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Delayed Activin A administration attenuates tissue death after transient focal cerebral ischemia and is associated with decreased stress-responsive kinase activation

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Abstract

Focal cerebral ischemia and reperfusion initiates complex cellular and molecular interactions that lead to either cell repair or destruction. In earlier work, we found that Activin A is an early gene response to cerebral ischemia and supports cortical neuron survival *in vitro*. In this study, the ability of exogenous activin A to attenuate injury from transient middle cerebral artery occlusion (MCAO) was tested in adult mice. Intracerebroventricular administration of activin A prior to MCAO reduced infarct volume apparent one day after experimental stroke. A single Activin A administration at 6 hr following ischemia/reperfusion reduced lesion volumes at 1 and 3 days and led to improved neurobehavior. Moreover, activin A treatment spared neurons within the ischemic hemisphere and led to a concomitant reduction in microglial activation. Activation of the stress-responsive kinases p38 and c-Jun N-terminal kinase implicated in neuronal apoptosis after stroke was reduced following activin A treatment. Together these findings suggest that activin A promotes tissue survival after focal cerebral ischemia/reperfusion with an extended therapeutic window.

Keywords

Activin A; ischemic reperfusion injury; neuroprotection; stress activated kinases

Introduction

Ischemic stroke is an enormous health burden with few current therapeutic options. Focal cerebral ischemia leads to the necrotic loss of neurons in a central region and a surrounding penumbra with a milder ischemic insult where some cells are viable due to collateral circulation. Occlusion poses an immediate threat to neuronal survival, but even when resolved, it is clear that reperfusion introduces additional injury due to apoptosis and increased inflammatory infiltration (Lo et al., 2003). Finding compounds that either activate survival pathways or inhibit the deleterious events after stroke is critical in discovering molecular-based therapeutic targets.

Transient focal cerebral ischemia can be reproduced with the filament MCAO model in rodents (Connolly et al., 1996). This model of ischemia/reperfusion results in damage to the ipsilateral hemisphere with an ischemic core encompassing the striatum and additional cell death in the dorsolateral cortex with apoptotic features (Carmichael, 2005). Cerebral ischemia also activates multiple intracellular signaling cascades that are crucial for maintaining cell survival. The phosphoinositide 3-kinase/protein kinase B signaling pathway supports cell survival and

has a role in blocking neuronal death after stroke (Zhao et al., 2006). Additionally, the mitogen-activated protein kinase family members, ERK1 and ERK2 are activated following ischemia and have neuroprotective effects, although their precise role in focal cerebral ischemia remains controversial (Nozaki et al., 2001). In parallel, cerebral ischemia triggers pro-apoptotic signals including the stress-activated protein kinases p38 and c-jun N-terminal kinases (JNK); (Borsello et al., 2003; Nito et al., 2008). A key component to the execution of apoptosis is the activation of caspase-3 which cleaves intracellular substrates required for cell function and caspase activity is present in the penumbra following transient cerebral ischemia (Namura et al., 1998). One approach to identify molecular targets for therapy in ischemic stroke is to identify factors that promote cell survival when exogenously administered after cerebral ischemia.

Growing evidence suggests that activin A, a member of the transforming growth factor-beta (TGF- β) family, is an endogenous neuronal survival factor increased after acute brain injury that may prove beneficial therapeutically. Activin A induces cellular responses by binding transmembrane serine/threonine kinase type I and type II receptors that stimulate smad2/3 phosphorylation (Harrison et al., 2004). Activin β A mRNA increases within hours of excitotoxic lesion (Tretter et al., 1996), permanent adult ischemia (Bottner et al., 2006), neonatal hypoxia-ischemic injury (Wu et al., 1999) and transient cerebral ischemia (Mukerji et al., 2007). Further, activin A administration increases neuronal survival after quinolinic or kainate acid and neonatal ischemia-hypoxia injury (Hughes et al., 1999; Wu et al., 1999; Tretter et al., 2000). Inactivation of activin receptors results in greater damage from excitotoxic lesion and altered glutamatergic transmission highlighting the requirement for normal activin functions in the brain (Muller et al., 2006). In this study, activin A is tested for its ability to attenuate ischemia-induced tissue death and mediate effects on the intracellular signals after focal cerebral ischemia/reperfusion.

Material and Methods

Animals and surgical procedure

Animal protocols were approved and performed in accordance with the Institutional Animal Care and Use Committee at Case Western Reserve University. Transient unilateral cerebral ischemia was produced in adult, male CD-1 mice (28-32g; Harlan, Indianapolis, IN) using middle cerebral artery occlusion (MCAO); (Endres et al., 1999). Mice were induced with 2% isoflurane in 30% oxygen and 70% nitrous oxide (Quantiflex VMC, Matrx Medical; Orchard Park, NY) and maintained at 1.5% isoflurane. Body temperature was kept at $37 \pm 0.1^\circ\text{C}$ with a heating lamp linked to a rectal thermometer (Harvard Apparatus; Holliston, MA). The left carotid artery was exposed and a 15 cm flame polished 6-0 monofilament nylon suture (Ethicon; Somerville, NJ) was introduced into the carotid lumen. The filament was left in place to block the middle cerebral artery for 60 minutes before removal, as described (Mukerji et al., 2007). Animals were tested for the presence of characteristic neurological deficits revealed by the neurological deficit score (Bederson et al., 1986; Mukerji et al., 2007). All animals were screened behaviorally with operator blinded to treatment groups for spontaneous movement without deficit (grade 0), forelimb flexion contralateral to injured hemisphere (grade 1), wire grip deficit with internal rotation (grade 2), circling to paretic side (grade 3), circling tightly with head tilt (grade 4), spinning or no movement (grade 5); only animals exhibiting grade 4 after stroke were included. Animals exhibiting grade 5 behavior were excluded from further study as previous experience indicated these animals die prior to 24 hrs of reperfusion. Ten percent of animals exhibited grade 5 behavior, and 20% exhibited behavior grades less than 4 and were also excluded. All surgeries and subsequent histological and behavioral assessments were done by operators blinded as to the treatment group.

Intracerebroventricular Injection

Recombinant human/mouse/rat Activin A (R & D Systems, Minneapolis, MN) was administered via intracerebroventricular (i.c.v.) injection of a solution containing either vehicle (0.1% mouse serum albumin diluted in saline; Sigma-Aldrich, St. Louis, MO) or activin A (2.5ug/kg or 15 ug/kg diluted in vehicle). A 26 gauge Hamilton syringe was used to deliver solutions at the coordinates of -0.2mm caudal, +1.0mm lateral to Bregma and 3.0mm deep to the skull surface (Stoelting Co., Wood Dale, IL). Earlier studies showed that stroked animals and stroked animals that received saline vehicle injection did not differ in infarct volume (Mukerji et al., 2007). Activin A or vehicle was prepared by lab personnel and provided in coded vials to operator.

Determination of Infarct Volume

After sacrifice, four 2mm coronal brain slabs were collected using a mouse brain matrix (WPI, Sarasota, FL) and incubated in 2% 2,3,5-triphenyltetrazolium chloride (TTC) solution. The region of infarct in each brain slab was digitized using OpenLab 3.7 image analysis system (Improvision; Lexington, MA). To correct for brain swelling after ischemia, volumes were calculated by summing the volumes of each slab using the following formula: contralateral hemisphere - noninfarcted ipsilateral hemisphere and multiplying by the distance between sections (Swanson et al., 1990; Endres et al., 1999). Infarct volume calculations were performed with operators blinded to the treatment group. Blood samples were obtained from a separate cohort of mice (n=3/group) that were subjected to 1 hr MCAO followed by 6 hr of reperfusion before activin A or vehicle injection. Blood gas measurements were obtained by withdrawing 150 µl of blood from the left ventricle with a heparinized syringe from isoflurane-anesthetized animals 30 minutes after activin A or vehicle injection and immediately analyzed using the automated blood gas analyzing system ABL5 (Radiometer Medical, Bronshoj, Denmark; Redel et. al 2006, Rigsby et. al 2007 and Jungebluth et. al 2008).

Immunoblot analyses

Brain sections of the principal areas supplied by the middle cerebral artery and ipsilateral to the site of injection (bregma +0.1mm to -1.9mm) were collected and homogenized on ice in RIPA buffer (20mM Tris, 150mM NaCl, 1mM NaVO₃, 10 mM NaF, 1mM EDTA, 1mM EGTA, 1% Triton X-100, 0.1% SDS, 0.5% deoxycholic acid), and protease inhibitor cocktail (Sigma). Protein concentrations in the supernatant were estimated by BCA protein assay (Pierce; Rockford, IL). Protein samples were diluted in sample buffer (0.125M Tris-HCL pH 6.8, 20% glycerol, 10% beta- mercaptoethanol, 4% SDS, and 0.001% bromophenol blue) and heated at 100°C for 10 minutes. Samples were separated on precast 10% acrylamide gels (Bio-Rad Laboratories, Hercules, CA) and transferred to nitrocellulose membrane (Bio-Rad) for 170V-hr (Mukerji et al., 2007). Nonspecific sites were blocked as per manufacturer's protocol before incubation overnight at 4°C in primary antibody: rabbit anti-psmad 2 (1:300), rabbit anti-pAkt (1:5000), rabbit anti-pERK 1/2 (1:2500), rabbit anti-phospho-p38 MAPK (1:1000), rabbit-anti-phospho-SAPK/JNK (1:2000), rabbit anti-cleaved caspase 3 (1:500) Cell Signaling, Danvers, MA). Following washes, membranes were incubated 1 hr at RT in goat anti-rabbit peroxidase IgG (1:2500, Jackson Immunological Research, West Grove, PA). Proteins were visualized using a chemiluminescent substrate for horseradish peroxidase (SuperSignal West Pico, Pierce Chemicals, Rockford, IL). The omission of primary antibody resulted in absence of specific signal. To estimate loading variations, membranes were stripped, and blots reprobed overnight at 4°C in mouse anti-Smad2 antibody (1:3000), rabbit anti-AKT (1:12000), rabbit anti-JNK (1:1000), and rabbit anti-p38 (1:1000), Cell Signaling; rabbit anti-ERK2 (1:60,000) and goat anti-actin antibody (1:5000) were purchased from Santa Cruz Biotechnology, Santa Cruz, CA. Flat bed densitometry (Versadoc 3000, Bio-Rad) was used to quantify the signal in arbitrary units.

Immunohistochemistry

Mouse brain sections were collected at either 1 hr or 18 hr after i.c.v. injection of activin A or vehicle (7 hr and 24 hr of reperfusion). Animals were perfused with 4% paraformaldehyde in 0.1M phosphate buffer, and brains removed and post-fixed at 4°C overnight. Brains were transferred to 30% sucrose in 0.1M phosphate buffer at 4°C and stored at -80°C in OCT before use. Cryosections were collected on gelatin-subbed slides and stored at -20°C. Slides were washed and blocked for 1 hr at RT in 5% goat serum in phosphate buffer saline/0.1% tween (PBST; dilution buffer). Tissue sections were incubated in rabbit anti-pSmad2 (1:250, Cell Signaling), mouse-anti NeuN (1:100, Chemicon), rabbit-anti Iba1 (1:500, Wako Pure Chemical Industries, Osaka, Japan), or rabbit-anti cleaved-caspase 3 (1:50, Cell Signaling) antibodies made in dilution buffer at 4°C overnight. After PBST rinses, sections were incubated in appropriate secondary antibodies (1:300; Jackson) in PBST for 1.5 hr at RT. A vectastain DAB kit (Vector Labs; Burlingame, CA) was used according to manufacturer's protocol and reaction time set for 4 minutes. For fluorescence visualization, sections were incubated in donkey anti-rabbit Cy3 or anti-rabbit Cy2 (1:250; Jackson) for 1 hr at RT. Slides were mounted in 2% n-propyl gallate (Sigma) in equal volumes of PBS and glycerol. Images were taken using a Leica (Nussloch, Germany) DMR microscope and a Hamamatsu (C4742-12G04; Hamamatsu, Shizuoka, Japan) digital CCD camera. In each case, omission of the primary antibody revealed no specific staining.

Statistical Analysis

In most cases, data were compared using the unpaired t-test was used for two-group comparisons and one-way analysis of variance (ANOVA) followed by Bonferroni/Dunn's test for multiple group comparisons. Data are presented as mean $\pm$ S.E.M and confidence is indicated with asterisks: *** $p < 0.0001$, ** $p < 0.001$, * $p < 0.05$ (Statview 4.1 software, Abacus Concepts, Inc., Berkeley, CA, USA). At the doses administered, the biological effects were large, and allowed us to acquire robust information from a relatively small number of animals. Thus, post hoc power tests were not relevant, as they are used to address any negative findings from studies with few subjects. Non-parametric ordinal behavioral test data were compared using Friedman's Test with repeated measures.

Results

Activin A was tested for neuroprotective effects in focal cerebral ischemia/reperfusion. Activin A administration prior to kainate acid excitotoxicity has been shown to protect hippocampal neurons from cell death *in vivo* (Tretter et al., 2000). To test for cell protective effects in ischemia/reperfusion injury, activin A (2.5ug/kg body weight (Tretter et al., 2000) or vehicle was injected into the lateral ventricle 1 hr prior to transient MCAO and tissue death was assessed 24 hr after reperfusion by TTC staining with the operator blinded to the material administered and the treatment groups during infarct volume analysis. The administration of vehicle followed by MCAO resulted in reproducible tissue death that encompassed approximately half the ipsilateral hemisphere in the cortex and striatum, and was similar to tissue death seen in stroked controls without i.c.v injection. By contrast, the administration of activin A 1 hr before ischemia sharply reduced the area of tissue death. Using standard approaches to estimate infarct volume (Endres et al., 1999), it was clear that a single activin A injection reduced infarct volume by 50% compared to vehicle-treated animals at 1 day (Figure 1A). These data suggest that activin A can protect brain tissue from cerebral ischemia/reperfusion injury.

To learn if activin A administration resulted in activated intracellular smad changes in the brain, activin A or vehicle was administered and the level of activated smad2 (pSmad2) was assayed at 1, 3 and 24 hr following injection in several animals at each timepoint. Activin A induces

cellular responses by binding transmembrane serine/threonine kinase type I and type II receptors that stimulate smad2/3 phosphorylation (Harrison et al., 2004). In the adult mouse brain, a single injection of activin A increased pSmad2 expression with relatively slow temporal dynamics. After activin injection, phosphorylated Smad2 was increased at 1 and 3 hr and returned to control levels at 24 hr (Figure 1B,C). Activin A receptors are broadly expressed on neurons in the central nervous system (Funaba et al., 1997). To learn which cells responded to activin, psmad2 immunoreactivity was localized 3 hr after vehicle or activin A administration. Activin A treatment increased psmad2 immunoreactivity compared to vehicle-treated animals, and psmad2 immunoreactive cells were confirmed to be neurons as they also expressed the neuronal marker, NeuN (Figure 1D). Similar immunoassays with psmad2 and GFAP to identify astrocytes and factor VIII for endothelial cells did not show double label (data not shown). These long-lasting increases in activated smad2 have been previously shown following activin A application (Xu and Hall, 2007) and suggest that activin A injection results in persistent intracellular smad responses.

While the pretreatment paradigm indicates that activin A can effectively reduce neural damage, it remained essential to learn if administration in a relevant therapeutic window following ischemia/reperfusion injury was neuroprotective. In this study, transient MCAO was followed 6 hr later by a single injection of activin A (2.5 ug/kg) or vehicle, and animals assayed at 24 hr post-stroke (Figure 2 A,B). Again, animals were randomized and operators were blinded to treatment group through surgery and histological analyses. Despite delaying activin A administration for 6 hrs, infarct volumes in these mice were reduced by 43% when compared to vehicle-treated animals (Figure 2A). As expected, after vehicle treatment, large portions of the ipsilateral striatum and cortex were damaged, with a broad rostrocaudal distribution along the MCAO territory (Figure 2B, top). TTC staining revealed that nearly all striatal and dorsolateral cortical tissue was damaged in these animals at 24 hr, and there was a distinct border in the cortex that demarked live and dead tissue. By contrast, activin A administration reduced in infarct size and spared portions of the dorsolateral parietal cortex (Figure 2B, bottom), with predominant activin A tissue effects occurring in regions that correspond to portions of the penumbra. In parallel animals, blood gases were tested to ensure that activin A treatment following ischemia/reperfusion. There were no differences in the pH, partial pressure of oxygen or carbon dioxide following ischemia/reperfusion and activin A or vehicle injection (Table 1), suggesting that protection by activin A tissue cannot be attributed to changes in these parameters.

To test whether activin A administration was sparing brain tissue or simply delaying tissue death, similar studies with the operator blinded to the treatment group were performed and survival was extended to 3 days post-ischemia/reperfusion. To learn if the initial dose of activin A was optimal for long-lasting effects, animals with transient MCAO received a single injection at 6 hrs reperfusion of activin A at 2.5ug/kg as above or at 15ug/kg, with corresponding vehicle controls, and infarct volumes were determined at 72 hrs (Figure 3 A,B). Extensive infarct maturation was observed in vehicle-treated animals at 72 hrs with evidence of hemispheric asymmetry (Figure 3C). Although the lower dose effectively reduced infarct volume when assayed at 24 hrs, this dose was less effective when damage was evaluated at 3 days (Figure 3 A,C). By contrast, a single injection of activin A (15ug/kg) reduced infarct volume by 41% compared to vehicle when assessed at 3 days (Figure 3B). Activin A administration resulted in distinct borders between live and dead tissues, and obvious preservation of cortical tissue (Figure 4C). These reductions in infarct maturation were associated with improved neurological behavior at 24 and 72 hrs (Figure 3D). Vehicle treated animals displayed body rotation to the paretic side when suspended in air, grip deficits with internal rotation and circling toward the paretic side at 1 day. When observations extended to 3 days, vehicle treated animals maintained their circling behavior in addition to internal body rotation when lifted by their tail. By contrast, activin A improved neurological behavior starting at day 1. Activin A-treated

animals did not circle tightly or exhibit head tilting at day 1 and demonstrated complete loss of circling behavior by day 3. These data indicate that activin A protection is effective when administered hours after ischemic onset and can reduce stroke neurodeficits.

To identify activin A effects on brain cells, neurons and microglial cells were evaluated in animals that received MCAO followed by activin A or vehicle treatment at 6 hrs and assayed at 24 hr reperfusion by immunohistochemistry. Reaction with the neuron-specific marker NeuN revealed many more neurons present in the ischemic territory after activin A treatment compared with vehicle alone (Figure 4B, top). In particular, many NeuN immunoreactive neurons were apparent in the dorsolateral cortical regions that appeared spared by activin A. These results were consistent with the general distribution of cell survival apparent by TTC tissue staining and suggest that activin A was indeed neuroprotective. Future studies are needed to test neuronal density in these regions after several days post-injury and treatment. Neural damage is followed by activation of macrophage/microglial cells that can be labeled by the ionized calcium-binding adaptor molecule 1 (Iba1) (Ito et al., 1998). Following MCAO and vehicle administration, numerous Iba1 immunoreactive cells with an activated, amoeboid microglial phenotype were detected in the infarct zone (Figure 4B, bottom). By contrast, after activin A treatment few activated macrophage/microglial cells were observed (Figure 4B). Immunostaining of the contralateral hemisphere in both groups demonstrated Iba1-positive cells with a nonactivated, ramified morphology (not shown). These observations suggest that activin A administration spares neurons after cerebral ischemia, and this reduction in neural damage is accompanied by a concomitant decrease in microglial activation.

Focal cerebral ischemia results in the release of apoptogenic factors such as activated caspase-3 that play critical roles in effecting cell death. To determine if activin A treatment reduced apoptotic factors, activated caspase-3 immunopositive cells were evaluated. Many cleaved caspase-3 immunoreactive cells were present in cortex following MCAO with vehicle administration at 24 hr of reperfusion (Figure 4C). By contrast, in the cortical regions spared by activin A that included many NeuN positive cells, there were few caspase-3 immunoreactive cells. Indeed, Western immunoblot for caspase 3 of the ipsilateral hemisphere suggested that activin A treatment had 30% less caspase activity compared to saline, at 24 hr. These data suggest that delayed activin A administration after ischemia/reperfusion interferes with apoptotic signals.

Brain cells activate multiple intercellular signaling cascades after cerebral ischemia/reperfusion such as the PI3k/Akt and the mitogen activated protein kinase (MAPK) pathways that are crucial for maintaining cell survival or initiating cell death (Nozaki et al., 2001; Zhao et al., 2006). To determine if activin A affected these intercellular signals after ischemia/reperfusion, animals with ischemia/reperfusion followed 6 hr later by activin A (15 ug/kg) or vehicle treatment were evaluated by immunoblot 1 hr after treatment. Activated forms of Akt and ERK 1/2 MAPK proteins levels were unchanged following Activin A injection, suggesting that the neuroprotective effects of Activin A are unlikely to rely on changes in either pathway (Figure 5 A,B). By contrast, phosphorylation at both the threonine and tyrosine residues of p38 (p-p38) and JNK1/2 (p-JNK1/2) required for full activation of either kinase were dramatically decreased after 1 hr following activin A treatment (Figure 5C,D (Nozaki et al., 2001). Activation of the JNK and p38 pathway is known to occur in response to stress stimuli and both play important roles in the induction of apoptosis and inflammatory cytokine production. The reduction in both p38 and p-JNK1/2 suggests that activin A administration inhibits the kinases upstream of p38/JNKs and intervenes in the early stages of the apoptotic cascade.

DISCUSSION

The balance between protective mechanisms and the signals that lead to cell death define the extent of tissue damage and functional impairment after cerebral ischemia/reperfusion (Lo et al., 2003). In this study, the TGF-beta family member activin A is shown to be effective in decreasing ischemia/reperfusion injury. Most notably, the level of protection remains high when activin A is administered as late as 6 hrs after transient cerebral ischemia. Delayed activin A administration specifically correlates with reduced activation of p38 and JNK1/2, suggesting its effects may be associated with inactivation of the apoptotic cascade involving stress kinases. These findings discussed below have important implications in the understanding of the potential mechanisms underlying activin A cellular effects as well as for the development of effective molecular targets in cerebral ischemia.

Delayed administration of activin A may not affect neurons that undergo necrosis in core regions in the early minutes after ischemia but is likely to target cells in the penumbra that have sustained mild ischemic injury and are vulnerable to further insult due to reperfusion events. Previous reports have shown that activin A promotes survival of neuron-like P19 cells (Schubert et al., 1990), neuron-like SH-SY5Y neuroblastoma cells (Kupersmidt et al., 2007), as well as primary dopaminergic (Kriegstein et al., 1995), hippocampal (Iwahori et al., 1997), and cortical (Mukerji et al., 2007) neurons *in vitro*. *In vivo*, activin A administration leads to the robust survival of striatal and hippocampal neurons following quinolinic (Hughes et al., 1999) and kainic acid (Tretter et al., 2000) excitotoxicity as well as hypoxic-ischemic injury in the perinatal rat (Wu et al., 1999). In the latter study, 1 µg activin A protected hippocampal neurons but failed to protect cortical neurons in the neonatal rat. By contrast in the present study, robust neuronal survival was observed in cortex following focal cerebral ischemia in the adult mouse. This discrepancy could be due to differences in activin A receptor expression by neurons at various developmental stages. For example, in 3 week rats, the activin type II receptor is weakly expressed in the parietal cortex with limited expression in layer IV (Wu et al., 1999) whereas by 7 weeks, there is robust protein expression in cortical layers II-IV (Funaba et al., 1997). In the present study, mice 6-8 weeks of age also demonstrated phosphorylated smad2 in cortical neurons following activin A administration, indicating activin A receptors are present in these animals and are able to transmit neuronal intracellular signals.

While activin A treatment one hour prior and six hours following ischemia/reperfusion was effective, it remains unclear when the optimal time period for treatment occurs. In other studies on erythropoietin treatment for ischemia/reperfusion, epo systemic administration at 3 and 6 hours after stroke reduced infarct volume following rat brain ischemia/reperfusion, although 3 hour treatment was most effective (Brines et al., 2000). A detailed timecourse as well as evaluation of administrative route in rodents will provide information as to optimal treatment window for activin A in future studies. It remains unclear if time periods in rodents are similar to those expected for humans, in light of considerable differences in brain size and potential perfusion.

Activin mRNA and protein increases accompany brain injury and inflammation. We have shown that activin A mRNA increases rapidly after the transient focal cerebral ischemia/reperfusion injury model used in this report (Mukerji et al., 2007), and Activin A increases in cerebrospinal fluid in a subset of patients with severe traumatic brain injury (Phillips et al., 2006). While it is difficult to directly compare the "robust responder" values in the previous study of greater than 0.2 ng/mL Activin A in human CSF with the "high" dose of activin at 15 µg/kg body weight given to mice in this report, the experimental doses in this study appear much higher than the endogenous levels responses *in vivo* (that are also ineffective for repair). Increased plasma concentrations of activin A are poor prognostic signs in preterm newborns

(Florio et al, 2006), and following heart surgery in children (Florio et al, 2007), perhaps because they reflect trauma or stress. Experimental models have also shown increases in circulating activin A after surgery or lipopolysaccharide (Jones et al, 2000, 2004). The endogenous increases are not likely to be highly therapeutic, but rather serve as biomarkers of injury or inflammation.

Ischemia/reperfusion is followed by an inflammatory response resulting in leukocyte infiltration, and the production of inflammatory cytokines. Iba1, the calcium binding protein, has restricted expression in the macrophage/microglial population and antibodies to Iba1 are thought to bind to “activated” microglia. Focal cerebral ischemia in rats was accomplished by increases in Iba1 immunoreactive cells beginning at 3.5 hrs after reperfusion, and peak at 7 days that were spread throughout the ischemic area (Ito et al., 1998). In the present study, vehicle-treated animals included many Iba1 immunopositive microglia/monocytes at 24 hr, while those treated with activin A had far fewer Iba1 immunoreactive cells. It is unclear whether this decrease results from a smaller infarct and decrease in injury-related molecules, or the direct inhibition of microglial cells by activin A. There are reports that activin A has anti-inflammatory effects on cultured microglial cells (Sugama et al., 2007) while other reports suggest activin promotes the differentiation of macrophages (Ogawa et al., 2000), and monocytes (Jones et al., 2007). Microglia themselves can also serve as a source of activin A if appropriately stimulated (Michel et al., 2003). Whether microglia/macrophages are deleterious after brain ischemia remains unclear. In other studies, activated microglia/macrophages and the cytokines released play key roles in the development of inflammatory lesions following ischemia, and agents inhibiting microglial activation result in functional neurological improvement and reduction of ischemic cell damage (Yrjanheikki et al., 1999; Kim et al., 2006). However, selective ablation of microglia/macrophages exacerbates ischemic tissue death (Lalancette-Hebert et al., 2007). Microglial activation is one of the fundamental responses to brain injury and the immune response is necessary for repair when appropriately controlled. It is plausible that agents that dampen the microglial/macrophage response after focal cerebral ischemia are tissue protective whereas their selective ablation impedes the necessary repair response. Understanding the sequence of molecular signals that recruit different leukocyte populations after stroke will be essential to interfere with pathogenic cells while preserving those involved in repair.

From a technical perspective, it was possible that the influx of numerous infiltrating cells might bias our assay of infarct volume at later time points. TTC relies upon the presence of live cells to stain normal tissue deep red, thereby delineating areas of normal versus abnormal tissue (Bederson et.al 1998). Significant infiltration of healthy immune cells into otherwise infarcted regions might lead to the erroneous conclusion that the infarct was smaller and the treatment was effective. Some have used TTC as an initial assay to measure infarct volume at 24 and 72 hr as in this study (Yanagisawa et al., 2008; Bardutzky et al., 2005; Swanson et al 1990) although TTC may not be as effective as cresyl violet-based histological analyses at long time periods after treatment. Lin and coworkers (1993) found a close correlation in infarct volumes with TTC and hematoxylin and eosin stained sections up to three days. Our data actually suggest that fewer infiltrating Iba1-immunoreactive cells are present after activin A treatment, not more, reducing the impact of this concern for the immediate study.

The mechanism by which activin A treatment results in neuronal preservation is not clear, although one working hypothesis is that Activin A may interfere with glutamate excitotoxicity. Until recently the function of activin A in the adult brain was largely unknown because animals with germline deletion of the gene that encodes activin A die perinatally (Matzuk et al., 1995). New insights into the role of activin A has come from recent work in which transgenic mice expressing a dominant negative activin A receptor IB construct under the calmodulin-kinase II-alpha promoter (Muller et al., 2006). The inhibition of activin A signaling in neurons

results in increased neuronal death after kainic acid (KA) lesion, confirming that one function of endogenous activin A is as a neuronal rescue factor. Glutamate excitotoxicity is a fundamental component of KA lesions as well as focal cerebral ischemia and it is possible that activin A neuroprotection may be due to decreased glutamate neurotransmission. However, in animals with the dominant negative activin A receptor, loss of activin signaling resulted in the unexpected reduction of glutamatergic transmission (Muller et al., 2006). Future studies to test whether and how activin A administration may alter glutamate signaling after ischemia/reperfusion remain important to understand if this potential mechanism underlies activin A effects, and which molecular signals may account for the protective function.

Inhibition of intracellular apoptotic signals presents a second molecular mechanism of activin A protective effects against *in vivo* brain injury. In primary mouse E14 striatal cell cultures, activin A application results in ERK1/2 phosphorylation but failed to alter p38 or pJNK expression (Bao et al., 2005). By contrast in the present study, activin A did not phosphorylate Akt or ERK1/2 1 hr after adult cerebral ischemia/reperfusion but rather, inhibited the p38 and JNK phosphorylation stimulated by stroke. This disparity in intracellular responses could be due to differences in activin A effects on developing versus adult neurons or due to cellular injury in cerebral ischemia/reperfusion. Indeed, JNKs and p38 MAPKs are activated in response to a variety of cell stress including reactive oxygen species, DNA damage and inflammatory-induced cytokines. Activation of p38 and JNK signals occurs in both cerebral and myocardial ischemia/reperfusion and inhibitors to these signals result in the robust attenuation of tissue infarction in models of adult cerebral ischemia/reperfusion (Borsello et al., 2003). Other studies have shown that TGF-beta-induced smad signaling prevents proinflammatory cytokine production through the inhibition of p38 and NF- κ B in macrophages (Jono et al., 2003). Simultaneous reduction in both p38 and JNK activation indicates that activin A administration potentially regulates a common upstream kinase such as apoptosis signal-regulating kinase 1 (ASK1; (Nozaki et al., 2001). Future studies to learn how activin A stimulation alters the stress kinase pathway after ischemia/reperfusion remain important as the specific modulation of these kinases is therapeutically attractive and allows molecular dissection of activin effects on apoptosis.

In summary, this report provides the first evidence that delayed activin A administration within hours after cerebral ischemia/reperfusion spares brain tissue and preserves function. The molecular mechanisms that underlie this effect are likely to be complex as initial characterization suggests that activin A may modulate intercellular neurotransmitter effects and abrogate intracellular cascades that lead to apoptosis. Future studies to test each of these potential mechanisms, as well as experiments to evaluate optimal administration routes and doses are warranted in light of the robust effects of Activin A treatment following cerebral ischemia/reperfusion.

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Figure 1. Activin administration protects against focal cerebral ischemia and activates intracellular signals

A) Infarct volumes following vehicle (grey bar) or activin A (black bar) injection. Mice received i.c.v. injections of Activin (2.5 ug/kg) or vehicle 1 hr prior to a transient MCAO. Twenty-four hours after reperfusion, brain tissues were assayed for infarct volumes by TTC staining. Activin A pretreatment reduced infarct volume by 50% compared to vehicle injected controls. Data represents the mean $\pm$ S.E.M; n=5/group, student t test, ***p<0.0001 when compared to vehicle treated animals. **B)** Adult mice received an i.c.v. injection of activin A (2.5 ug/kg) or vehicle. Brain tissue from the injected hemisphere was isolated at the various times indicated and immunoblot performed with an anti-phospho-Smad2 followed by reprobe for Smad2 and actin proteins. Each lane represents different animals. **C)** Optical density of bands (phospho-smad2/total smad2) for the indicated times after activin A injection. Phosphorylation of Smad2 was increased at 1 hr with prolonged activation at 3 hr. Each bar represents mean $\pm$ S.D., student t test *p<0.05, **p<0.001 comparing activin A psmad2/smard2

ratios at 1 hr and 3 hr with vehicle 1 hr, respectively. **D)** Activin A induces phosphorylated smad2 in neurons. Brain sections were prepared from adult mice 3 hr after i.c.v injection of either vehicle (top) or activin A (bottom). Brain sections from cortex were labeled with anti-phospho-smad2 antibody (*red*) and anti-NeuN (*green*). Merged overlay reveals activin A-induced smad2 activation occurs in neurons (*yellow*). Scale bar, 50 μ m.

Figure 2. Activin A administered after cerebral ischemia prevented tissue death

A) Adult mice underwent a 1 hr MCAO followed by vehicle or activin A (2.5ug/kg) i.c.v. injection into the lateral ventricle at 6 hr of reperfusion. Infarct volumes were assayed with TTC staining at 24 hr of reperfusion. Activin A reduced infarct volume by 43% compared to the corresponding vehicle treated group. Each bar represents mean $\pm$ S.E.M (vehicle, n=11, grey bar; activin A, n=12, black bar), student t test ***p<0.001 when compared to vehicle treated animals. **B)** Representative TTC pictures of vehicle-injected animals (top) demonstrate clear striatal and dorsolateral cortical tissue death in these animals at 24 hr and a distinct border between live (red) and dead tissue (white). By contrast, activin A administration reduced infarcted tissue, sparing large portions of the dorsolateral parietal cortex (bottom).

Figure 3. Activin A tissue protective effects persist

Exogenous activin A reduces tissue damage up to 3 days after MCAO. Following transient MCAO and 6 hr reperfusion, i.c.v. injection of vehicle or activin A (2.5ug/kg or 15ug/kg), infarct volumes were calculated at 72 hr of reperfusion with TTC staining. **A)** Low dose activin A (2.5ug/kg) reduced infarct volume at 3 days but differences between groups were not significant. Each bar represents mean $\pm$ S.E.M (vehicle n=4, grey bar; Activin n=4, black bar). **B)** High dose of activin A (15ug/kg) resulted in a 41% reduction in infarct volumes when compared to the vehicle treated group. Each bar represents mean $\pm$ S.E.M (vehicle n=6, grey bar; activin A n=5, black bar), student t test $**p < 0.01$ when compared to vehicle treated animals. **C)** Representative TTC sections from vehicle (top), activin A (2.5ug/kg, middle) and activin A (15ug/kg, bottom) treated animals. **D)** Evaluation of stroke neurological deficits indicates activin A (15ug/kg) injected animals have improved neurological scores at 24 hr and 72hr (Friedman's Test, $p = 0.008$ Activin). There was no improvement in vehicle treated animals over 24 or 72 hr ($p = 0.135$ vehicle)

Figure 4. Delayed Activin A delivery spares neurons and reduces apoptosis

Mice received a transient MCAO followed by 6 hr reperfusion before receiving an i.c.v. injection of activin A (15ug/kg) or vehicle and brain sections collected at 18 hr after injection (24 hr of reperfusion) for immunohistochemical studies. **A**) Schematic representation of brain region included immunostaining. **B-C**) Photomicrographs of sections with vehicle and activin A (15ug/kg) for: anti-NeuN antibody; Scale bars: 100µm, inset 50 µm (B, top) ; anti-Iba1 antibody; Scale bar; 50 µm (B, bottom); and anti-cleaved-caspase 3 antibody; Scale bar; 50 µm (C). Delayed activin A administration resulted in more neurons present in cortex at 24 hr after MCAO with a parallel decrease in Iba-1-immunopositive microglia. More cleaved-caspase 3 immunoreactivity was present in vehicle (*arrows*) than in activin A-treated animals.

D. Western immunoblot for cleaved caspase 3 in animals stroked and treated at 6 hr. Each lane represents material from one hemisphere from one animal. Cleaved caspase 3 had Mr about 17kd, and protein loading was normalized with actin. Numbers indicate arbitrary units following densitometry. Animals treated with activin A had about 30% less cleaved caspase 3 in the stroked hemisphere at 24 hr.

Figure 5. Activin A reduces p38 and JNK MAPK activation but does not alter Akt or ERK1/2 activation after focal ischemia

Adult mice were subjected to MCAO and 6 hr of reperfusion followed by activin A (15ug/kg) or vehicle injection. Brain tissues from the ischemic hemisphere were prepared 1 hr after injection for immunoblotting with phospho-specific antibodies recognizing **A**) phosphorylated Akt (ser473, p-Akt), **B**) phosphorylated ERK1/2 (p-ERK1/2), **C**) phosphorylated p38 MAPK (p-p38) or **D**) phosphorylated JNK/SAPK (p-JNK1 and p-JNK2) protein levels. Blots were stripped and reprobed for total Akt, ERK1/2, p38, JNK/SAPK and β -actin protein. Quantification of band density (phosphorylated protein/total protein) from activin A (n=6) or Vehicle (n=5) treated animals is shown. Graph represents the mean $\pm$ S.D. student t test

*** $p < 0.001$ for phospho-p38 and ** $p < 0.01$ for p-JNK1 and p-JNK2 when compared to appropriate control values. Activin A delivery did not affect activated levels of either Akt or ERK2 but robustly decreased activated p38 and pJNK1 and pJNK2 at this timepoint.

Table I**Blood Gas Analysis**

Blood samples were obtained from mice that were subjected to 1 hr MCAO followed by 6 hr of reperfusion before single activin A (15 ug/kg) or vehicle injection. Blood gas measurements were obtained by withdrawing 150 μ l of blood from the left ventricle with a heparinized syringe from isoflurane-anesthetized animals 30 minutes after activin A or vehicle injection and immediately analyzed. Data were compared by unpaired t-test, and in no comparison were values for Activin A and vehicle treated animals different.

Parameter	Vehicle	Activin A
pH	7.27 $\pm$ 0.01	7.26 $\pm$ 0.02
PaO ₂ (mmHg)	98 $\pm$ 1	113 $\pm$ 10
PaCO ₂ (mm Hg)	51 $\pm$ 5	41 $\pm$ 6
Weight (gm)	29.7 $\pm$ 1.2	30.3 $\pm$ 1.2