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Progesterone is neuroprotective against ischemic brain injury through its effects on the PI3K/Akt signaling pathway

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Abstract

We tested the hypothesis that the phosphatidylinositol-3 kinase (PI3K/Akt) pathway mediates some of the neuroprotective effects of progesterone (PROG) after ischemic stroke. We examined whether PROG acting through the PI3K/Akt pathway could affect the expression of vascular endothelial growth factor (VEGF) and brain-derived neurotrophic factor (BDNF). Rats underwent permanent focal cerebral ischemia (pMCAO) by electro-coagulation and received intraperitoneal injections of PROG (8mg/kg) or vehicle at 1h post-occlusion and subcutaneous injections at 6, 24, and 48h. pAkt/Akt levels, apoptosis and apoptosis-related proteins (pBAD, BAD, caspase-3, and cleaved caspase-3) were analyzed by TUNEL assays, Western blotting and immunohistochemistry at 24h post-pMCAO. VEGF and BDNF were analyzed at 24, 72h and 14 days post-pMCAO with Western blots. Following pMCAO, PROG treatment significantly ($p < 0.05$) reduced ischemic lesion size and edema. Treatment with PROG significantly ($p < 0.05$) decreased VEGF at 24 and 72h but increased VEGF expression 14d after injury. The treatment also increased BDNF, and attenuated apoptosis by increasing Akt phosphorylation compared to vehicle-alone. The selective PI3K inhibitor Wortmannin compromised PROG-induced neuroprotective effects and reduced the elevation of pAkt levels in the ischemic penumbra. Our findings lead us to suggest that the PI3K/Akt pathway can play a role in mediating the neuroprotective effects of PROG after stroke by altering the expression of trophic factors in the brain.

Keywords

apoptosis; growth factors; ischemic stroke; neuroprotection; phosphoinositide-3-kinase/Akt pathway; progesterone

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DISCLOSURE/CONFLICT OF INTEREST

DGS is entitled to royalty payment (~6.5%) 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 (~60.0%) in accordance with its conflict of interest policies. The abstract was presented at the AHA/ASA International Stroke Conference, February 9–11, 2011, Los Angeles, CA.

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1.0 Background: The P13/Akt Pathway and Stroke Neuroprotection

Because of its complex pathology, treatment of ischemic stroke remains a serious challenge. Tissue plasminogen activator (tPA) is still the only drug approved for the acute treatment of stroke, and it has therapeutic limitations because of its neurotoxic effects (Lansberg et al., 2007; Marder et al., 2010). A safe and effective therapy that can target multiple pathways to neuroprotection is urgently needed.

The phosphoinositide 3-kinase/Protein kinase B (PI3K/Akt) is a central mediator in the signal transduction pathways that help to regulate cell growth, metabolism, inflammation and cell survival in response to growth factors (Brazil et al., 2004; Zhao et al., 2006). The neuroprotective role of the PI3K/Akt pathway after ischemic stroke has already been demonstrated (Xu et al., 2008; Wang et al., 2009). Following cerebral ischemia, levels of pAkt transiently increase within hours in neurons (Noshita et al., 2001; Shibata et al., 2002), and this elevation is believed to be a neuroprotective response (Noshita et al., 2001; Zhao et al., 2006). Activated Akt phosphorylates a number of downstream proteins, including several associated with apoptosis including *BAD*, glycogen synthase kinase-3 (GSK-3), caspases and others (Srivastava and Pandey, 1998; Luo et al., 2003).

The neurosteroid progesterone (PROG) is a pleiotropic hormone shown to have neuroprotective effects in various models of neuronal injury (Stein, 2008; Sayeed and Stein, 2009; Ishrat et al., 2010; Djebaili et al., 2005; Cutler et al., 2007). Recent studies from our laboratory have demonstrated robust neuroprotective effects of PROG on infarct volume and functional outcomes in a number of ischemic stroke models mimicking the clinical condition (Sayeed et al., 2006; 2007; Ishrat et al., 2009). Furthermore, PROG is safe and has been well tolerated in recent clinical trials for the treatment of traumatic brain injury (TBI) (Wright et al., 2007; Xiao et al., 2008). PROG acts in the brain in part through the classical steroid nuclear PROG receptors (PRs) (Brinton et al., 2008). Ligand binding to PRs results in their activation, allowing the recruitment of transcriptional coregulators to control the transcription of target genes. Further, PROG activates membrane PROG receptors dx-25, PR-A, PR-B, and PR-C (Brinton et al., 2008; Guennoun et al., 2008), which are involved in the regulation of membrane/cytoplasmic signaling and the activation/phosphorylation of different kinases such as extracellular signal-regulated kinase (ERK), PI3K/Akt, and GSK3 (Singh, 2001; Guerra-Araiza et al., 2007; Guerra-Araiza et al., 2009). These multiple actions of PROG may then help to modulate neuronal differentiation, neuronal function, and neuroprotection (Cai et al., 2008; Stein, 2008; Zhang et al., 2010). Studies continue to demonstrate the variety of mechanisms and pathways by which PROG provides neuroprotection in TBI and stroke (Stein and Hurn, 2009). However, other molecular mechanisms and signaling pathways underlying PROG's neuroprotective effects after stroke remain to be identified.

In light of the neuroprotective role of the PI3K/Akt pathway in cerebral ischemia, we tested the hypothesis that PROG's neuroprotective effect after ischemic injury involves activation of the PI3K/Akt pathway. We further hypothesized that treatment with wortmannin, a selective PI3K inhibitor (Ui et al., 1995), could compromise the beneficial effects of PROG and thus demonstrate that PROG's actions on the activation of the PI3K/Akt pathway is of the important mechanisms involved in improving stroke outcome.

2.0 Experimental Procedures

2.1 Animals and drug administration

Adult male Sprague-Dawley rats (350–400g; Charles River Laboratories, Wilmington, MA) were used according to procedures approved by the Institutional Animal Care and Use

Committee, Emory University (protocol # 306–2008). The rats were separated into three groups: sham-operated vehicle-treated control (S, n=20); permanent middle cerebral artery occlusion (pMCAO) + vehicle (L, n=20); and pMCAO + PROG (8 mg/kg; LP, n=20). PROG (P-0130; Sigma-Aldrich Co., St. Louis, MO) was dissolved in 22.5% 2-hydroxypropyl- β cyclodextrin and administered by intraperitoneal (IP) injection 1h post-occlusion, and then subcutaneously at 6, 24, and 48h post-occlusion. The PROG dose used in this experiment was determined from previous studies showing that 8 mg/kg provides consistent protective effects following central nervous system (CNS) injuries (Djebaili et al., 2005; Ishrat et al., 2010). In a separate experiment, wortmannin was given (15 μ g/kg, intravenously (IV)) at 30 min post-pMCAO to L and LP groups (LW and LPW, n=10 for each group) to determine whether inhibition of the PI3K/Akt pathway was involved in attenuating the neuroprotective effects of PROG.

2.2 Induction of pMCAO

Prior to pMCAO, anesthesia was induced by 5% isoflurane and then maintained during surgery at 1.5–2% in 2:1 nitrous oxide and oxygen. The left MCA was exposed and electrocoagulated as described previously (Ishrat et al., 2010). Sham-operated rats were subjected only to MCA exposure 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% (Table 1).

Permanent MCAO is considered a clinically relevant model of human stroke. Permanent or thromboembolic occlusion of cerebral arteries, especially MCAO, is the commonest type (>80%) of focal stroke in humans (Delcker et al., 1993; Hacke et al., 1996; Kassem-Moussa and Graffagnino, 2002). The Stroke Therapy Academic Industry Roundtable (STAIR) endorsed the testing of novel agents in permanent models of ischemia to simulate better the typical human stroke without reperfusion (Fisher, 2003).

2.3 Assessment of infarct size

Cerebral infarct size was evaluated according to previously applied methods (Ishrat et al., 2010). Twenty-four hours after pMCAO, rats were given an overdose of Nembutal (2 ml/kg, IP) and then transcardiacally perfused with cold saline followed by 4% paraformaldehyde in phosphate-buffered sucrose (PBS) (pH 7.4) via the ascending aorta. Brains were removed and post-fixed in 4% paraformaldehyde for 48h 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 20 μ m-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. The infarct area was measured on each brain section using NIH imaging software (Image J, version 1.38, NIH, Rockville, MD). Infarct size was then calculated by multiplying the infarct area on each section by the distance between sections and represented as a percentage of the size of the contralateral hemispheric side \pm SEM. The calculations and presentation of infarct sizes were corrected for hemispheric edema index. The percentage of brain edema formation was calculated with the formula [(contralateral side – ipsilateral side)/ contralateral side] \times 100%.

2.4 Tissue collection

Rats were overdosed with Nembutal (2 ml/kg; IP) at 24 and 72h and 14 days after pMCAO, and then decapitated while anesthetized. We used a brain matrix for sampling peri-infarct (penumbra) cortical regions. Brain tissue was rapidly dissected and then cut 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.

2.5 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 1h on 4–20% 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-Akt, pAkt, *BAD*, *pBAD*, caspase-3, brain-derived neurotrophic factor (BDNF) (1:1000; Cell Signaling, Danvers, MA), and vascular endothelial growth factor (VEGF) (1:500; Abcam, Cambridge, MA). 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).

2.6 TUNEL staining

For TUNEL staining, rats were overdosed and anesthetized with Nembutal at 24h post-pMCAO and then perfused transcardially with cold phosphate-buffered saline (PBS) and 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 72h. After cryopreservation, brains were cut into two slices at 2 mm posterior from bregma (bregma -2 mm). Each slice was cut on a cryostat to obtain five sections (12 µm). TUNEL-staining was performed using an *In Situ* Cell Death Detection Kit (Roche Applied Science, Mannheim, Germany) according to the manufacturer's protocol. Briefly, sections were permeabilized with 0.01% Triton X-100 (Sigma) in 0.1% sodium citrate (2 min on ice), and then incubated (60 min, 37°C) with the TUNEL reaction mixture in a humidified atmosphere. Every fifth section of each slice was collected. We then counted TUNEL-positive cells in three randomly selected areas of the damaged hemisphere using a light microscope with 200 x magnification.

2.6 Immunohistochemistry

For immunohistochemistry, the brains were sectioned in the coronal plane at a thickness of 12 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. The antibody used was cleaved caspase-3 (1:16000; Cell Signaling). 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.

2.7 Statistics

Data were presented as mean ± 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.

3.0 RESULTS

3.1 Effects of PROG on pAkt/Akt

Consistent with a previous study (Gao et al., 2008), Western blot analysis post-pMCAO showed up-regulated pAkt in the vehicle-treated animals compared to sham-operated controls (Figure 1). We confirmed our hypothesis that PROG treatment significantly ($p<0.05$) increased the *ratio* of pAkt/Akt expression at 24h after pMCAO compared to the vehicle-treated pMCAO and sham-operated groups. Administration of wortmannin significantly ($p<0.05$) reduced the elevation of pAkt induced by PROG.

3.2 Effects of PROG on infarct size and edema

Our analysis of cresyl violet-stained brain tissue slices showed that, compared to vehicle-treated controls, PROG administration significantly ($p<0.05$) reduced total infarct size and edema formation by 45.54% and 39.72% respectively at 24h after pMCAO (Figure 2). We further investigated whether the protective effect of PROG after pMCAO could be blocked by wortmannin, a PI3K inhibitor. The infarct size of groups treated with wortmannin alone or in combination with PROG was comparable to that of the vehicle-treated control group. Wortmannin treatment eliminated the protection induced by PROG.

3.3 Effects of PROG on TUNEL

TUNEL staining was used to assess the typical DNA laddering pattern of neurons. We observed a significant increase in the number of TUNEL-positive cells in the penumbra of the injury at 24h in pMCAO rats compared to shams (Figure 3). PROG treatment significantly ($p<0.05$) decreased the number of TUNEL-labeled apoptotic cells compared to the vehicle-treated pMCAO group. This effect was significantly compromised by treatment with wortmannin.

3.4 Effects of PROG on pBAD, BAD, caspase-3 and cleaved caspase-3

Western blotting was used to evaluate the effect of PROG on the apoptosis-related protein *BAD* (Figure 4.1). PROG administration significantly ($p<0.05$) inhibited the pBAD decrease caused by the pMCAO. After pMCAO the expression of caspase-3 was increased significantly ($p<0.05$) in the cortical penumbra compared to sham-operated rats. PROG treatment significantly ($p<0.05$) decreased caspase-3 compared to the vehicle-treated pMCAO group (Figure 4.2).

The immunohistochemical expression of cleaved caspase-3 immunopositive signals in the peri-infarct area of brain sections was markedly increased at 24h following pMCAO+vehicle compared to the sham-operated+vehicle group (Figure 4.3). Following pMCAO, PROG treatment significantly ($p<0.05$) reduced the immunopositive signals of cleaved caspase-3 compared to the vehicle-treated pMCAO group. Treatment of wortmannin partly reduced the protective effects of PROG on these markers.

3.5 Effects of PROG on VEGF and BDNF

Inhibition of VEGF is thought to be neuroprotective in the acute stage of injury (Zhang et al., 2000; Chiba et al., 2008). In contrast, at the later stages of stroke, an increase in the expression of VEGF is considered to be beneficial because it can stimulate angiogenesis (Jin et al., 2002; Whitaker et al., 2007). PROG treatment significantly ($p<0.05$) reduced VEGF expression at 24 and 72h compared to vehicle-treated pMCAO rats, but increased it significantly ($p<0.05$) at 14 days after pMCAO. Effects of PROG on the level of VEGF in the cortical penumbra at 24h, 72h and 14 days following pMCAO are shown in Figure 5.

BDNF levels did not change significantly at 24h after pMCAO, but significantly ($p < 0.05$) decreased at 72h and 14 days in the vehicle-treated pMCAO group compared to shams. PROG treatment significantly ($p < 0.05$) increased BDNF levels compared to the vehicle-treated pMCAO group (Figure 5).

4.0 DISCUSSION

PROG is known to have multiple neuroprotective effects in various models of neuronal injury including stroke. However, not all the mechanisms of PROG's actions are known and they may be somewhat different for stroke compared to penetrating or blunt traumatic injury to the brain. The aim of the present study was to learn more about some of PROG's signaling mechanisms of action in preventing brain damage after ischemic stroke. We now show for the first time that PROG can attenuate neuronal damage after permanent ischemic stroke by activating the Akt-mediated signaling pathway. Inhibition of this pathway by giving wortmannin compromised PROG's protective effects, an effect which might have been even more pronounced with a larger dose of this antagonist. The effects of PROG on the pAkt pathway might have important implications for the regulation of the actions of growth factors in the brain known to mediate neuroprotection and repair following injury. In the present study, ischemic injury induced an increase of VEGF and a decrease of BDNF, while PROG administration, through its effects on the pAkt/Akt signaling pathway, significantly reduced VEGF and enhanced BDNF levels.

4.1 Akt Phosphorylation

Phosphorylation of Akt (pAkt), an important physiologic mediator of the PI3K pathway, promotes cell survival and prevents apoptosis by regulating several downstream targets, including *BAD*, GSK-3, caspases and others (Srivastava and Pandey, 1998; Luo et al., 2003; Zhao et al., 2006). Activation of the PI3K/Akt pathway protects brain tissue from ischemic injury by inhibiting the expression of genes that promote vascular permeability, apoptosis, and inflammation (Blum et al., 2001; Williams et al., 2006; Zhao et al., 2006). For example, cerebral ischemia activates Akt, which in turn blocks *BAD* and Bax translocation into the mitochondrial membrane, subsequently preventing mitochondrial cytochrome c release and the initiation of detrimental caspase activity (Noshita et al., 2001; Zhao et al., 2006). Previous studies suggest that these pathways are directly implicated in the mechanisms of PROG-mediated protection after CNS injury (Kaur et al., 2007; Guerra-Araiza et al., 2009).

Our present experiment focused on the effect of PROG on the activation of Akt, *BAD* and caspase-3 in response to ischemic injury-induced cell death. In the presence of survival factors, activated Akt phosphorylates specific residues of pro-apoptotic proteins such as *BAD*, leading to suppression of their apoptotic activity and thereby promoting cell survival (Zhao et al., 2006). Apoptotic stimuli have been shown to induce dephosphorylation of *BAD*, which activates Bax and caspase-3 (Yang et al., 1995). An increase in the level of phosphorylated *BAD* plays an important role in the inhibition of apoptosis (Yang et al., 1995). Consistent with previous studies (Xu et al., 2008; Wang et al., 2009), our results showed a significant change in the apoptotic cell death markers (pBAD, caspase-3 and TUNEL staining) at 24h after ischemic stroke.

Moreover, our data show that PROG increases the levels of pAkt, attenuates its downstream targets (p*BAD*), and decreases caspase-3, thereby reducing ischemic neuronal death, as observed via TUNEL staining and reduced infarct size. Inhibiting PI3K/Akt with wortmannin attenuated the activation of Akt and thus compromised the neuroprotective effects of PROG administration. Our results can be taken to suggest that at least some the neuroprotective effects of PROG are mediated through the activation of the PI3K/Akt pathway after ischemic stroke. Although our results showing some upregulation of pAkt

following pMCAO seem puzzling, our results showing decrease in pBAD are consistent with earlier findings (Noshita et al., 2001; Zhao et al., 2006). One can argue that there has to be a direct and proportional relation between pAkt and pBAD expression, but it is conceivable that, in the penumbral region (sampled for the present study) as opposed to the core of the stroke, Akt is activated because the damage inflicted by the stroke model is relatively moderate. Studies have shown that phosphorylation of Akt depends on the severity of the injury/stress. In a global ischemia model, Yano et al. (Yano et al., 2001) demonstrated that in the CA1 region of the hippocampus, phospho-Akt was dephosphorylated immediately after ischemia and rephosphorylated above control levels for 6h after only 5 minutes of global ischemia. These investigators also demonstrated that the extent of ischemic injury could make a difference in the phospho-Akt level in the CA1 region—lethal damage reduced phospho-Akt in the CA1 region, whereas sublethal damage increased phospho-Akt 7 days after global ischemia. We think it is conceivable that there is not enough Akt upregulation to trigger BAD phosphorylation at 24h post-injury.

4.2 The role of VEGF and BDNF

VEGF and BDNF are neurotrophic factors with important functional implications in the brain's vascular remodeling, neurogenesis, neuronal survival and plasticity (Chen et al., 2005; Greenberg et al., 2009). Both are involved in PROG's mechanisms of action in the CNS (Gonzalez et al., 2004; Kaur et al., 2007; Swiatek-De Lange et al., 2007). VEGF can mediate both neuroprotection and vascular permeability through the VEGF/VEGFR2/FIk1 and PI3K/Akt pathways (Zhang et al., 2000; Kilic et al., 2006; Chiba et al., 2008). At a later stage of stroke, VEGF has been shown to stimulate angiogenesis, and this can be beneficial (Jin et al., 2002). However, in animal models and clinical stroke, during the acute stage of ischemic injury, upregulation of VEGF can result in the exacerbation of ischemic cell damage (Krupinski et al., 1994; Zhang et al., 2000), increased BBB leakage, risk of hemorrhagic transformation, and increased size of infarction (Zhang et al., 2000). Inhibition of endogenous VEGF by topical application of anti-VEGF antibody in the ischemic cortex has been shown to decrease BBB disruption (Zhang et al., 2000). We have previously reported that PROG treatment attenuated BBB permeability following pMCAO (Ishrat et al., 2010). Our current results showing acute reduction of VEGF in PROG-treated animals after ischemic injury can be taken to suggest that PROG preserved the functional and structural integrity of the BBB in part through its early inhibition of VEGF.

More studies on Akt signaling and the mechanism by which PROG mediates VEGF expression after stroke are needed. In this proof-of-principle experiment, our focus was to analyze the expression pAkt/Akt and its related apoptotic cell death markers in brain rather than on role of PI3K/Akt signaling on growth factor regulation. It is possible that the downregulation of VEGF we observed in the present study may be from activation of Akt or through another yet unknown pathway. VEGF triggers angiogenesis at a later stage of stroke and this response may be independent of Akt actions on VEGF. Additional studies will be needed to evaluate the timing and spatial localization of VEGF to shed light on how PROG mediates VEGF expression and angiogenesis in the ischemic brain.

BDNF acts specifically to protect tissue from insult and to promote neuronal plasticity through various mechanisms including the PI3K/Akt-pathway (Kaur et al., 2007; Greenberg et al., 2009). PROG induces the expression of BDNF in various neuronal injuries (Gonzalez et al., 2004; Kaur et al., 2007). The interaction of PROG with the intracellular signaling of these growth factors via regulation of the Akt pathway may modulate the actions of BDNF in neuronal development, cell survival and functional response to brain injury (Mizuno et al., 2003; Kaur et al., 2007). Post-treatment with BDNF has been shown to be neuroprotective after ischemic stroke (Zhang and Pardridge, 2001). Our results show that

ischemic injury induced a significant decrease in BDNF expression which was counteracted following PROG treatment.

4.3 Conclusion

In this one experiment we focused on the acute-stage treatment of ischemic injury. Further studies are needed to look at PROG's possible long-term effects, including its effects on vascular endothelial apoptosis and neuronal survival. Although stroke remains a complex systemic disease that activates many signaling pathways, our current data can be interpreted to suggest that the Akt pathway activated by PROG during the acute stage of the stroke-induced injury cascade mediates some of its neuroprotective effects. Whether the acute downregulation of VEGF observed in the present study resulted from activation of Akt or from a direct effect of PROG remains to be determined.

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References

- Blum S, Issbrucker K, Willuweit A, Hehlhans S, Lucerna M, Mechtcheriakova D, Walsh K, von der Ahe D, Hofer E, Clauss M. An inhibitory role of the phosphatidylinositol 3-kinase-signaling pathway in vascular endothelial growth factor-induced tissue factor expression. *J Biol Chem.* 2001; 276:33428–33434. [PubMed: 11445586]
- Brazil DP, Yang ZZ, Hemmings BA. Advances in protein kinase B signalling: AKTion on multiple fronts. *Trends Biochem Sci.* 2004; 29:233–242. [PubMed: 15130559]
- Brinton RD, Thompson RF, Foy MR, Baudry M, Wang J, Finch CE, Morgan TE, Pike CJ, Mack WJ, Stanczyk FZ, Nilsen J. Progesterone receptors: form and function in brain. *Front Neuroendocrinol.* 2008; 29:313–339. [PubMed: 18374402]
- Cai W, Zhu Y, Furuya K, Li Z, Sokabe M, Chen L. Two different molecular mechanisms underlying progesterone neuroprotection against ischemic brain damage. *Neuropharmacology.* 2008; 55:127–138. [PubMed: 18572204]
- Chen J, Zhang C, Jiang H, Li Y, Zhang L, Robin A, Katakowski M, Lu M, Chopp M. Atorvastatin induction of VEGF and BDNF promotes brain plasticity after stroke in mice. *J Cereb Blood Flow Metab.* 2005; 25:281–290. [PubMed: 15678129]
- Chiba Y, Sasayama T, Miyake S, Koyama J, Kondoh T, Hosoda K, Kohmura E. Anti-VEGF receptor antagonist (VGA1155) reduces infarction in rat permanent focal brain ischemia. *Kobe J Med Sci.* 2008; 54:E136–146. [PubMed: 18772615]
- Cutler SM, Cekic M, Miller DM, Wali B, VanLandingham JW, Stein DG. Progesterone improves acute recovery after traumatic brain injury in the aged rat. *J Neurotrauma.* 2007; 24:1475–1486. [PubMed: 17892409]
- Delcker A, Diener HC, Timmann D, Faustmann P. The role of vertebral and internal carotid artery disease in the pathogenesis of vertebrobasilar transient ischemic attacks. *Eur Arch Psychiatry Clin Neurosci.* 1993; 242:179–183. [PubMed: 8461343]
- Djebaili M, Guo Q, Pettus EH, Hoffman SW, Stein DG. The neurosteroids progesterone and allopregnanolone reduce cell death, gliosis, and functional deficits after traumatic brain injury in rats. *J Neurotrauma.* 2005; 22:106–118. [PubMed: 15665606]
- Fisher M. Recommendations for advancing development of acute stroke therapies: Stroke therapy academic industry roundtable 3. *Stroke.* 2003; 34:1539–1546. [PubMed: 12750546]
- Gao X, Zhang H, Takahashi T, Hsieh J, Liao J, Steinberg GK, Zhao H. The Akt signaling pathway contributes to postconditioning's protection against stroke; the protection is associated with the MAPK and PKC pathways. *J Neurochem.* 2008; 105:943–955. [PubMed: 18182053]

- Gonzalez SL, Labombarda F, Gonzalez Deniselle MC, Guennoun R, Schumacher M, De Nicola AF. Progesterone up-regulates neuronal brain-derived neurotrophic factor expression in the injured spinal cord. *Neuroscience*. 2004; 125:605–614. [PubMed: 15099674]
- Greenberg ME, Xu B, Lu B, Hempstead BL. New insights in the biology of BDNF synthesis and release: implications in CNS function. *J Neurosci*. 2009; 29:12764–12767. [PubMed: 19828787]
- Guennoun R, Meffre D, Labombarda F, Gonzalez SL, Deniselle MC, Stein DG, De Nicola AF, Schumacher M. The membrane-associated progesterone-binding protein 25-Dx: expression, cellular localization and up-regulation after brain and spinal cord injuries. *Brain Res Rev*. 2008; 57:493–505. [PubMed: 17618691]
- Guerra-Araiza C, Amorim MA, Camacho-Arroyo I, Garcia-Segura LM. Effects of progesterone and its reduced metabolites, dihydroprogesterone and tetrahydroprogesterone, on the expression and phosphorylation of glycogen synthase kinase-3 and the microtubule-associated protein tau in the rat cerebellum. *Dev Neurobiol*. 2007; 67:510–520. [PubMed: 17443805]
- Guerra-Araiza C, Amorim MA, Pinto-Almazan R, Gonzalez-Arenas A, Campos MG, Garcia-Segura LM. Regulation of the phosphoinositide-3 kinase and mitogen-activated protein kinase signaling pathways by progesterone and its reduced metabolites in the rat brain. *J Neurosci Res*. 2009; 87:470–481. [PubMed: 18752305]
- Hacke W, Schwab S, Horn M, Spranger M, DeGeorgia M, von Krummer R. “Malignant” middle cerebral artery territory infarction: Clinical course and prognostic signs. *Arch Neurol*. 1996; 53:309–315. [PubMed: 8929152]
- Ishrat T, Sayeed I, Atif F, Stein DG. Effects of progesterone administration on infarct volume and functional deficits following permanent focal cerebral ischemia in rats. *Brain Res*. 2009; 1257:94–101. [PubMed: 19135987]
- Ishrat T, Sayeed I, Atif F, Hua F, Stein DG. Progesterone and allopregnanolone attenuate blood-brain barrier dysfunction following permanent focal ischemia by regulating the expression of matrix metalloproteinases. *Exp Neurol*. 2010; 226:183–190. [PubMed: 20816826]
- Jin K, Zhu Y, Sun Y, Mao XO, Xie L, Greenberg DA. Vascular endothelial growth factor (VEGF) stimulates neurogenesis in vitro and in vivo. *Proc Natl Acad Sci U S A*. 2002; 99:11946–11950. [PubMed: 12181492]
- Kassem-Moussa H, Graffignino C. Nonocclusion and spontaneous recanalization rates in acute ischemic stroke: A review of cerebral angiography studies. *Arch Neurol*. 2002; 59:1870–1873. [PubMed: 12470173]
- Kaur P, Jodhka PK, Underwood WA, Bowles CA, de Fiebre NC, de Fiebre CM, Singh M. Progesterone increases brain-derived neurotrophic factor expression and protects against glutamate toxicity in a mitogen-activated protein kinase- and phosphoinositide-3 kinase-dependent manner in cerebral cortical explants. *J Neurosci Res*. 2007; 85:2441–2449. [PubMed: 17549730]
- Kilic E, Kilic U, Wang Y, Bassetti CL, Marti HH, Hermann DM. The phosphatidylinositol-3 kinase/Akt pathway mediates VEGF’s neuroprotective activity and induces blood brain barrier permeability after focal cerebral ischemia. *FASEB J*. 2006; 20:1185–1187. [PubMed: 16641198]
- Krupinski J, Kaluza J, Kumar P, Kumar S, Wang JM. Role of angiogenesis in patients with cerebral ischemic stroke. *Stroke*. 1994; 25:1794–1798. [PubMed: 7521076]
- Lansberg MG, Albers GW, Wijman CA. Symptomatic intracerebral hemorrhage following thrombolytic therapy for acute ischemic stroke: a review of the risk factors. *Cerebrovasc Dis*. 2007; 24:1–10. [PubMed: 17519538]
- Luo HR, Hattori H, Hossain MA, Hester L, Huang Y, Lee-Kwon W, Donowitz M, Nagata E, Snyder SH. Akt as a mediator of cell death. *Proc Natl Acad Sci U S A*. 2003; 100:11712–11717. [PubMed: 14504398]
- Marder VJ, Jahan R, Gruber T, Goyal A, Arora V. Thrombolysis with plasmin: implications for stroke treatment. *Stroke*. 2010; 41:S45–49. [PubMed: 20876504]
- Mizuno M, Yamada K, Takei N, Tran MH, He J, Nakajima A, Nawa H, Nabeshima T. Phosphatidylinositol 3-kinase: a molecule mediating BDNF-dependent spatial memory formation. *Mol Psychiatry*. 2003; 8:217–224. [PubMed: 12610654]

- Noshita N, Lewen A, Sugawara T, Chan PH. Evidence of phosphorylation of Akt and neuronal survival after transient focal cerebral ischemia in mice. *J Cereb Blood Flow Metab.* 2001; 21:1442–1450. [PubMed: 11740206]
- Sayed I, Wali B, Stein DG. Progesterone inhibits ischemic brain injury in a rat model of permanent middle cerebral artery occlusion. *Restor Neurol Neurosci.* 2007; 25(2):151–9. [PubMed: 17726274]
- Sayed I, Guo Q, Hoffman SW, Stein DG. Allopregnanolone, a progesterone metabolite, is more effective than progesterone in reducing cortical infarct volume after transient middle cerebral artery occlusion. *Ann Emerg Med.* 2006; 47(4):381–9. [PubMed: 16546625]
- Sayed I, Stein DG. Progesterone as a neuroprotective factor in traumatic and ischemic brain injury. *Prog Brain Res.* 2009; 175:219–237. [PubMed: 19660659]
- Shibata M, Yamawaki T, Sasaki T, Hattori H, Hamada J, Fukuuchi Y, Okano H, Miura M. Upregulation of Akt phosphorylation at the early stage of middle cerebral artery occlusion in mice. *Brain Res.* 2002; 942:1–10. [PubMed: 12031847]
- Singh M. Ovarian hormones elicit phosphorylation of Akt and extracellular-signal regulated kinase in explants of the cerebral cortex. *Endocrine.* 2001; 14:407–415. [PubMed: 11444439]
- Srivastava AK, Pandey SK. Potential mechanism(s) involved in the regulation of glycogen synthesis by insulin. *Mol Cell Biochem.* 1998; 182:135–141. [PubMed: 9609122]
- Stein DG. Progesterone exerts neuroprotective effects after brain injury. *Brain Res Rev.* 2008; 57:386–397. [PubMed: 17826842]
- Stein, DG.; Hurn, PD. Effects of sex steroids on damaged neural systems. In: Pfaff, DW.; Arnold, AP.; Etgen, AM.; Fahrbach, SE.; Rubin, RT., editors. *Hormones, brain and behavior.* 2. Vol. 4. Oxford: Elsevier; 2009. p. 2223-2258.
- Swiatek-De Lange M, Stampfl A, Hauck SM, Zischka H, Gloeckner CJ, Deeg CA, Ueffing M. Membrane-initiated effects of progesterone on calcium dependent signaling and activation of VEGF gene expression in retinal glial cells. *Glia.* 2007; 55:1061–1073. [PubMed: 17551930]
- Ui M, Okada T, Hazeki K, Hazeki O. Wortmannin as a unique probe for an intracellular signalling protein, phosphoinositide 3-kinase. *Trends Biochem Sci.* 1995; 20:303–307. [PubMed: 7667888]
- Wang HY, Wang GL, Yu YH, Wang Y. The role of phosphoinositide-3-kinase/Akt pathway in propofol-induced postconditioning against focal cerebral ischemia-reperfusion injury in rats. *Brain Res.* 2009; 1297:177–184. [PubMed: 19703434]
- Whitaker VE, Cui L, Miller S, USO, Wei L. Whisker stimulation enhances angiogenesis in the barrel cortex following focal ischemia in mice. *J Cereb Blood Flow Metab.* 2007; 27:57–68. [PubMed: 16670699]
- Williams DL, Ozment-Skelton T, Li C. Modulation of the phosphoinositide 3-kinase signaling pathway alters host response to sepsis, inflammation, and ischemia/reperfusion injury. *Shock.* 2006; 25:432–439. [PubMed: 16680006]
- Wright DW, Kellermann AL, Hertzberg VS, Clark PL, Frankel M, Goldstein FC, Salomone JP, Dent LL, Harris OA, Ander DS, Lowery DW, Patel MM, Denson DD, Gordon AB, Wald MM, Gupta S, Hoffman SW, Stein DG. ProTECT: A Randomized Clinical Trial of Progesterone for Acute Traumatic Brain Injury. *Ann Emerg Med.* 2007; 49:391–402. [PubMed: 17011666]
- Xiao G, Wei J, Yan W, Wang W, Lu Z. Improved outcomes from the administration of progesterone for patients with acute severe traumatic brain injury: a randomized controlled trial. *Crit Care.* 2008; 12:R61. [PubMed: 18447940]
- Xu X, Chua CC, Gao J, Chua KW, Wang H, Hamdy RC, Chua BH. Neuroprotective effect of humanin on cerebral ischemia/reperfusion injury is mediated by a PI3K/Akt pathway. *Brain Res.* 2008; 1227:12–18. [PubMed: 18590709]
- Yang E, Zha J, Jockel J, Boise LH, Thompson CB, Korsmeyer SJ. Bad, a heterodimeric partner for Bcl-XL and Bcl-2, displaces Bax and promotes cell death. *Cell.* 1995; 80:285–291. [PubMed: 7834748]
- Yano S, Morioka M, Fukunaga K, Kawano T, Hara T, Kai Y, Hamada J, Miyamoto E, Ushio Y. Activation of Akt/protein kinase b contributes to induction of ischemic tolerance in the Ca1 subfield of gerbil hippocampus. *J Cereb Blood Flow Metab.* 2001; 21:351–360. [PubMed: 11323521]

- Zhang Y, Pardridge WM. Neuroprotection in transient focal brain ischemia after delayed intravenous administration of brain-derived neurotrophic factor conjugated to a blood-brain barrier drug targeting system. *Stroke*. 2001; 32:1378–1384. [PubMed: 11387502]
- Zhang Z, Yang R, Cai W, Bai Y, Sokabe M, Chen L. Treatment with progesterone after focal cerebral ischemia suppresses proliferation of progenitor cells but enhances survival of newborn neurons in adult male mice. *Neuropharmacology*. 2010; 58:930–939. [PubMed: 20079361]
- Zhang ZG, Zhang L, Jiang Q, Zhang R, Davies K, Powers C, Bruggen N, Chopp M. VEGF enhances angiogenesis and promotes blood-brain barrier leakage in the ischemic brain. *J Clin Invest*. 2000; 106:829–838. [PubMed: 11018070]
- Zhao H, Sapolsky RM, Steinberg GK. Phosphoinositide-3-kinase/akt survival signal pathways are implicated in neuronal survival after stroke. *Mol Neurobiol*. 2006; 34:249–270. [PubMed: 17308356]

- PROG neuroprotection after pMCAO involves the Akt signaling pathway.
- Ischemic brain injury increases VEGF and decreases BDNF.
- PROG, acting on the pAkt/Akt signaling pathway, reduces VEGF and enhances BDNF.
- PROG increases pAkt and reduces pBAD, caspase-3, ischemic cell death and infarct size.
- PROG inhibits VEGF, preserving the integrity of the BBB to improve stroke outcome.

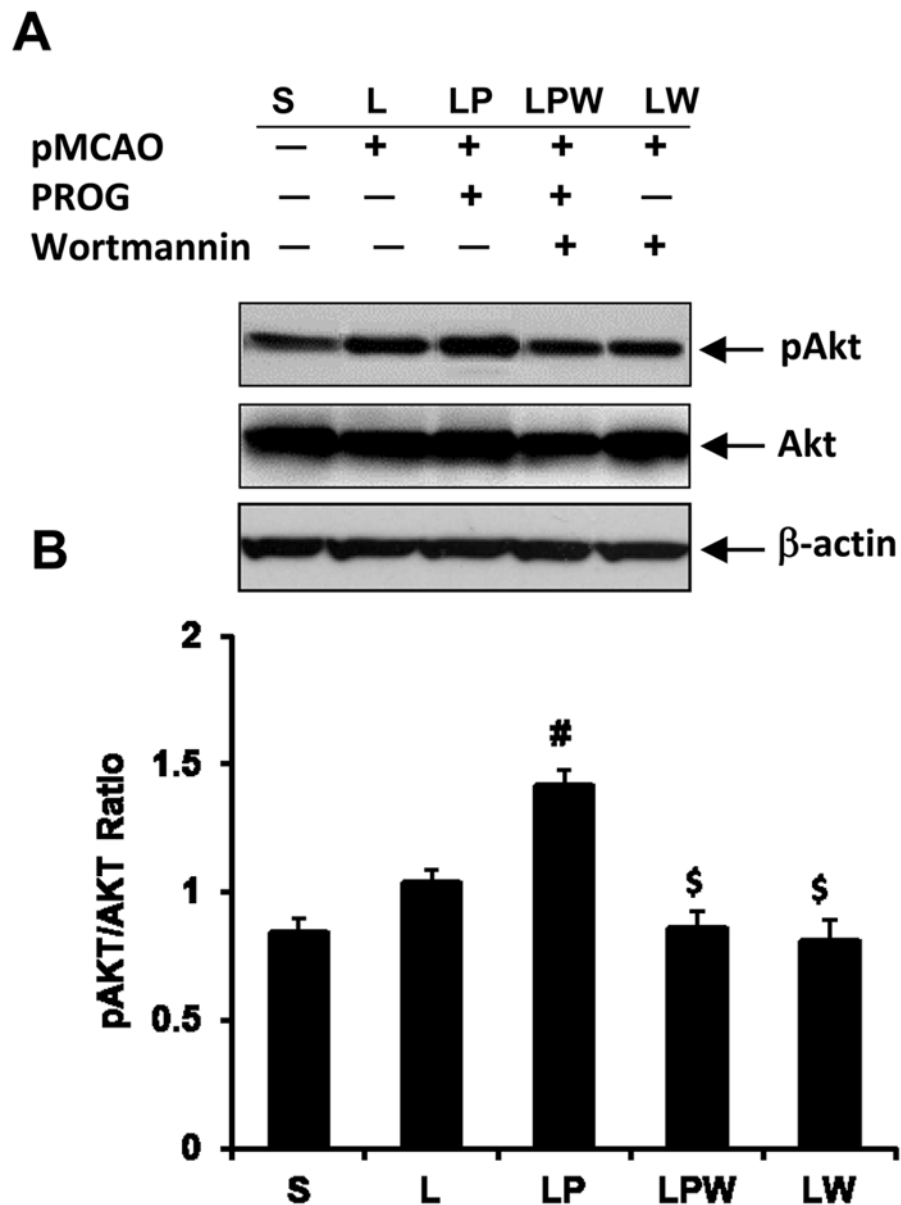


Figure 1. Effect of PROG on Akt levels at 24h post-pMCAO. **A.** Western blot analysis of phosphorylated and total Akt from representative sham (S), pMCAO-vehicle (L), pMCAO-PROG (LP), LP-wortmannin (LPW) and L-wortmannin (LW) treated rats. **B.** Bar graph shows quantitative data for phosphorylated Akt from each group. Values are mean \pm SE ($^{\#}p < 0.05$ L vs. LP, $^{\$}p < 0.05$ LP vs. LPW and LW; $n=5$).

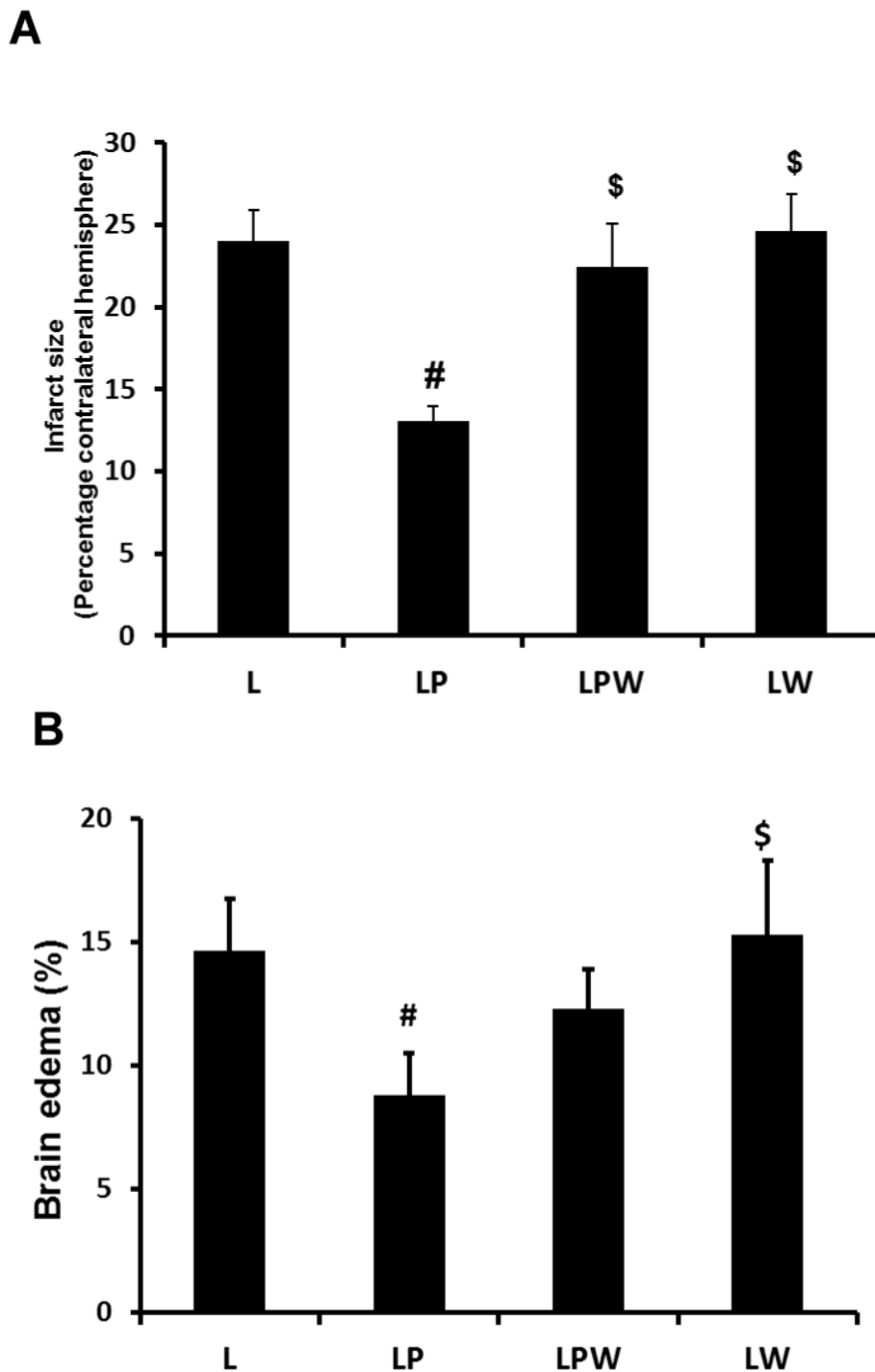


Figure 2.
A. PROG reduces infarct size at 24h post-pMCAO. The graph shows the percent area distribution of infarction (% contralateral side) in L, LP, LPW and LW groups. **B.** PROG reduces brain edema at 24h post-pMCAO. The graph shows the percent brain edema in L, LP, LPW and LW groups. Values are mean \pm SE ([#] $p < 0.05$ L vs. LP, ^{\$} $p < 0.05$ LP vs. LPW and LW).

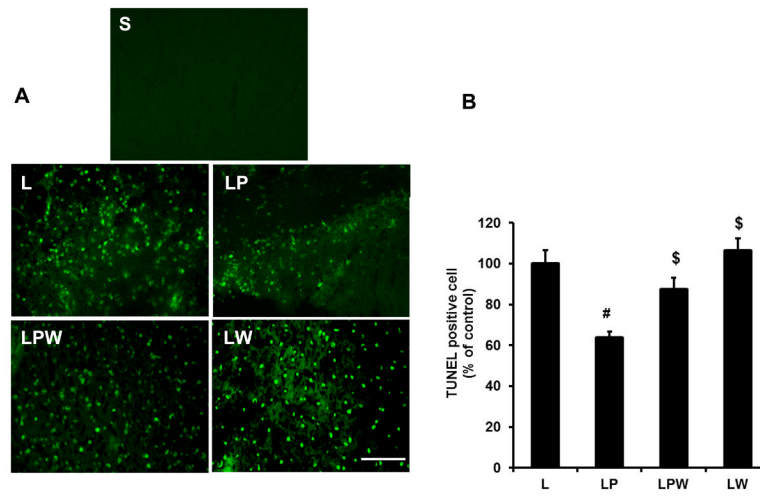
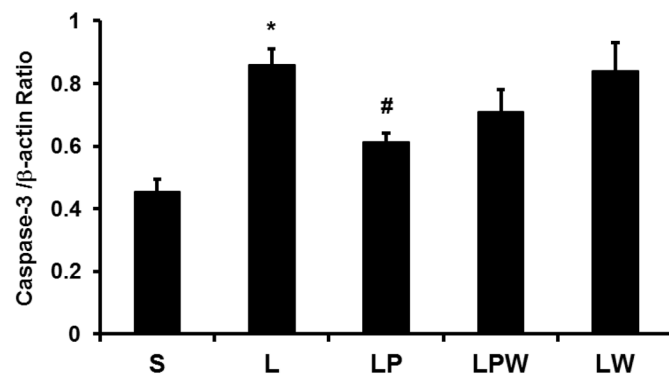
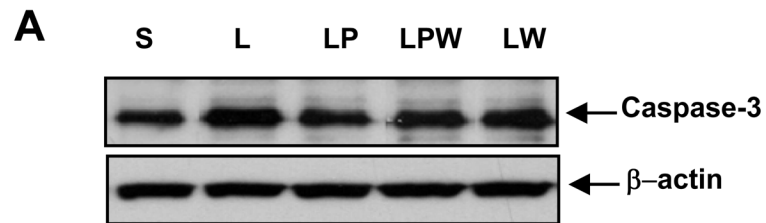
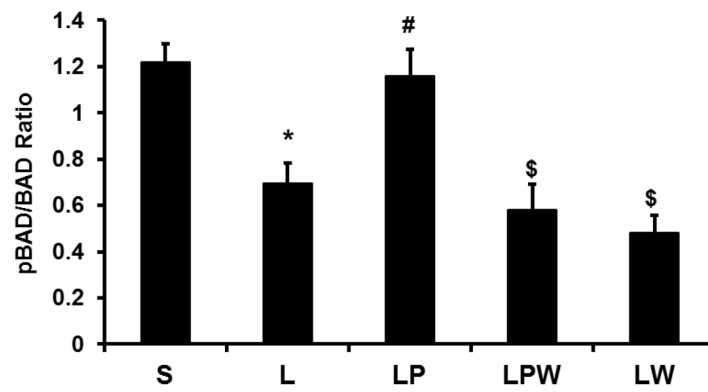
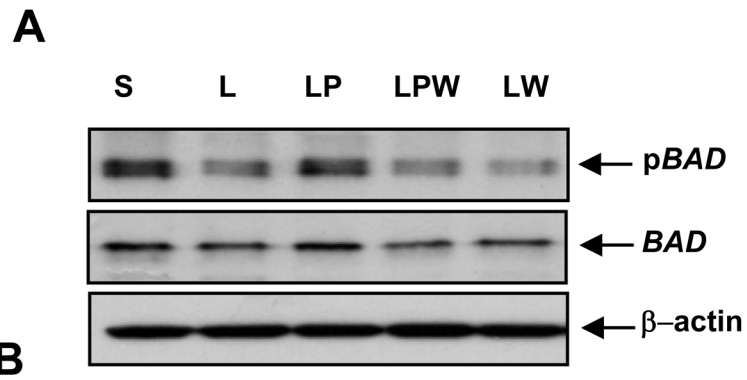


Figure 3. **A.** Representative photomicrographs of TUNEL staining in the peri-infarct cortex ($\times 400$). **B.** Percent of TUNEL-positive cells ($n=5$) in treated versus untreated rats. Values are mean \pm SE (# $p<0.05$ L vs. LP; \$ $p<0.05$ LP vs. LPW and LW).



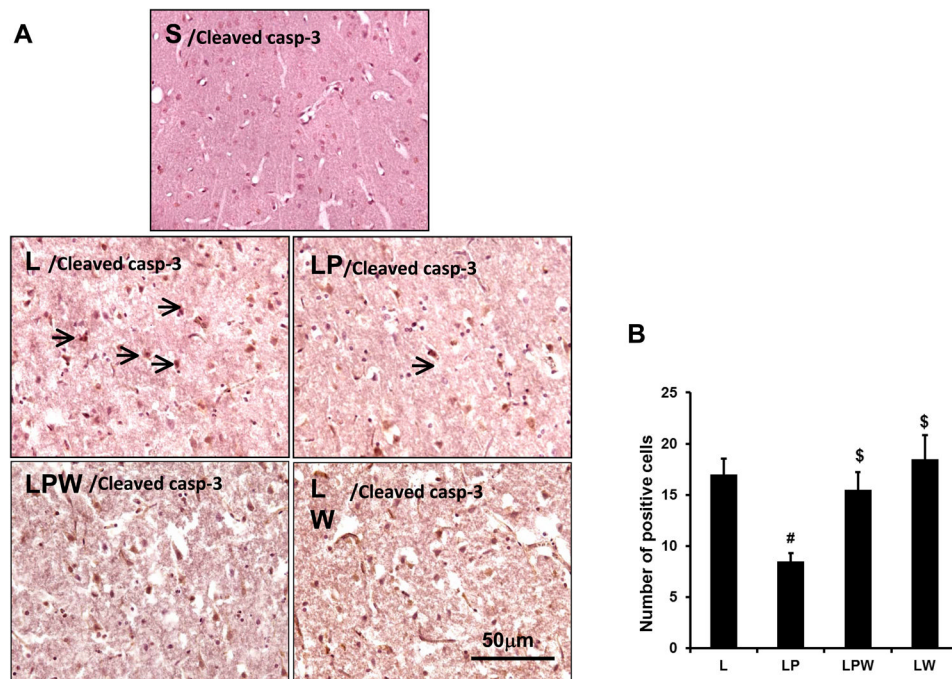


Figure 4.

Effect of PROG on p*BAD*, *BAD*, caspase-3 and cleaved caspase-3 at 24h post-pMCAO.

4.1.A. Western blot analysis of phosphorylated and total *BAD* from representative S, L, LP, LPW and LW rats. **4.1.B.** Bar graph shows quantitative data for phosphorylated *Bad* from each group (n=5). **4.2.A.** Western blot analysis of caspase-3 from representative S, L, LP, LPW and LW rats. **4.2.B.** Bar graph shows quantitative data for caspase-3 from each group (n= 5). Values are mean ±SE. **4.3.A.** Representative photomicrographs of immunohistochemical representation of the expression of cleaved caspase-3 in the peri-infarct cortex of brain sections at 24h after pMCAO. Cleaved caspase-3 immunopositive signal is increased in the peri-infarct cortex after pMCAO compared to Shams, and decreased by PROG treatment. **4.3. B.** Bar graph shows the number of cleaved caspase-3-positive cells in treated versus untreated rats (n=5). Values are mean ±SE (*p<0.05 L vs. S; #p<0.05 L vs. LP; \$p<0.05 LP vs. LPW and LW).

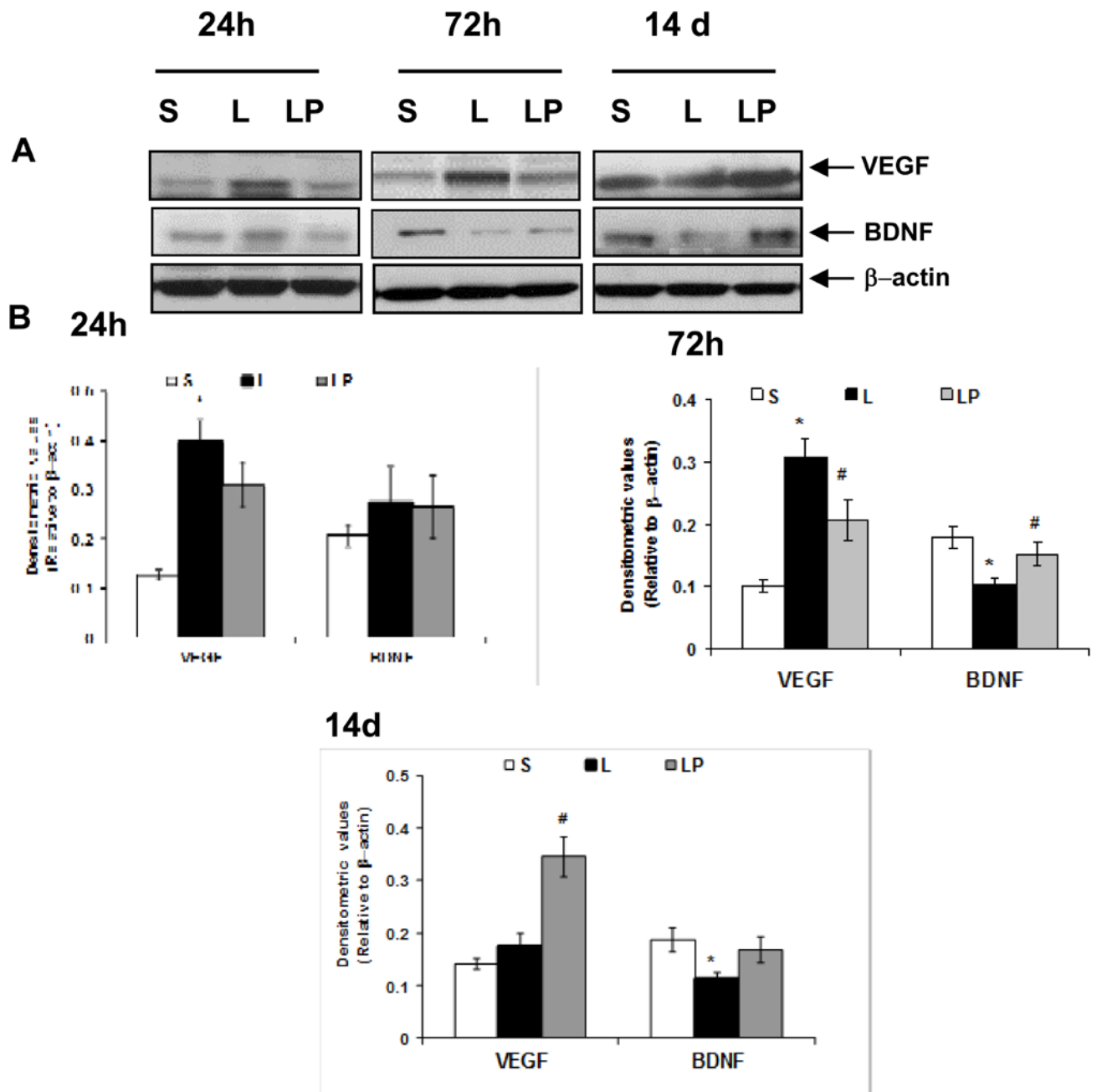


Figure 5. Effect of PROG on VEGF and BDNF expression at 24h, 72h and 14 days post-pMCAO. **A.** Western blot analysis of VEGF and BDNF expression from representative S, L and LP rats. **B.** Bar graph shows quantitative data for VEGF and BDNF from each group. Values are mean \pm SE (* p <0.05 L vs. S; # p <0.05 L vs. LP, n =5).

Table 1

Physiological Monitoring

GROUP	SPO2	HEART BEATS	TEMPERATURE
Sham (S)	95.14 ± 1.16	354.0 ± 2.12	36.64 ± 0.54
	94.54 ± 1.11	352.48 ± 2.22	36.76 ± 0.42
Lesion (L)	94.64 ± 1.25	355.74 ± 1.96	36.92 ± 0.38
	94.86 ± 1.24	355.12 ± 2.32	36.76 ± 0.72
L + PROG	93.90 ± 1.15	354.55 ± 2.44	35.95 ± 0.67
	94.50 ± 1.35	355.15 ± 2.51	36.89 ± 0.84

10 minutes before occlusion; 90 minutes after occlusion

Values are expressed as mean ± SE. 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.