may be affected by the outcome of this research. The terms of this arrangement have been reviewed and approved by Emory University, which receives the largest share of any benefits in accordance with its conflict of interest policies. The abstract was accepted for poster presentation in the AHA/ASA annual "International Stroke Conference" February 2010, San Antonio, TX.

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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, Sayeed, et al., 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 3 α -5 α reduced neuroactive metabolite, is produced by the neuron-glia functional unit by the 5 α -reductase-3 α -hydroxysteroid oxydoreductase (5 α R-3 α -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 (300–340g; 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 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, Sayeed, et al., 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 ($37 \pm 2^\circ\text{C}$) 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 beats per minute 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 claudin 5 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.

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 (75 mg/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 $\pm$ 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 mm and -3.5 mm from bregma). The peri-infarct cortical regions were snap-frozen in liquid nitrogen and then stored at -80°C until needed (Figure 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 10,000g. A bicinchoninic acid protein assay (Pierce, 23225) was performed for protein equalization. Forty µg of total protein was separated at 200V for 1 h on 8–14% SDS gel and transferred onto PVDF membrane at 100 V for 30 min. After blocking with 5% milk, membranes were probed with primary antibodies followed by incubation with peroxidase-conjugated secondary antibodies (1:5,000; KPL, Gaithersburg, MD). The peroxidase reaction was developed with an ECL-plus detection kit (Amersham BioSciences, Piscataway, NJ). The following primary antibodies were used: anti-MMP-9, MMP-2 (1:500, Oncogene Research Products, San Diego, CA), occludin1 (1:500, Santa Cruz), claudin5 (1:1000, Abcam, Cambridge, MA), TNF-α (1:250, Santa Cruz) and IL-6 (1:200, Santa Cruz). All blots were stripped and re-incubated with β-actin antibodies (1:10,000, Sigma) as a loading control. Intensity of the bands was measured by densitometry and quantified using Quantity One software (BioRad Laboratories, Hercules, CA).

Immunohistochemistry

For immunohistochemistry, rats were overdosed and anesthetized with Nembutal at 72 h after pMCAO and then perfused transcardially with cold PBS and 200 mL of 4% paraformaldehyde in PBS. Brains were removed and postfixed in the same fixative overnight at 4°C and then with 30% sucrose in PBS for 72 h. The brains were sectioned in the coronal plane at a thickness of 15 µm. Sections were blocked with 3% horse serum and then incubated with primary antibodies overnight at 4°C. After washing three times with PBS, sections were incubated with biotinylated secondary antibodies (1:400, DAKO, Carpinteria, CA), washed with PBS, incubated in avidin-biotin horseradish peroxidase complex solution (DAKO) for 10 min, and the reaction product visualized with diaminobenzidine (DAB Kit, Vector Laboratories, Burlingame, CA) as the final chromogen. Antibodies used were MMP-9 and MMP-2 (1:100). Sections without primary antibodies were similarly processed to control for unspecific peroxidase staining of the secondary antibodies in the damaged tissue, as a negative control. Sham brain sections were used as a positive control.

Gelatin zymography

Substrate-specific zymography for determination of gelatinolytic activity of MMP-9 and MMP-2 was performed on brain homogenates taken 72 h after injury because previous work reported a substantial increase in MMP-2 and 9 expression 72 h after pMCAO (Koistinaho, et al., 2005). The concentration of protein was adjusted equally in all the tissue samples. Samples were then mixed 1:1 with loading buffer (80 mmol/L Tris-HCl [pH 6.8], 4% SDS, 10% glycerol, and 0.01% bromophenol blue) and left standing for 10 min at room temperature. Proteins were separated by electrophoresis in a 10% SDS-PAGE gel containing 0.1% gelatin (BioRad) at 125 volts constant current. Gels were then washed to remove SDS with 2.5% Triton X-100 (Sigma) for 1 h and incubated at 37°C with developing buffer (50 mmol/L Tris-HCl [pH 7.5], 10 mmol/L CaCl₂, 0.02% NaN₃) for 36 h. Enzymatic bands were visualized after staining for 1h with Coomassie blue (BioRad) R-250 for 30 min, and de-stained with three

changes of methanol: acetic acid: water (50:10:40). The gel was scanned and the bands of activity were quantified using Quantity One software (BioRad).

Statistics

Data were presented as mean $\pm$ SEM. Calculations were obtained using GraphPad Prism. One-way analysis of variance (ANOVA) was followed by Tukey's test to assess statistical differences among groups. $P < 0.05$ was considered significant.

RESULTS

Effects of PROG and ALLO on BBB permeability

At 72 h following pMCAO, the vehicle-treated pMCAO group demonstrated a significant ($p < 0.05$) increase in BBB permeability to Evans blue compared to sham-operated controls (shams). Administration of either PROG or ALLO significantly ($p < 0.05$) inhibited Evans Blue extravasation compared to the pMCAO group, indicating a reduced BBB opening in response to neurosteroid treatment (Figure 2). ALLO's effect was not significantly more pronounced than that of PROG.

Effects of PROG and ALLO on infarct size

Figure 3 illustrates the effects of PROG and ALLO on infarct size (expressed as percent of intact contralateral structure). Figure 3A shows representative brain slices stained with CV 72 h after pMCAO in vehicle-treated, PROG-treated and ALLO-treated rats. Figure 3B shows the distribution of percent area of infarct in serial brain sections stained with CV at 72 h after pMCAO in vehicle-treated, PROG-treated and ALLO-treated rats. PROG and ALLO administration significantly ($p < 0.05$) reduced total infarct size (41.15% 35.45% respectively) after pMCAO.

Effects of PROG and ALLO on MMP-9 and MMP-2

Western blot analysis done 72 h after pMCAO showed significantly ($p < 0.05$) up-regulated expression of MMP-9 and MMP-2 in the vehicle-treated pMCAO group compared to shams (Figure 4.1A). PROG and ALLO treatments significantly ($p < 0.05$) decreased MMP-9 and MMP-2 expression after pMCAO compared to the vehicle-treated pMCAO group (Figure 4.1B).

The immunohistochemical expression of MMP-9 and MMP-2 immunopositive signals in the peri-infarct area of brain sections was markedly increased at 72 h following pMCAO in the vehicle-treated compared to the sham-operated vehicle-treated group (Figure 4.2). Following pMCAO, repeated treatments with PROG and ALLO reduced the immunopositive signals of MMP-9 and MMP-2 compared to the vehicle-treated pMCAO group.

The inhibitory effects of PROG and ALLO on MMP-9 and MMP-2 activation were also demonstrated by gelatin zymography assay. MMP-9 and MMP-2 levels were significantly ($p < 0.05$) increased in the vehicle-treated pMCAO group compared to shams, and significantly ($p < 0.05$) down-regulated by treatments with PROG and ALLO (Figure 4.3 A and B).

Effects of PROG and ALLO on claudin5 and occludin1

Effects of PROG and ALLO on the level of junction proteins at 72 h following pMCAO are shown in Figure 5. The level of claudin5 and occludin1 was significantly ($p < 0.05$) decreased in the vehicle-treated pMCAO group compared to shams. Administration of PROG and ALLO significantly ($p < 0.05$) restored these protein levels compared to the pMCAO group. This

finding demonstrates reduced degradation of extracellular proteins after neurosteroid treatment.

Effects of PROG and ALLO on TNF- α and IL-6

Western blotting revealed that TNF- α and IL-6 were expressed at very low levels in the sham group, highly expressed after injury in the vehicle-treated pMCAO group (Figure 6), and reduced following pMCAO in the PROG- and ALLO-treated groups. Densitometry analysis showed that TNF- α and IL-6 were significantly ($p < 0.05$) expressed in the vehicle-treated pMCAO compared to the sham group, and significantly reduced ($p < 0.05$) in the PROG- and ALLO-treated groups.

DISCUSSION

BBB compromise, inflammation and edema

Cerebral stroke can produce substantial brain injury, triggering inflammation, MMP elevation, BBB dysfunction and passive diffusion of water leading to vasogenic edema (Rosenberg and Yang, 2007). Inflammation plays an important role in the pathogenesis of ischemic stroke and other forms of ischemic brain injury. Experimentally and clinically, the brain responds to ischemic injury with an acute and prolonged inflammatory process characterized by rapid activation of resident cells (mainly microglia), production of proinflammatory mediators, and infiltration of various types of inflammatory cells (including neutrophils, different subtypes of T cells, monocyte/macrophages, and other cells) into the ischemic brain tissue (McColl, et al., 2009). Recent experimental studies have shown that systemic inflammation exacerbates neutrophil infiltration and immune-mediated (T-helper type and CD8 T cells) brain vascular permeability, and thus alters the kinetics of BBB tight junction proteins (e.g., claudin 5 and occludin 1) after brain injury, including stroke (Argaw, et al., 2009, Kebir, et al., 2007, McColl, et al., 2007, McColl, et al., 2008, Suidan, et al., 2008). In contrast, inhibition of the inflammatory response decreases infarct size, and reduces neurological deficits after experimental stroke (Wang, 2005, Yilmaz and Granger, 2008).

The post-ischemic neuroinflammatory response is characterized by microglial and astrocytic activation and increased expression of inflammatory mediators (McColl, et al., 2007, Nilupul Perera, et al., 2006). In particular, pro-inflammatory cytokines such as TNF- α and certain other interleukins can trigger the early events that cause BBB breakdown and subsequent development of cerebral edema (Meistrell, et al., 1997, Nilupul Perera, et al., 2006). The causes of pathologic BBB opening are not fully characterized, but studies have suggested that proteolytic degradation of the vascular basement membrane leads to breakdown of the BBB (Asahi, et al., 2001, Ballabh, et al., 2004). Stabilization of the BBB between adjacent endothelial cells involves a complex network of tight junction proteins, including occludins and claudins that link transmembrane proteins to the actin cytoskeleton. Expression of these tight junction proteins is modified in several neurological diseases including stroke (Zlokovic, 2008), and they are vulnerable to attack by MMPs (Yang, et al., 2007).

Previous studies have suggested a positive correlation between elevated expression of MMPs and inflammation-mediated BBB breakdown (de Vries, et al., 1996, Rosenberg and Mun-Bryce, 2004, Rosenberg and Yang, 2007). MMPs are zinc-dependent endopeptidases that can promote breakdown of the BBB by direct degradation of components of the extracellular matrix and basement membrane of the cell, and are implicated in ischemic injury (Gidday, et al., 2005, Rosenberg and Mun-Bryce, 2004). MMP inhibitors have been shown to attenuate infarct volume (Svedin, et al., 2007) and reduce BBB disruption (Rosenberg, et al., 1998) caused by ischemic stroke.

In the present study, three days of PROG or ALLO treatment *after* ischemic stroke resulted in substantial restoration of BBB integrity, possibly by inhibiting inflammation (TNF- α and IL-6), reducing the expression of MMPs (MMP-9 and MMP-2), and preventing the degradation of claudin5 and occludin1. It is possible that the observed inhibition in MMP expression is attributable either to restricted passage of MMPs from the blood into the brain through the restored BBB, or to the down-regulation of MMP gene expression by neurosteroid treatment (Yepes, et al., 2003). Our previous studies on TBI and stroke (Djebaili, et al., 2005, Sayeed, et al., 2006, Sayeed, et al., 2009) show that lower doses of ALLO are as effective as higher doses of PROG (0.5x). In the present study, however, ALLO's effect was not significantly better than PROG's at a similar dose in facilitating the repair of BBB integrity.

Neurosteroid treatments improve stroke outcomes

Our earlier papers show that both PROG and ALLO decrease infarct volume and improve functional outcome after ischemic injury (Ishrat, et al., 2009, Sayeed, et al., 2006). In this study we also looked at the effects of these neurosteroids on infarct size. A short course of treatment with PROG or ALLO significantly reduced infarct size following pMCAO in rats. Overall, we found that the average total size of the infarctions were reduced with PROG and ALLO by 41.15% and 35.45% respectively, compared to the vehicle-treated group. These data corroborate our previous finding that PROG attenuates infarct volume in transient and permanent rat models of stroke (Ishrat, et al., 2009, Sayeed, et al., 2006, Sayeed, et al., 2007), and the reports of others (Chen, et al., 1999, Gibson and Murphy, 2004, Jiang, et al., 2009, Jiang, et al., 1996, Kumon, et al., 2000), who have also been demonstrated the protective effect of PROG in reducing infarct volume following stroke. PROG and ALLO treatments consistently inhibit inflammatory reactions (Djebaili, et al., 2005, Gibson, et al., 2009) and reduce edema (Jiang, et al., 2009, Wright, et al., 2001) following TBI and stroke. Many studies show that PROG, a natural steroid hormone present in both males and females, exerts its neuroprotective actions in a number of ways: it has potentiating effects on GABA, inhibits and attenuates excitatory amino acid responsiveness, amplifies adenosine's inhibitory action on cerebral cortical neuronal activity, is anti-apoptotic, antioxidant and anti-inflammatory, and acts as a free-radical scavenger (Sayeed and Stein, 2009, Stein, 2008). PROG also modulates the expression of aquaporin 4 (Guo, et al., 2006), a water channel protein shown to be critically involved in the formation and resolution of brain edema following ischemic brain injury (Frydenlund, et al., 2006, VanLandingham, et al., 2007); and increases the expression of CD-55, a cell surface protein that can reduce complement factor convertases which trigger the inflammatory cascade (VanLandingham, et al., 2007).

PROG and ALLO work through different receptor pathways

Previous research has also shown that while PROG can act as a ligand to the classical PR, the sigma-1 receptor, and the putative PROG dx-25 membrane binding site, ALLO has not demonstrated any significant binding or activity at these locations (Monnet, et al., 1995, Rupprecht, et al., 1993). Research on ALLO in brain injury is relatively new, so we do not yet know which of the ALLO pathways mediate its neuroprotective effects when given after ischemic stroke. While neurosteroids can have pleiotropic actions in the brain (Stein and Wright), one hypothesis suggests that ALLO may enhance GABA_A and thereby reduce the secondary excitotoxicity induced by ischemic or traumatic injury (Schumacher, et al., 2003). Further, ALLO has been shown to delay the onset and severity of neurodegeneration (Griffin, et al., 2004), stimulate myelination (Schumacher, et al., 2003), and influence different neural survival functions including neurogenesis and synaptic stability (Brinton and Wang, 2006, Wang, et al., 2008).

Another possible mechanism underlying neurosteroid neuroprotection may be through the increased expression of neuroserpin, a serine protease inhibitor (serpin) that is released from

neurons and inhibits tPA (Miranda and Lomas, 2006, VanLandingham, et al., 2008, Yepes and Lawrence, 2004). Following ischemic stroke, endogenous tPA activity increases significantly within the ischemic hemisphere and correlates with the development of cerebral edema (Wang, et al., 1998, Yepes, et al., 2003). In laboratory studies, both genetic deficiency of tPA and its inhibition with neuroserpin are associated with neuronal survival and decreased infarct volume (Cinelli, et al., 2001, Wang, et al., 1998, Yepes, et al., 2000). These findings suggest that some tPA inhibition within the brain may be beneficial after ischemic stroke. Moreover, treatment with tPA is associated with increased vascular permeability, infarct volume and activity of MMP-9 following cerebral stroke (Lapchak, et al., 2000, Lee, et al., 2007, Yepes, et al., 2003, Zhang, et al., 2002). Under pathological conditions there may be a correlation between tPA activity and changes in vascular permeability, and any increases in vascular permeability could result in vascular tPA crossing into the brain, where it may intensify the effect of ischemia on excitotoxic cell death. Our recent results demonstrate that both PROG and ALLO can significantly increase the expression of neuroserpin (VanLandingham, et al., 2008). It is likely that this increase in neuroserpin by the neurosteroids inhibits endogenous tPA expression and causes a decrease in MMP-9 activity and vascular permeability.

To evaluate “proof of principle,” the present study looked at the acute effects of PROG and ALLO at a single survival timepoint of 72 hours. Because ischemic stroke maturation occurs over several days, in future studies it will be important to compare multiple survival timepoints and long-term effects of neurosteroids on stroke outcome. Animal and human studies have provided strong evidence linking MMP-9 induction and tPA-induced hemorrhagic transformation in ischemic stroke (Montaner, et al., 2003, Sumii and Lo, 2002). Our present study can be taken to demonstrate that PROG and ALLO treatments attenuate MMP-9 and MMP-2 induction in the ischemic brain. The neurovascular protective effects of these neurosteroids strongly suggest the need for future studies looking at a combinatorial treatment approach to improve the safety and efficacy of tPA thrombolysis.

CONCLUSIONS

When given after ischemic stroke, PROG and ALLO treatments inhibit the inflammatory response and down-regulate MMP induction while preserving the functional and structural integrity of the BBB. These findings will support the development of new therapeutic approaches for stroke and other types of injury.

Acknowledgments

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Figure 1.

Damage to the left cortical region (unstained core area) at 72 h after pMCAO is shown by 2, 3, 5-triphenyltetrazolium chloride (TTC)-staining. The box shows the penumbral region (peri-infarct cortical tissue) selected for Western blotting and immunostaining.

Figure 2.

Evans blue leakage is significantly increased at 72 h after ischemic injury. PROG and ALLO treatments significantly attenuate Evans blue leakage in ischemic cortex. *Lesion (L) vs. Sham (S); L vs. #Lesion + Progesterone (LP) and #Lesion + Allopregnanolone (LA); $p < 0.05$; $n = 6$.

Figure 3.

PROG and ALLO reduce infarct size in a rat model of pMCAO. (A) CV-stained coronal sections from representative rats given vehicle, PROG or ALLO, brains harvested at 72 h post-occlusion. Infarcts are shown as unstained regions. The infarct area in PROG- and ALLO-treated rats is substantially reduced. (B) Bar diagram shows the percent area distribution of infarction compared to the area of the contralateral side in pMCAO, pMCAO plus PROG, and pMCAO plus ALLO groups.

Figure 4.

Figure 4.1. (A) Representative Western blots of MMP-9 and MMP-2 in peri-infarct cortex at 72 h after pMCAO. (B) Densitometric analysis shows that expression of MMP-9 and MMP-2 is significantly up-regulated after pMCAO, and decreased by PROG and ALLO treatments (n = 6). **4.2** Immunohistochemical representation of secondary antibody staining for endogenous peroxidase activity as a negative control for the expression of MMP-9 and MMP-2 in the peri-infarct cortex 72 h after pMCAO (n = 3). Compared to shams, MMP-9 and MMP-2 signals increased at 72 h after pMCAO, and were decreased by PROG and ALLO treatments. **4.3.** (A) Representative zymography shows effects of PROG and ALLO on MMP-9 and MMP-2. (B) Densitometric analysis shows that MMP-9 and MMP-2 levels are enhanced at 72 h after pMCAO, and decreased by PROG and ALLO treatments. *L vs. S; L vs. #LP and #LA; p<0.05; n = 6.

Figure 5.

(A) Representative Western blots showing that junction protein levels were decreased in peri-infarct cortex after pMCAO compared to Shams, and increased after treatment with PROG and ALLO. (B) Quantitative analysis showing significantly lower levels of junction proteins following stroke compared to Shams. The level of claudin5 and occludin1 is significantly increased after treatment with PROG and ALLO following stroke. *L vs. S; L vs. #LP and #LA; $p < 0.05$; $n = 6$.

Figure 6.

(A) Representative Western blot showing that TNF- α and IL-6 are highly expressed in injured brain compared to Shams, and are reduced by treatment with PROG and ALLO. (B) Quantitative analysis with significant expression of TNF- α and IL-6 following stroke compared to Shams. Expression of TNF- α and IL-6 is significantly reduced after treatment with PROG and ALLO following stroke. *L vs. S; L vs. #LP and #LA; $p < 0.05$; $n = 6$.

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 minutes before occlusion; 90 minutes after occlusion			

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