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Bryostatin extends tPA time window to 6 hours following middle cerebral artery occlusion in aged female rats

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Abstract

Background and Purpose—Blood-brain barrier (BBB) disruption and hemorrhagic transformation (HT) following ischemic/reperfusion injury contributes to post-stroke morbidity and mortality. Bryostatin, a potent protein kinase C (PKC) modulator, has shown promise in treating neurological injury. In the present study, we tested the hypothesis that administration of bryostatin would reduce BBB disruption and HT following acute ischemic stroke; thus, prolonging the time window for administering recombinant tissue plasminogen activator (r-tPA).

Methods—Acute cerebral ischemia was produced by reversible occlusion of the right middle cerebral artery (MCAO) in 18–20-month-old female rats using an autologous blood clot with delayed r-tPA reperfusion. Bryostatin (or vehicle) was administered at 2 hours post-MCAO and r-tPA was administered at 6 hours post-MCAO. Functional assessment, lesion volume, and hemispheric swelling measurements were performed at 24 hours post-MCAO. Assessment of BBB permeability, measurement of hemoglobin, assessment of matrix metalloproteinase (MMP) levels by gel zymography, and measurement of PKC ϵ , PKC α , PKC δ expression by western blot were conducted at 24 hours post-MCAO.

Results—Rats treated with bryostatin prior to r-tPA administration had decreased mortality and hemispheric swelling when compared with rats treated with r-tPA alone. Administration of bryostatin also limited BBB disruption and HT and down-regulated MMP-9 expression while up-regulating PKC ϵ expression at 24 hours post-MCAO.

Conclusions—Bryostatin administration ameliorates BBB disruption and reduces the risk of HT by down-regulating MMP-9 activation and up-regulating PKC ϵ . In this proof-of-concept study, bryostatin treatment lengthened the time-to-treatment window and enhanced the efficacy and safety of thrombolytic therapy.

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Keywords

Bryostatin; blood-brain barrier; hemorrhagic transformation; Protein kinase C; MMP-9

1 Introduction

Recombinant tissue plasminogen activator (r-tPA) remains the only Federal Drug Administration approved therapeutic for ischemic stroke. The time window for r-tPA administration is 3–4.5 hours for intravenous administration; consequently, less than 5% of stroke victims are eligible to receive r-tPA due to an elevated risk of hemorrhagic transformation (HT). The elevated vascular risk is due, in part, to blood-brain barrier (BBB) disruption and increased basement membrane remodeling from matrix metalloproteinase (MMP) activation (Wang et al., 2003b; Wang and Lo, 2003). Thus, a pressing need exists for identifying therapeutics that can work in combination with r-tPA to reduce complications and extend the time window for administration.

In a prior study, we demonstrated that bryostatin, a protein kinase C (PKC) modulator, reduced ischemic brain injury in aged-females when administered at 6 hours following middle cerebral artery occlusion (MCAO) (Tan et al., 2013). In a separate study, we showed that bryostatin provided neuroprotection in young-adult males by modulating PKC activity at the BBB following neural injury (Lucke-Wold et al., 2014). We used aged-females in the current study because aged-males are drastically larger than their young counterparts. We expect bryostatin to have beneficial properties despite gender as evidenced in prior studies. Increased PKC activity has been shown to affect structural and functional integrity of the BBB following ischemic brain injury (Willis et al.). PKC ϵ enhances BBB integrity by upregulating claudin-5 expression, while PKC δ increases BBB permeability through alterations in tight junction protein localization (Selvatici et al., 2003). Following MCAO/reperfusion, upregulated expression of PKC α occurs concurrently with the downregulation of tight junction proteins zonula occludens-1, occludin, and VE-cadherin leading to increased BBB permeability and HT (Yu et al., 2012). PKC δ and PKC α also initiate the ERK1/2 mitogen-activated protein kinase (MAP kinase) pathway, which is known to regulate MMP-9 activation (Arai et al., 2003).

Following the onset of ischemia/reperfusion with delayed r-tPA, MMP enzymes are excessively activated and expressed (Romanic et al., 1998). The activation and overexpression of MMPs cause endothelium damage and leads to cleavage of extracellular matrix (ECM) proteins such as collagen, proteoglycan, and basal laminin. Ultimately, these perturbations trigger migration of inflammatory cells and large toxic molecules into the brain (Arai et al., 2003). Acute inhibition of MMP-9 could be beneficial following cerebral ischemic/reperfusion injury with a later upregulation beneficial for remodeling (Zlokovic, 2006).

In this proof-of-concept study, we reversed the treatment paradigm of our previous study and sought to determine if administration of bryostatin at 2 hours could reduce the BBB disruption and HT associated with r-tPA administration at 6 hours after MCAO. We did this using a clinically relevant ischemic stroke model that utilized r-tPA reperfusion in aged

female rats for reasons previously outlined. Results of this study demonstrate that administration of bryostatin extended the time-to-treatment window of r-tPA with reduced mortality and swelling, decreased BBB disruption, and attenuated HT.

2 Materials and Methods

2.1 Animals, drug treatment, and inclusion/exclusion criteria

Fifty-six female Sprague-Dawley rats (18–20 months old) were purchased from Hilltop Laboratories (Scottsdale, PA) and housed under 12 hour light-dark conditions with food and water available ad libitum. The West Virginia University Animal Care and Use Committee approved all work involving rats. Study 1 (N=27 rats) assessed mortality, neurological score, infarct volume, hemispheric swelling, and hemoglobin concentration at 24 hours post-MCAO. Study 2 (N=15 rats) measured changes in BBB permeability to vascular impermeant markers, Evans blue (EB) albumin and sodium fluorescein (NaF), at 24 hours post-MCAO. Study 3 (N=14 rats) identified changes in MMP activity and PKC expression at 24 hours post-MCAO. All the studies included two treatment groups: Group 1 (r-tPA): MCAO, 0.9% vehicle (saline + 10% dimethylsulfoxide) at 2 hours post-MCAO, r-tPA (5 mg/kg; 30% bolus infused over 30 minutes) at 6 hours post-MCAO and Group 2 (r-tPA + bryostatin): MCAO, bryostatin (2.5 mg/kg; i.p. dissolved in 0.9% saline) at 2 hours post-MCAO, r-tPA at 6 hours post-MCAO. For the third study, a sham was added to compare immunohistochemistry stains between groups. A sham group was necessary to observe basal activation of PKC levels without MCAO. The sham group received anesthesia and surgery without the MCAO.

2.2 Surgical Procedure for MCAO

Rats underwent a modified reversible embolic MCAO with r-tPA reperfusion (Tan et al., 2013). MCAO was confirmed by measuring a drop in cerebral perfusion over the MCA region >80% of initial baseline with continuous laser Doppler monitoring. Recanalization was successful if cerebral blood flow in the MCA region increased to 75% of baseline. Rats not meeting these criteria were excluded.

Rats were given a reversible embolic MCAO as previously described (Dinapoli et al., 2006; Tan et al., 2009). Briefly, rats were anesthetized with 2% isoflurane in a mixture of 30% oxygen and 70% nitrous oxide. A servo-controlled homeothermic heating blanket, equipped with a rectal thermometer, was used to maintain body temperature at 37 °C. Cerebral blood flow was monitored with a laser Doppler probe precisely positioned over the area supplied by the middle cerebral artery (MCA). A modified PE 50 microcatheter was inserted into the external carotid artery stump and advanced into the MCA. Placement in the proximal MCA was verified by a sudden decrease in cerebral blood flow as measured by laser Doppler. The microcatheter was withdrawn ~1 mm, allowing cerebral blood flow to return to baseline, and then a 25 mm fibrin-enriched clot was injected. Successful MCA occlusion was confirmed by a drop in perfusion greater than 80% of baseline flow. Recanalization was achieved when cerebral blood flow increased to at least 75% of initial baseline. Rats not achieving these standards were excluded from the study.

2.3 Neurological Functional Assessment

Neurological functional assessments were measured at 24 hours post-MCAO by an investigator blinded to treatment groups. The modified Neurological Severity Scores (mNSS) evaluated motor, sensory, balance and reflex measures. The scoring range was from 0 to 17, with higher scores indicating greater neurological injury (Seyfried et al., 2004).

2.4 Measurement of infarction volume by 2,3,5-triphenyltetrazolium chloride (TTC)

At 24 h following MCAO, rats were sacrificed and infarct size and volume were measured by 2,3,5-triphenyltetrazolium chloride (TTC; 2%) staining. 2 mm coronal brain slices were stained for 15 min at 37 °C. Hemispheres were scanned on a flatbed scanner and analyzed using Image J software. On each slice, the non-stained area (ischemic brain) was outlined, and the infarct volume was calculated. The corrected infarction volume (CIV) was calculated using the following equation: $CIV = (CHV - [IHV - IHI]) \times d$, where CHV was the area of the contralateral cortex or striatum in mm^2 , IHV was the area of the ipsilateral cortex or striatum in mm^2 , IHI was the infarct area (cortical or striatal) in mm^2 , and d was slice thickness (2 mm). Edema index was calculated as follows: $Edema\ index = ([volume\ of\ ipsilateral\ hemisphere - volume\ of\ contralateral\ hemisphere] / volume\ of\ contralateral\ hemisphere) \times 100\%$ (Schielke et al., 1998).

2.5 Spectrophotometric Assay of Hemoglobin

HT was quantified using a spectrophotometric assay (Quantichrom Bioassay Systems) of hemoglobin content. At 24 hours after MCAO, rats were anesthetized with ketamine/xylazine (90 mg/kg and 5 mg/kg; i.p.) and perfused transcardially with 0.1 M phosphate-buffered saline (PBS). MCA area in both hemispheres were dissected, weighed, and homogenized in 0.1 M PBS. After 30 minutes of centrifugation ($13,000 \times g$), 50 μ l aliquots of sample were transferred into a 96 well plate with 200 μ l of reagent. After 15 minutes at room temperature, optical density was measured at a wavelength of 400 nm per manufacturer's instructions. A standard curve was generated using absorbance from serial dilutions of hemoglobin added in incremental aliquots from control rats to the homogenized brain tissue from control rats. Hemoglobin content was expressed as mg/g tissue.

2.6 Assessment of BBB permeability

BBB permeability was assessed using EB and NaF as vascular markers. Saline (0.9%) containing EB (2%: 5 ml/kg) and NaF (2%: 5 ml/kg) was administrated intravenously to anesthetized (ketamine/xylazine) rats. After 30 minutes, anesthetized rats were perfused transcardially with 0.9% saline. Brains were excised, meninges and ependymal organs removed, hemispheres separated, and tissue from MCA area weighed and homogenized in 1 ml of 50% trichloroacetic acid. The suspension was divided into two 0.5 ml aliquots. One aliquot was incubated for 24 hours at 37°C, centrifuged at $10,000 \times g$ for 10 minutes, and the supernatant measured by absorbance spectroscopy at 620 nm for EB albumin determination. The other aliquot was centrifuged at $10,000 \times g$ for 10 minutes and neutralized with 5 N sodium hydroxide. For NaF determination, the supernatant was measured with a fluorometer at 485 nm excitation and 535 nm emission wavelengths.

Standard curves using absorbance from serial dilutions of EB and NaF were used to determine concentration.

2.7 MMP Zymography

Rats were anesthetized (ketamine/xylazine) and perfused transcardially with 0.1 M PBS at 24 hours post-MCAO. Using gel zymography, protein isolated from ipsilateral hemispheres was assessed for MMP-2 and MMP-9 activity. Equal concentrations and volumes (50 µg / 20 µl) of proteins were loaded and separated on a 10% SDS-PAGE gel with 0.1% gelatin. After electrophoresis, gels were washed in 2.5% (w/v) Triton X-100 for 1 hour and then incubated in a developing buffer (50 mM Tris-HCl, 50 mM NaCl, 5 mM CaCl₂, 2 µM ZnCl₂ and 0.02% Brij-35; pH 7.6) for 48 hours at 37°C. Gels were stained with 1% Coomassie blue and de-stained in a buffer containing 30% methanol and 10% glacial acetic acid. Images of gelatinolytic activities were scanned and analyzed by an individual blinded to experimental groups using Image J software.

2.8 Measurement of changes in PKC ϵ , PKC α , PKC δ protein expression at 24 hours after MCAO

Rats were anesthetized (ketamine/xylazine) and perfused transcardially with 0.1 M PBS at 24 h post-MCAO. Brain tissue from the ipsilateral hemisphere was used for analysis. Brain tissue was homogenized in 1% SDS (95°C) and protein concentration was determined using a Bradford assay kit (Pierce; Rockford, IL). Samples were loaded with 2X lamelli buffer at 20 µg of protein/well on 4–12% 10-well gels (Life Technologies, Carlsbad, CA). Gels were run with the Bolt® Mini tank system (Bio-Rad, Contra Costa, CA) and equilibrated for 15 min in Towbin's transfer buffer prior to transfer to polyvinylidene fluoride (PVDF) membranes (Bio-Rad) with semidry electrophoretic transfer cells (Bio-Rad). Membranes were blocked by 5% fat-free milk/tris buffered saline and Tween 20 (TBS-T) for 2 h and incubated with primary antibodies against PKC ϵ , PKC α , & PKC δ raised in mouse (Santa Cruz Biotechnologies, Santa Cruz, CA) each at 1:200 overnight. A β -actin rabbit monoclonal antibody (Cell Signaling) was used as an endogenous control at concentration 1:10,000. Anti-mouse and anti-rabbit IgG HRP-linked antibodies (Cell Signaling) were used as secondary antibodies at 1:2000 with gentle shaking for 2 h. Molecular weight was determined using the SeeBlue® Plus2 Pre-stained Standard (Life Technologies). Imaging was conducted using 20X LumiGLO chemiluminescent substrate (Cell Signaling). Images were analyzed using densitometry with ImageJ.

2.9 Immunohistochemistry

Animals were anesthetized with 4% isoflurane and transcardially perfused with cold 0.9% saline. 10% formalin was then perfused for a total of ten minutes. Immediately following perfusion the brain was extracted and placed into formalin. The brain was sliced into sections using a brain block and was paraffin embedded using the Tissue-Tek TEC 5 embedding system (Sakura Finetek). Tissues were then sliced with a Leica RM2235 microtome (Leica Microsystems) and mounted on slides for staining. Standard fluorescent staining protocols were utilized for PKC α , PKC δ (Santa Cruz), PKC ϵ (Invitrogen), and Calcium and Rack2 (Abcam). Images were acquired from the MCA region of the cortex from a blinded observer. Imaging for fluorescence was performed using a Zeiss Axio

Observer, and the *Just Another Co-localization plugin* for ImageJ was used to determine Pearson's coefficient with background threshold adjusted to baseline.

2.10 Statistical Analysis

All data were compiled and analyzed by an investigator blinded to treatment group and presented as mean $\pm$ SEM. Functional data and band densities were compared by Student's t-test or analysis of variance (ANOVA). Mortality data were compared using Fisher's exact test. Level of significance was set at $p < 0.05$. Co-localization was reported as a Pearson's r -value for immunofluorescent staining.

3 Results

3.1 Bryostatin decreased mortality and brain swelling in rats at 24 h post-MCAO

Bryostatin administration significantly ($p = 0.049$) reduced mortality from 50% in r-tPA alone group ($n = 18$) to 11% in the r-tPA + bryostatin group ($n = 9$) (Figure 1A). No difference ($p = 0.4$) in mNSS was noted between rats in the r-tPA group (10 ± 1 ; $n = 9$) and the r-tPA + bryostatin group (10 ± 1 ; $n = 8$) (Figure 1B). No difference in cortical ($p = 0.06$), striatal ($P = 0.11$), or total hemispheric ($p = 0.59$) infarction volume was observed between rats in the r-tPA ($34 \pm 3\%$, $56 \pm 5\%$, $41 \pm 3\%$; $n = 9$) and r-tPA + bryostatin ($45 \pm 4\%$, $42 \pm 6\%$, $44 \pm 4\%$; $n = 8$, cortical, striatal, total, respectively) groups (Figure 1C). A significant ($p = 0.0005$) reduction in cerebral swelling was measured in rats from the r-tPA + bryostatin group ($19 \pm 2\%$; $n = 8$) compared to the r-tPA alone group ($37 \pm 3\%$; $n = 9$) at 24 hours post-MCAO (Figure 1D).

3.2 Bryostatin decreased HT in cortex and total hemisphere of rats at 24 h post-MCAO

Hemoglobin concentration in the cortex, striatum, and total cortical hemisphere was measured at 24 hours post-MCAO (Figure 2A). A significant decrease in relative hemoglobin concentrations in cortex ($p = 0.04$), striatum ($p = 0.049$), and total cerebral hemisphere ($p = 0.03$) were observed in rats in the r-tPA + bryostatin group (0.056 ± 0.008 , 0.072 ± 0.014 , 0.064 ± 0.009 ; $n = 8$) compared to rats in the r-tPA group (0.096 ± 0.015 , 0.111 ± 0.030 , 0.104 ± 0.014 ; $n = 8$). Representative coronal sections show that administration of bryostatin reduced the severity of hemorrhage at 24 hours post-MCAO (Figure 2 B).

3.3 Bryostatin reduced BBB disruption in cortex, striatum, and total hemisphere of rats at 24 h post-MCAO

Diffusion of EB-bound albumin into cortex, striatum, and total hemisphere was measured at 24 hours post-MCAO (Figure 3A). A significant decrease in relative EB-bound albumin concentration within cortex ($p = 0.03$), striatum ($p = 0.049$) and total hemisphere ($p = 0.03$) was observed in the r-tPA + bryostatin group ($37 \pm 15\%$, $47 \pm 18\%$, $42 \pm 16\%$; $n = 4$) compared to the r-tPA alone group ($100 \pm 20\%$, $100 \pm 8\%$, $100 \pm 13\%$; $n = 5$). NaF diffusion into cortex, striatum, and total hemisphere was measured at 24 hours post-MCAO (Figure 3B). A significant decrease of relative NaF concentrations in cortex ($p = 0.002$), striatum ($p = 0.02$), and total hemisphere ($p = 0.008$) was noted in rats from the r-tPA + bryostatin group ($43 \pm 6\%$, $25 \pm 6\%$, $30 \pm 5\%$; $n = 4$) compared to the r-tPA alone group ($100 \pm 11\%$, $100 \pm 27\%$, $100 \pm 21\%$; $n = 5$). Representative coronal slices without TTC staining showed that rats in the r-tPA group had extensive EB albumin leakage compared to rats in the r-tPA + bryostatin group (Figure 3C).

3.4 Bryostatin decreased pro-MMP-9 and activated-MMP-9 activities but not MMP-2 levels in ischemic striatum of rats at 24 h post-MCAO

Band intensity was quantified for pro-MMP-9 and activated MMP-9 (Figure 4A and 4B). A significant ($p=0.01$) decrease in pro-MMP-9 levels was observed in rats from the r-tPA + bryostatin group ($27\pm3\%$; $n=4$) compared to the r-tPA group ($100\pm24\%$; $n=3$). A significant ($p=0.03$) decrease in activated-MMP-9 levels was observed in rats from the r-tPA + bryostatin group ($69\pm6\%$; $n=4$) compared to the r-tPA group ($100\pm12\%$; $n=3$). Band intensity was quantified for MMP-2 (Figure 4C) with no difference ($p=0.54$) between rats in the r-tPA group ($100\pm11\%$; $n=3$) and the r-tPA + bryostatin group ($109\pm8\%$; $n=4$). A representative gelatin zymogram shows MMP-2/9 levels for both groups (Figure 4D).

3.5 Bryostatin increased PKC ϵ protein expression with no change in PKC δ and PKC α expression in rats at 24 h post-MCAO

A significant ($p=0.03$) increase in PKC ϵ expression was observed in rats from the r-tPA + bryostatin group ($194\pm21\%$; $n=4$) compared to the r-tPA group ($100\pm22\%$; $n=3$) (Figures 5A & 5B). No difference ($p=0.23$) in PKC δ expression was observed between rats in the r-tPA group ($100\pm11\%$; $n=3$) compared to the r-tPA + bryostatin group ($68\pm18\%$; $n=4$) (Figures 5C & 5D). No difference ($p=0.96$) in PKC α expression was observed between rats in the r-tPA group ($100\pm25\%$; $n=3$) and the r-tPA + bryostatin group ($113\pm18\%$; $n=4$) (Figures 5E & 5F).

3.6 Bryostatin increased PKC ϵ activity with no effect on PKC δ activity

Calcium serves as an important co-activator for PKC α and PKC δ , and is necessary for these isozymes to function. We co-stained for calcium in conjunction with these isozymes and quantified co-localization with an image J plug-in. A weak correlation was seen for PKC α and calcium in the control sham group with a Pearson's coefficient $r=0.258$ (Figure 6A). A strong correlation was seen for the r-tPA group $r=0.773$ (Figure 6B). Although a visible increase in PKC α was observed in the r-tPA + bryostatin group co-localization was weak $r=0.255$ indicative of reduced activation (Figure 6C). A weak correlation was also seen for PKC δ and calcium in the control sham group $r=0.155$ with strong correlations for the r-tPA group $r=0.77$ and r-tPA + bryostatin group $r=0.761$ (Figure 6D-F). Bryostatin had no effect on PKC δ activity. In order for PKC ϵ to exert its neuroprotective activity, it must be translocated by its chaperone receptor for activated PKC 2 (RACK2). We co-stained for PKC ϵ and RACK2 and found a weak correlation for the control sham group $r=0.307$, modest correlation for the r-tPA group $r=0.451$, and strong correlation for the r-tPA + bryostatin group $r=0.819$ (Figure 6G-I). Bryostatin increased PKC ϵ translocation and activity.

4 Discussion

The primary finding of this study was that bryostatin administration extended the time window for r-tPA administration out to 6 hours post-MCAO. The mortality rate in this study with bryostatin and extended r-tPA administration was similar to previous studies where r-tPA was administered at 2 hours (Kelly et al., 2009). Results of the study demonstrate that bryostatin improved survival and reduced cerebral swelling in aged female rats that received

bryostatin at 2 hours and r-tPA at 6 hours following MCAO. While infarct volumes were not significantly reduced, the reduction in cerebral hemisphere swelling is particularly significant as edema formation is one of the most important signs of poor prognosis (Strbian et al., 2013). Additionally, the increased risk for BBB breakdown and development of HT is a dangerous complication observed clinically and in rodent models of ischemic stroke when r-tPA is administered past the recommended time window (Fan et al., 2014). In this study, the significant increase in hemoglobin measured in the brain parenchyma of rats administered r-tPA only compared to rats administered bryostatin + r-tPA following MCAO show the benefit of improved BBB function.

Activation or inhibition of specific PKC isozymes has been shown to confer a vasculo-protective effect following ischemia. PKC ϵ can prevent the generation of reactive oxygen species and limit intracellular damage (Sun et al., 2013). Bryostatin has been shown previously to decrease levels of PKC α and increase levels of PKC ϵ (Tan et al., 2013). Although we report in the current paradigm that bryostatin did not decrease PKC α when administered prior to r-tPA, it did reduce co-localization with the calcium co-activator on fluorescent IHC. Bryostatin also enhanced translocation of PKC ϵ by its chaperone RACK2. Bryostatin is a potent modulator of PKC activity and ongoing research is needed to discover how different dosing strategies facilitate recovery post-ischemia. The timing, localization, and which PKC isozymes are activated play critical, and sometimes contrasting, roles in how the injury process progresses (Hongpaisan and Alkon, 2007). Previous studies demonstrate that an initial increase in PKC ϵ activity initiates cell survival signaling pathways following ischemic stroke and reperfusion (Bright and Mochly-Rosen, 2005). PKC α and PKC δ activation however deregulates the structural and functional integrity of BBB tight junctions (Qi et al., 2008). Our results show that bryostatin significantly up-regulated PKC ϵ activation early without significant regulation of PKC α and PKC δ activation. It is likely that PKC α activity would be down-regulated if bryostatin was administered at a later time point. The current study suggests that up-regulation of PKC ϵ activation by bryostatin may play a critical role in decreasing damage to tight junctions within the BBB, and decrease the risk of hemorrhagic transformation following reperfusion by r-tPA.

A strong relationship between MMP-9 and cerebral hemorrhage and BBB disruption in ischemia/ reperfusion injury has been demonstrated in animals and humans (Wang et al., 2003a). After activation, MMP-9 degrades matrix proteins of the basal lamina and the proteins of the extracellular matrix (Woessner, 1991), such as collagen, fibronectin, and laminin. This degradation leads to BBB disruption and swelling formation (Rosenberg et al., 1998). Also, activation of MMP-9 likewise causes the degradation of critical neurovascular units predisposing to the development of hemorrhage (Hamann et al., 1996). MMPs are therefore terminal enzymes in the remodeling cascade that result in deleterious brain swelling and hemorrhage. We demonstrated that bryostatin treatment can decrease the up-regulation of active MMP-9 but not MMP-2 following ischemic stroke. Our results support that inhibition of MMP-9 may be one potential mechanism by which bryostatin improves stroke outcomes following ischemic stroke through reducing BBB disruption, hemorrhagic transformation, and swelling. Bryostatin's effect on MMP-9 is independent of PKC δ

because no modulation of this isozyme was observed. PKC ϵ has recently been shown to regulate MMP-9, which might account for our findings (Ding et al., 2011).

A particular limitation of this study is that the r-tPA (5 mg/kg; i.v.) dosage used in this study is 5 times higher than the clinical dose (0.9 mg/kg) administered. The reason is that the fibrinolytic system in rats has been determined to be 10 times less responsive to r-tPA than humans; thus most preclinical studies use a dose of 10 mg/kg to reperfuse the rodent following MCAO (Jin et al., 2014). In developing our stroke model, we determined that due to the small clot used and the precise placement of the clot into the MCA (90%), we can use a dose that is half that of other labs. This reduction in dose has had the beneficial effect of reducing the likelihood of HT in our model following MCAO (Tan et al., 2013). The animals in the r-tPA alone group, exhibited extensive hemorrhagic transformation and enlarged infarcts. The higher mortality rate seen in the r-tPA group may introduce a survival bias in that the most severely infarcted and impaired animals died and were excluded from further analysis. Thus, the difference in parameters between the r-tPA alone and r-tPA + bryostatin groups is likely even larger than reported.

5 Conclusion

Our study shows that bryostatin treatment reduces mortality, brain swelling, BBB disruption and cerebral hemorrhage with delayed r-tPA treatment for focal ischemic stroke in aged rats. Activation and enhanced translocation of PKC ϵ as well as inhibition of PKC α activity and MMP-9 levels may explain some of the mechanisms underlying bryostatin's BBB protection and hemorrhagic reduction. We suggest that the neurovascular protective effects of bryostatin may support a new therapeutic strategy to reduce BBB disruption and hemorrhage. EMT personnel could potentially administer bryostatin to suspected stroke patients on the way to the hospital. This proof-of concept study suggests that bryostatin may lengthen the time-to-treatment window and enhance the efficacy and safety of thrombolytic therapy in stroke patients.

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Figure 1.

Measurement of mortality, behavior, stroke volume, and edema post-stroke (A) At 24 hours post-MCAO, the mortality rate was significantly reduced from 50% (9/18) in the r-tPA group to 11% (1/9) in the r-tPA + bryostatin group. (B) Assessment of neurological function at 24 hours post-MCAO showed no difference in mNSS scores between the r-tPA group (n=9) and the r-tPA + bryostatin group (n=8). (C) Measurement of infarct volumes in the cortex, striatum, and total hemisphere at 24 hours post-MCAO found no difference in volume sizes between the r-tPA (n=9) and r-tPA + bryostatin (n=8) groups. (D) Measurement of cerebral swelling in the infarcted hemisphere at 24 hours post-MCAO revealed a significant decrease in the r-tPA group (n=9) as compared to r-tPA + bryostatin group (n=8). * = $p < 0.05$, *** = $p < 0.001$

Figure 2.

Hemoglobin staining post-stroke (A) Measurement of relative hemoglobin concentration at 24 hours post-MCAO showed a significant reduction of hemoglobin in the cortex, striatum, and total hemisphere of the r-tPA + bryostatin group (n=8) as compared with the r-tPA group (n=8). (B) Representative coronal slices without TTC staining showed that rats in the r-tPA group had extensive HT with parenchymal hemorrhage noted in the lateral caudate-putamen and somatosensory cortex as compared to rats in the r-tPA + bryostatin group. * = $p < 0.05$

Figure 3.

Changes in BBB post-stroke (A) Measurement of changes in BBB permeability to EB albumin at 24 hours post-MCAO showed a significant reduction in the cortex, striatum, and total hemisphere of rats in the r-tPA + bryostatin group (n=5) compared to rats in r-tPA alone group (n=4). (B) Results showed a significant decrease in relative EB concentration in cortex, striatum and total hemisphere in r-tPA + bryostatin group (n=5) compared with r-tPA group (n=4). (B) Measurement of relative sodium fluorescein (NaF) concentration in cortex, striatum, and total hemisphere at 24 hours post-MCAO. Results showed a significant decrease in relative NaF concentration in cortex, striatum, and total hemisphere in the r-tPA + bryostatin group (n=5) compared with the r-tPA group (n=4). (C) Representative coronal slices without TTC staining showed that r-tPA at 6 hours after the onset of ischemia had extensive EB leakage while pretreatment with bryostatin at 2 hours had less EB leakage. The leakage of EB is noted in the lateral caudo-putamen and the somatosensory cortex within the MCA territory. * = $P < 0.05$, ** = $P < 0.01$.

Figure 4.

Regulation of MMP activity with bryostatin (A) Band intensity was quantified for pro-MMP-9, and the relative quantity of pro-MMP-9 level was expressed as pro-MMP-9 ratio. Results show a significant decrease in pro-MMP-9 levels in the r-tPA + bryostatin group (n=4) compared to the r-tPA group (n=3). (B) Band intensity was quantified for activated-MMP-9, and the relative quantity of activated-MMP-9 level was expressed as activated-MMP-9 ratio (r-tPA group as 100%). Results showed a significant decrease in activated-MMP-9 levels in r-tPA + bryostatin group (n=4) compared to the r-tPA group (n=3). (C) Band intensity was quantified for MMP-2, and the relative quantity of MMP-2 level was expressed as an MMP-2 ratio (r-tPA group as 100%). No difference ($P>0.05$) in MMP-2 levels was observed between the r-tPA (n=3) and r-tPA + bryostatin (n=4) groups. (D) A representative gelatin zymogram showing MMP-2/9 levels in the r-tPA and r-tPA + bryostatin groups. Both pro-MMP-9 (92 kDa) and activated-MMP-9 levels (82 kDa) were decreased in the r-tPA + bryostatin group, while no change in MMP-2 activity level was observed between the groups. * = $P<0.05$.

Figure 5.

Changes in PKC levels with bryostatin administration (A) Band intensity was quantified for PKCε, and the relative quantity of PKCε level was expressed as PKCε ratio (r-tPA group as 100%). Results showed a significant difference in PKCε levels between the r-tPA (n=3) and r-tPA + bryostatin group (n=4) groups. (B) Representative western blots show different PKCε protein expression levels between the r-tPA and r-tPA + bryostatin groups. (C) Band intensity was relatively quantified for PKCδ (r-tPA group as 100%). Results showed no difference ($P < 0.05$) in PKCδ expression levels between the r-tPA group (n=3) and r-tPA + bryostatin (n=4) groups. (D) Representative western blots show PKCδ levels in the r-tPA and r-tPA + groups. (E) Band intensity was relatively quantified for PKCα (r-tPA group as 100%). Results show no difference ($P < 0.05$) in PKCα expression levels between the r-tPA (n=3) and r-tPA + bryostatin (n=4) groups. (F) Representative western blots show PKCα levels in the r-tPA and r-tPA + bryostatin groups. * = $P < 0.05$.

Figure 6.

PKC isozyme activity was altered by bryostatin administration in the MCA region. PKCα was weakly co-localized with its co-activator calcium in the sham control group (A), was strongly co-localized in the r-tPA group (B), and was weakly co-localized in the r-tPA + bryostatin group (C). PKCδ was weakly co-localized with its co-activator calcium in the sham control group (D), and strongly co-localized in the r-tPA group (E) and r-tPA + bryostatin group (F). In order for PKCε to be active, it must translocate by binding to its chaperone RACK2. PKCε was weakly co-localized with RACK2 in the sham control group (G), moderately co-localized in the r-tPA group (H), and strongly co-localized in the r-tPA + bryostatin group (I).