Thrombolytic treatment with recombinant tissue plasminogen activator (tPA) remains the only US Food and Drug Administration–approved drug for treatment of acute ischemic stroke. However, <3% of people with ischemic stroke receive tPA, because of increased risk of secondary cerebral hemorrhage and edema formation.1 Based on the aging population and increased stroke burden, an unmet need exists to develop alternative approaches to treat acute ischemic stroke, either independently of tPA or in an effort to increase the number of patients eligible for tPA.

Preclinical studies of proposed therapeutics for ischemic stroke have largely failed to consider the greatest risk factor for stroke—age.2 Previous studies suggest that aged rats represent a more clinically relevant model of ischemic stroke as compared with younger animals.3,4 These studies illustrate an increased severity of ischemic injury and altered neural injury progression.1–6 Thus, use of aged animals may increase clinical relevance and the likelihood of bench-to-bedside therapeutic translation.

Protein kinase C (PKC) plays a critical role in storage of associative memory and related synaptogenesis in normal animals7; reducing the accumulation of β-amyloid, synaptic loss, cognitive deficits, and neurodegeneration in preclinical models of Alzheimer disease8,9; and protecting neurons against ischemic pathology10,11 and traumatic brain injury in rodents.12 PKC isozymes α and ε regulate synaptogenic and antiapoptotic signaling pathways,13 as well as critical functional pathways in the multicellular response to ischemia/reperfusion injury.14 Individual PKC isoforms play differing, and sometimes opposing, roles in injury response that often depend on cell types and degree of pathophysiology.15,16 Observations that PKC activation mediates both protective and harmful messages results from different PKC isoforms being activated by different signals that play concentration- and concentration-dependent roles in injury response.

Background and Purpose—Bryostatin, a potent protein kinase C (PKC) activator, has demonstrated therapeutic efficacy in preclinical models of associative memory, Alzheimer disease, global ischemia, and traumatic brain injury. In this study, we tested the hypothesis that administration of bryostatin provides a therapeutic benefit in reducing brain injury and improving stroke outcome using a clinically relevant model of cerebral ischemia with tissue plasminogen activator reperfusion in aged rats.

Methods—Acute cerebral ischemia was produced by reversible occlusion of the right middle cerebral artery (MCAO) in 18- to 20-month-old female Sprague–Dawley rats using an autologous blood clot with tissue plasminogen activator–mediated reperfusion. Bryostatin was administered at 6 hours post-MCAO, then at 3, 6, 9, 12, 15, and 18 days after MCAO. Functional assessment was conducted at 2, 7, 14, and 21 days after MCAO. Lesion volume and hemispheric swelling/atrophy were performed at 2, 7, and 21 days post-MCAO. Histological assessment of PKC isozymes was performed at 24 hours post-MCAO.

Results—Bryostatin-treated rats showed improved survival post-MCAO, especially during the first 4 days. Repeated administration of bryostatin post-MCAO resulted in reduced infarct volume, hemispheric swelling/atrophy, and improved neurological function at 21 days post-MCAO. Changes in αPKC expression and εPKC expression in neurons were noted in bryostatin-treated rats at 24 hours post-MCAO.

Conclusions—Repeated bryostatin administration post-MCAO protected the brain from severe neurological injury post-MCAO. Bryostatin treatment improved survival rate, reduced lesion volume, salvaged tissue in infarcted hemisphere by reducing necrosis and peri-infarct astrogliosis, and improved functional outcome after MCAO. (Stroke. 2013;44:00-00.)

Key Words: aging ■ blood-brain barrier ■ infarction ■ protein kinase C ■ tissue plasminogen activator

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Bryostatin, an ultrapotent PKC activator, may provide substantial benefit in the treatment of acute ischemic stroke. Studied extensively as an antitumorogenic agent, recent studies demonstrate that administration of bryostatin after global ischemic insult resulted in curative neurogenesis, synaptogenesis, and cognitive enhancement. The purpose of this study was to investigate the pharmacological potential of repeated administration of bryostatin to improve outcome after acute ischemic stroke in aged rats.

Materials and Methods

Animals and Treatment Protocol

Sixty-six female Sprague-Dawley rats (18–20 months old) were purchased from Hilltop Laboratories (Scottdale, PA) and housed under 12-hour light–12-hour dark conditions with food and water ad libitum. All work involving rats was approved by the West Virginia University Animal Care and Use Committee. For study 1, rats were randomly divided into 2 treatment groups (N=56). Group 1 underwent a 2-hour middle cerebral artery occlusion (MCAO) with tPA-mediated (5 mg/kg, i.v.) reperfusion as previously described and at 6 hours was administered bryostatin (2.5 mg/kg, i.v.) with subsequent doses every 3 days for a total of 7 doses over 21 days. Animals in group 2 served as the control and underwent the same procedures except they were administered 0.9% saline, instead of bryostatin, at 6 hours post-MCAO. Rats were assayed at 2, 7, and 21 days after MCAO. A second set of rats (N=10; 6 saline and 4 bryostatin-treated rats) underwent MCAO with bryostatin/saline treatment at 6 hours post-MCAO. Rats were assayed at 2, 7, and 21 days after MCAO. A second set of rats (N=10; 6 saline and 4 bryostatin-treated rats) underwent MCAO with bryostatin/saline treatment at 6 hours and were then euthanized at 24 hours to visualize changes in PKC isozyme (α and ε) expression in neurons, endothelial cells, and astrocytes. Ischemia and reperfusion were defined as a minimum of 80% decrease in cerebral blood flow at onset of ischemia and return to 80% of baseline blood flow measurement, respectively. Rats not achieving these standards were excluded from the study.

Neurological Functional Assessment

The modified Neurological Severity Scores (mNSS) was used to assess neurological function at 2, 7, 14, and 21 days after MCAO (n=7 saline and 10 bryostatin-treated rats). mNSS is a composite score of motor, sensory, balance, and reflex measures ranging from scores of 1 to 17, with higher scores indicating greater neurological injury.

TUNEL Staining, Immunohistochemistry, and Infarct Volume Measurement

TUNEL staining was performed at 7 (n=5 rats per treatment) and 21 (n=7 saline and 10 bryostatin-treated rats) days after MCAO according to manufacturer’s instructions (Roche Applied Science). After mounting and nuclear staining with DAPI, slices were visualized for apoptotic tissue.

Using alternating slices from the same brain, immunohistochemistry using a modified ABC procedure was performed with anti-GFAP (1:1000) for evaluation of astrocyte morphology (n=7 saline and 10 bryostatin-treated rats). At 2 (n=4 rats per treatment), 7 (n=5 rats per treatment), and 21 (n=7 saline and 10 bryostatin-treated rats) days after MCAO, lesion volumes were determined using creyl violet staining. Degree of cerebral hemispheric swelling/atrophy at 2, 7, and 21 days after MCAO was calculated using the formula: cerebral hemispheric swelling/atrophy=(LV−RV)/LV×100%, where LV was volume (mm3) of left hemisphere and RV was volume of right hemisphere.

Trilabeled confocal microscopy was performed to determine immunolocalization of εPKC (1:100) and αPKC (1:100) with neurons (1:150), endothelial cells (1:100), and astrocytes (1:100) at 24 hours after MCAO (n=4 rats per treatment). Nuclear staining was performed on sections using DAPI, and secondary antibodies were incubated at 1:200 dilution.

Expression levels of DNA damage (TUNEL), tPKC, and εPKC were evaluated based on immunofluorescence intensity by an observer blinded to treatment group. Confocal images were quantified using ImageJ as previously described.

Water Maze Test

Spatial acquisition was determined using 6 training days with a probe test on day 7. Training began the 15th day post-MCAO and the probe test was conducted at 21 days post-MCAO (n=3 saline and 4 bryostatin-treated rats). Learning trials were conducted over 6 consecutive days with 4 trials per day. Time interval between trials was 60 seconds, and maximum swim time to find platform was 120 seconds. Start location used N, S, E, and W designations for the start of each trial. Start locations for each trial were semirandomly selected so that all directions were used for each rat on each training day. Each training session and probe test was conducted by investigators blinded to treatment. During the training period and probe test, latency speed, as well as distance traveled to platform and 300% of platform, was measured.

Statistical Analysis

All data were compiled and analyzed by an investigator blinded to treatment and presented as mean±SEM. Physiological parameters, functional data, and immunofluorescence intensity were compared by Student t test or ANOVA grouped by treatment (saline versus bryostatin) or days post-MCAO (2, 7, and 21 days). Mortality data were compared using Fisher exact test. P<0.05 was considered statistically significant.

Results

Administration of Bryostatin After MCAO in Aged Rats Improved Survival Rate and Neurological Function

A total of 66 rats were used in this study. Fifty-six rats were evaluated for effects of bryostatin on ischemic brain injury over 21 days after MCAO. Two rats from this cohort, both saline-treated, were excluded from study for not meeting ischemia/reperfusion criteria. Survival rates were documented daily from 1 to 21 days (Figure 1A). Administration of bryostatin post-MCAO improved survival rates through 21 days with positive effects most evident during the first 4 days and a significant increase in survival achieved from 2 to 17 days post-MCAO. Although survival decreased in bryostatin-treated rats after day 4, improved survival was demonstrated at 7 days (68% bryostatin, n=13, versus 41% saline-treated rats, n=7), 14 days (59% bryostatin, n=11, versus 41% saline-treated rats, n=7), and 21 days (53% bryostatin, n=10, versus 41% saline-treated rats, n=7) post-MCAO. The dosing schedule used in this study was selected based on previous preclinical studies showing cognitive enhancement in rats, therapeutic efficacy in young rats after global ischemia, and neuroprotection in transgenic mice displaying Alzheimer disease–like symptoms. Based on the results of this study, future studies will need to use dose–response measurements to determine the optimal dosing schedule for acute ischemic stroke in aged rats. Functional assessment revealed a significant improvement in mNSS scores at 21 days post-MCAO in bryostatin-treated rats (2.6±0.3; n=10) compared with saline-treated rats (3.7±0.2; n=7; Figure 1B). No difference (P>0.05) in mNSS scores was observed at 2 (n=15 saline and 18 bryostatin-treated rats),
Bryostatin Resulted in Diminished Lesion Volume Post-MCAO

Quantification of lesion volume for cortex, striatum, and total cerebral hemisphere was measured at 2, 7, and 21 days post-MCAO using cresyl violet–stained coronal sections. No difference \((P>0.05)\) in lesion volume was observed at 2 days post-MCAO (Figure 2A). At 7 days, bryostatin administration resulted in a significant reduction in lesion volume in the striatum and total hemisphere as compared with saline-treated rats (Figure 2A). At 21 days, a significant decrease in lesion volume was observed in cortex, striatum, and total hemisphere as compared with saline-treated rats (Figure 2A). Measurements of cortical hemispheric swelling or atrophy revealed a significant decrease in hemispheric swelling at 2 and 7 days after MCAO (Figure 2B). At 21 days after MCAO, the swelling phase of ischemic brain injury had subsided and atrophy of the saline-treated rats was visually evident by cavitation and necrosis of the infarcted hemisphere (Figure 3B and 3D). A significant decrease in atrophy was measured in bryostatin-treated rats at 21 days after MCAO (Figure 2C).

Administration of Bryostatin Decreases Astroglialosis and Reduces Cellular Apoptosis in Aged Rats at 7 and 21 Days After MCAO

A significant reduction in TUNEL-positive cells, representing those cells undergoing apoptosis because of DNA fragmentation, was detected in the peri-infarct region surrounding the infarct in bryostatin-treated rats at 7 \((75\pm6\%\) of control) and 21 \((43\pm3\%\) of control) days after MCAO (Figure 4). This finding was reinforced by a profound reduction in reactive astrocytes, identified by GFAP expression, observed in
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bryostatin-treated rats, in which not only was astrogliosis reduced, but also the degree of tissue destruction was markedly attenuated (Figure 3). At both 7 and 21 days, astrocytic hypertrophy with enlarged processes was evident in tissue surrounding the necrotic zone around the infarct in saline-treated aged rats, consistent with the astrocytic response. Bryostatin attenuated this response and reduced tissue damage in the peri-infarct area (Figure 3).

αPKC Expression Was Reduced in Neurons in the Penumbral Region of Bryostatin-Treated Rats at 24 Hours After MCAO

Colocalization of αPKC with neurons (NeuN), endothelial cells (CD31), and astrocytes (GFAP) was investigated in penumbra surrounding the infarct in aged rats at 24 hours post-MCAO (n=4 rats per treatment group). αPKC immunoreactivity was observed in neurons and endothelial cells (Figures 5A and 6A). Expression of αPKC was diffusely localized throughout the cytoplasm of the cell. No colocalization of αPKC was shown with GFAP-positive astrocytes (data not shown). Rats treated with bryostatin showed a significant decrease in immunoreactivity of αPKC in neurons at 24 hours after MCAO (Figure 5A and 5C). No change (P=0.23) in αPKC expression was observed in endothelial cells at 24 hours after MCAO (Figure 6A and 6C).

Bryostatin Increased εPKC Within Neurons in the Penumbral Area at 24 Hours After MCAO and tPA Reperfusion

Colocalization of εPKC with NeuN and CD31 in the penumbral area at 24 hours after MCAO was investigated (n=4 rats per treatment group). εPKC immunoreactivity was observed in neurons and endothelial cells (Figures 5B and 6B). Expression of εPKC was punctate along cytoplasmic edge of the plasma membrane of the cell. No colocalization of εPKC was shown with GFAP-positive astrocytes. Rats treated with bryostatin showed a significant increase in immunoreactivity of εPKC in neurons at 24 hours after MCAO (Figure 5B and 5C).

Discussion

The primary finding of this study was that administration of bryostatin after MCAO with tPA reperfusion decreased mortality over 21 days, with marked improvement during the first 4 days. Additionally, to the best of our knowledge, we are the first to report that bryostatin administration improved recovery from ischemic brain injury and functional outcome up
to 21 days after MCAO in aged animals. Use of bryostatin attenuated both necrotic and apoptotic cell death. The reduced hemispheric swelling/atrophy observed at 2 and 7 days after MCAO and the significant attenuation of ipsilateral hemispheric atrophy seen in aged rats treated with bryostatin at 21 days suggests the potential for decreased inflammation and reduced cellular death after MCAO and tPA reperfusion.

A marked improvement in neurological function was observed in bryostatin-treated rats at 21 days after MCAO. In this study, we also evaluated changes in poststroke cognition using the Morris water maze test. Results showed that bryostatin-treated rats had improved spatial cognition as compared with saline-treated rats at 21 days after MCAO. Extinguishment of the cognitive deficit in saline-treated rats in the 300% platform

Figure 3. A. Representative photomicrographs of GFAP-positive cells at 7 and 21 days after middle cerebral artery occlusion (MCAO). Astroglialosis was observed in saline-treated brain at 7 and 21 days after MCAO; bryostatin mitigated the degree of astroglialosis. B. At 21 days after MCAO, cavitation and necrosis was evident in saline-treated rats. Administration of bryostatin decreased lesion volume and reduced atrophy as compared with saline-treated rats. Cell nuclei were stained using DAPI.

Figure 4. Comparison of TUNEL staining in aged rats showed a marked reduction in TUNEL-positive cells in bryostatin-treated rats at 7 and 21 days after middle cerebral artery occlusion as compared with saline-treated rats. Cell nuclei were stained using DAPI.
trial suggests that discrimination of environment is improved in aged rats treated with bryostatin.

Effects measured in this study were associated with changes in expression and localization of PKC isozymes in neurons and endothelial cells within the peri-infarct region.

Regulation of PKC activity is the primary reported mechanism of action for bryostatin. Inhibition of PKC isozymes blocked the synaptogenic and cognitive-enhancing effects of bryostatin on normal animals. Therefore, it is plausible that pathological mechanisms initiated after ischemic brain injury involve PKC-mediated pathways. Results from this study clearly show increased εPKC expression and a modest decrease in αPKC expression in neurons of bryostatin-treated rats at 24 hours post-MCAO. Roles of PKC activity after ischemic stroke are complex and poorly defined. What is known is that PKC activity mediates protective and damaging signaling cascades during the injury and reparative phases of ischemic stroke, suggesting that timing, localization, and the PKC isozyme activated play critical, and sometimes contrasting, roles in injury response and progression of neuronal damage and regeneration after stroke. Previous studies report that increased εPKC activity initiates proliferative and cell survival signaling pathways after ischemic stroke and reperfusion, whereas increased αPKC activity exacerbates neuronal damage after poststroke brain injury through initiation of antiproliferative and proapoptotic pathways; thus, although still inconclusive, our results suggest that changes in PKC activity in endothelial cells and neurons may play a vital role in the acute neuroprotective actions of bryostatin.

It should be noted that although bryostatin initially activates both αPKC and εPKC, it only causes prolonged binding of εPKC to the critically required anchoring protein, RACK 1, that is required for prolonged activation (unpublished data).

Of particular interest especially during the acute phase of stroke injury is the role different PKC isozymes play in alterations of endothelial cell function during pathophysiological conditions. Increased PKC activity has been shown to affect structural and functional integrity of the blood-brain barrier after ischemic brain injury with αPKC and βPKC increasing blood-brain barrier opening through alterations in tight junction protein localization, and εPKC enhancing blood-brain barrier integrity by upregulating the expression of claudin 5. Our study showed that αPKC and εPKC were localized to both neurons and cerebral endothelial cells. No colocalization of εPKC or αPKC was observed with GFAP-positive astrocytes; however, that does not mean that changes in PKC activity do not have a significant influence on astrocyte reactivity as demonstrated by our study, which showed a reduction in astrogliosis and tissue damage in bryostatin-treated rats. In fact, in relation to chronic brain response after ischemic injury, attenuation of glial response may end up being the most significant finding of this study. A recent study shows that PKC activation leads to a significant decrease in calcium wave propagation between astrocytes by attenuating astroglial gap junction communication. Gaining a better understanding of the critical mediators and timing of this response will be a primary focus of follow-up studies. A limitation of this study and immunofluorescent imaging, in general, was the effect of...
bryostatin on PKC activation. Future studies will probe for changes in PKC activity by measuring cytosolic to membrane translocation and phosphorylation of specific PKC isozymes at different time points postischemia.

In conclusion, administration of bryostatin after MCAO and tPA reperfusion has neuroprotective effects on the magnitude of ischemic brain injury. A notable finding in this study was the marked improvement in survival observed in bryostatin-treated rats, especially during the first week after ischemic insult. Although mortality increased in the bryostatin-treated group during the 21 days, it must be emphasized that PKC activator treatment, depending on dose and duration, causes 3 sequential consequences: activation, downregulation (and thus inhibition), and de novo protein synthesis. Thus, careful selection of treatment dosing and duration has a profound impact on PKC-mediated phosphorylation of downstream substrates and associated therapeutic benefits. The beneficial effects of bryostatin administration after stroke is most likely because of attenuation of inflammatory cell activity; thus, bryostatin may act as a potent adjuvant with other proven methods, such as hypothermia, for reduction of inflammatory reaction during the acute phase of ischemia/reperfusion injury. In summary, bryostatin-treated rats displayed reduced ischemic brain injury after MCAO, characterized by increased survival, reduced infarct volume, decreased hemispheric swelling/atrophy, and improved neurological function.

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Disclosures
None.

References


Bryostatin Improves Survival and Reduces Ischemic Brain Injury in Aged Rats After Acute Ischemic Stroke

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