RESEARCH PAPER

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Angiotensin II induces cell growth and IL-6 mRNA expression through the JAK2-STAT3 pathway in rat cerebellar astrocytes

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The pleiotrophic effects of angiotensin II (Ang II) play important roles in astrocyte growth and inflammatory responses. We investigated whether Ang II induces astrocyte growth and interleukin-6 (IL-6) mRNA expression in rat cerebellar astrocytes through Janus kinase 2-signal transduction activator of transcription (JAK2-STAT3). Ang II increased JAK2 and STAT3 phosphorylation in a time- and a dose-dependent manner. One hundred nanomolar Ang II induced maximal phosphorylation of both JAK2 and STAT3 between 15 min and 30 min. The Ang II-mediated phosphorylation of both JAK2 and STAT3 was blocked by AG490, a selective JAK2 inhibitor. Losartan, a selective AT1 receptor antagonist, inhibited Ang II-mediated JAK2 and STAT3 phosphorylation, while pretreatment with an AT2 receptor blocker, PD123319, was ineffective. Ang II increased the mRNA expression of IL-6 in a concentration-and time-dependent manner. Maximal IL-6 mRNA expression occurred with 100 nM Ang II, and the peak effect occurred in a biphasic manner at 3 h and between 12 and 24 h. Moreover, pretreatments with AG490 attenuated Ang II-induced IL-6 mRNA levels, and Ang II-induced astrocyte growth. This study has demonstrated that Ang II induced the phosphorylation of both JAK2 and STAT3 via the AT1 receptor in cerebellar astrocytes. In addition, our results suggest that JAK2 and STAT3 are upstream signals that mediate Ang II-induced IL-6 mRNA expression and astrocyte growth. These findings represent a novel non-classical mechanism of Ang II signaling in cerebellar astrocytes.

Introduction

The renin-angiotensin system (RAS) plays a major role in regulating physiological processes of the cardiovascular system. Angiotensin II (Ang II) is the primary effector peptide of the RAS and it is produced by cleavage of the precursor molecule angiotensinogen. Ang II has emerged as a critical hormone, growth factor and proinflammatory molecule that affects the function of virtually all organs and structures including heart, kidney, the vasculature and the brain.[1] In brain, the peptide triggers a myriad of physiological responses such as increased salt appetite, increased sympathetic outflow, and many other responses implicated in physiological as well as pathological consequences of the peptide.[2,3] Centrally, astrocytes are the major sources of angiotensinogen the precursor molecule of Ang II suggesting an important role of astrocytes in central RAS effects.[4]

Ang II exerts its action through two pharmacological classes of G protein-coupled receptors, known as the Ang AT1 and Ang AT2 receptors.[5,6] In astrocytes, Ang II interaction with the AT1 receptor causes activation of several intracellular signaling pathways involving mitogen activated protein (MAP) kinases, tyrosine kinases, protein kinase C (PKC), immediate early response genes and others.[7-12] These intracellular pathways are involved in widely diverse effects of Ang II including cell growth, proliferation and inflammatory actions.[13,14] The Janus kinase-signal transducer and signal transduction activator of transcription (JAK-STAT) pathway is a characteristic signal transduction pathway that plays a crucial role in development and homeostasis.[15] JAK-STAT signaling mediates several Ang II-induced physiological and pathological responses.[16-18] It has been shown that Ang II, via AT1 receptor activation, stimulates JAK2, a key member of the Janus family of kinases. This leads to phosphorylation and activation of a group of transcription factors collectively called STATs.[19] Phosphorylated STATS translocate into the nucleus, where they bind to specific DNA sequences called ST-domains that are present in the promoter regions of targeted genes. These transcription factors have been shown to mediate Ang II-specific vascular smooth muscle growth, migration and remodeling as well as cardiac muscle hypertrophy.[16,20] The JAK-STAT signaling cascade was shown to be an important link

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between activation of the AT1 receptor and nuclear transcriptional changes leading to cell growth.[21]

Thus in peripheral systems, it is well established that the JAK-STAT pathway is a key player in Ang II-mediated physiological and pathological responses.[19] In addition, Ang II-induced activation of the JAK-STAT pathway regulates smooth muscle function, leading to increased production of angiotensinogen and IL-6.[16,19,20] IL-6 is an inflammatory cytokine whose expression in plasma is elevated through activation of the JAK-STAT pathway.[21] IL-6 has multiple biological activities such as induction of cell growth and development, and inflammation; it also plays a role in several disease processes as well.[21,22] Ang II interaction with the AT1 receptor and subsequent to IL-6 production may be a critical factor contributing to many cardiovascular diseases including heart attack, stroke and hypertension.[17,23-27] Moreover, central production of IL-6 mediates the synthesis of C-reactive protein (CRP), an important risk factor for myocardial infarction.[23,27] Further, in cardiomyocytes and smooth muscle cells, Ang II induces the upregulation of angiotensinogen via IL-6/JAK-STAT-dependent mechanisms.[1,28] Our previous studies showed that in rat brainstem astrocytes, Ang II acts on AT1 receptors to induce the secretion of IL-6 via JAK2-STAT3 pathway.[29] In this study, we examined the role of JAK2-STAT3 signaling on cell growth and IL-6 mRNA expression in cerebellar astrocytes. Cerebellar astrocytes were selected since it has been shown that Ang II activates distinct signaling pathways leading to cellular proliferation, prostacyclin release, tyrosine kinase activation and other effects in cerebellar astrocytes.[9,12]

Results

JAK2 activation by Ang II. The optimum dose of the peptide to cause JAK2 protein phosphorylation was determined by incubating cerebellar astrocytes for 15 min with Ang II ranging in concentrations from 0.1 nM to 1 μM. Our study revealed that phosphorylation of JAK2 protein by Ang II occurred in a concentration-dependent manner (**Fig. 1A**). Maximal phosphorylation was observed with 100 nM Ang II (2.23 ± 0.2-fold over basal). As shown in **Figure 1B**, significant phosphorylation of JAK2 by Ang II occurred by 10 min and was maximal by 15 min. Selective Ang receptor blockers were used to identify the receptor subtype that mediated the phosphorylation of JAK2 protein by Ang II. Pretreatment with the AT1 receptor blocker, Losartan, followed by Ang II stimulation blocked Ang II-induced JAK2 phosphorylation by 88% (**Table 1**). Pretreatment with 10 μM PD123319, the selective AT2 receptor antagonist failed to prevent Ang II-mediated JAK2 phosphorylation (**Table 1**).

To determine the specificity of this Ang II effect, quiescent astrocytes were pretreated for 15 min with 50 μM AG490, the JAK2 inhibitor, followed by stimulation with Ang II. As shown in **Table 1**, pretreatment with AG490 completely prevented Ang II-induced JAK2 phosphorylation suggesting that this is a selective effect of Ang II in cerebellar astrocytes to induce JAK2 phosphorylation.

STAT3 activation by Ang II. Cultured neonatal cerebellar astrocytes were incubated with increasing concentrations of Ang II (0.1 nM to 1 μM) for 15 min to determine whether the peptide increased STAT3 phosphorylation in these cells. As shown in **Figure 2A**, maximal phosphorylation was observed with 100 nM Ang II. Treatment of cerebellar astrocytes with 100 nM Ang II for

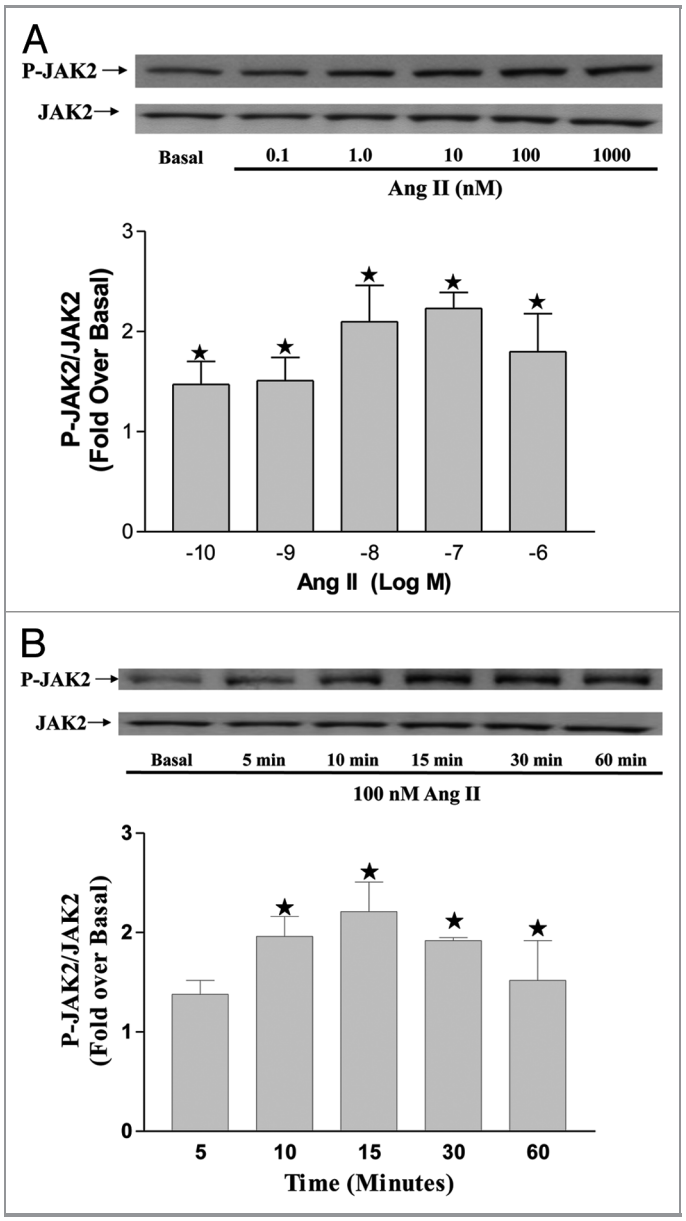

Figure 1. Effect of concentration and time on Ang II-induced JAK2 protein phosphorylation. Quiescent monolayers of cerebellum astrocytes were incubated for 15 min with increasing concentrations of Ang II (A) or with 100 nM Ang II for 5 min to 60 min (B). JAK2 protein phosphorylation was measured by western blot analysis using an antibody specific for the phosphorylated form of JAK2. Protein loading was visualized using a non-phosphorylated JAK2 antibody. The data were analyzed by densitometry and the amount of phosphorylation was calculated as the fold increase over basal in the presence of vehicle. Each value represents the mean ± SEM of preparations of cerebellar astrocytes isolated from six or more litters of neonatal rat pups. *denotes $p < 0.05$ as compared with basal levels for JAK2 phosphorylation in astrocytes prepared from the cerebellum

Table 1. Effects of AG490, Losartan and PD123319 on Ang II-induced JAK2 phosphorylation

Treatment	Fold over basal
100 nM Ang II	2.3 ± 0.42
10 μM Losartan	1.20 ± 0.03
10 μM Losartan + 100 nM Ang II	1.16 ± 0.2*
10 μM PD123319	1.0 ± 0.4
10 μM PD123319 + 100 nM Ang II	1.9 ± 0.4
50 μM AG490	1.2 ± 0.4
50 μM AG490 + 100 nM Ang II	0.95 ± 0.3*

*Denotes $p < 0.05$ as compared with Ang II stimulation of JAK2 phosphorylation. Basal and stimulated astrocytes were treated with DMSO, the vehicle for the inhibitors. Data were quantified by densitometric analysis and are the results of individual experiments of 6 or more preparation of astrocytes.

5 min to 60 min showed that Ang II stimulated STAT3 phosphorylation in a time-dependent manner (**Fig. 2B**) with maximal Ang II phosphorylation observed at 15 min (1.71 ± 0.23-fold over basal). Taken together, these findings suggest that Ang II induces both JAK2 and STAT3 phosphorylation in cerebellar astrocytes.

To determine the receptor mediating Ang II phosphorylation of STAT3, quiescent astrocytes were pretreated with either 10 μM Losartan (AT1 receptor antagonist) or 10 μM PD123319, the selective AT2 receptor antagonist. As shown in **Table 2**, Losartan treatment inhibited by over 75% Ang II-mediated STAT3 phosphorylation. On the other hand, inhibition of the AT2 receptor by PD123319 did not significantly affect Ang II phosphorylation of STAT3. These findings suggest that Ang II induce STAT3 phosphorylation through activation of the AT1 receptor.

Previous studies showed that activation of JAK2 leads to phosphorylation of STAT proteins.[18,19] Thus, in these studies we determined whether JAK2 mediates Ang II phosphorylation of STAT3. Quiescent astrocytes were pretreated for 15 min with 50 μM AG490, the selective JAK2 inhibitor. Astrocytes were subsequently stimulated with 100 nM Ang II for 15 min. Pretreatment with the JAK2 inhibitor completely abolished Ang II-induced phosphorylation of STAT3 (**Table 2**). These findings suggest that JAK2 is an upstream mediator of Ang II-induced STAT3 phosphorylation.

Ang II upregulation of IL-6 expression. IL-6 mRNA is expressed in activated astrocytes[30] and our previous studies have shown that activation of the JAK-STAT pathway leads to stimulation of IL-6 release from astrocytes isolated from brainstems.[29] Thus in these studies, using qPCR, we determined whether Ang II induces the expression of IL-6 mRNA in cerebellar astrocytes. Quiescent cerebellar astrocytes were treated with Ang II ranging in concentrations from 0.1 nM to 1 μM range. As shown in **Figure 3A**, Ang II upregulated IL-6 mRNA expression in a concentration-dependent manner. Maximal IL-6

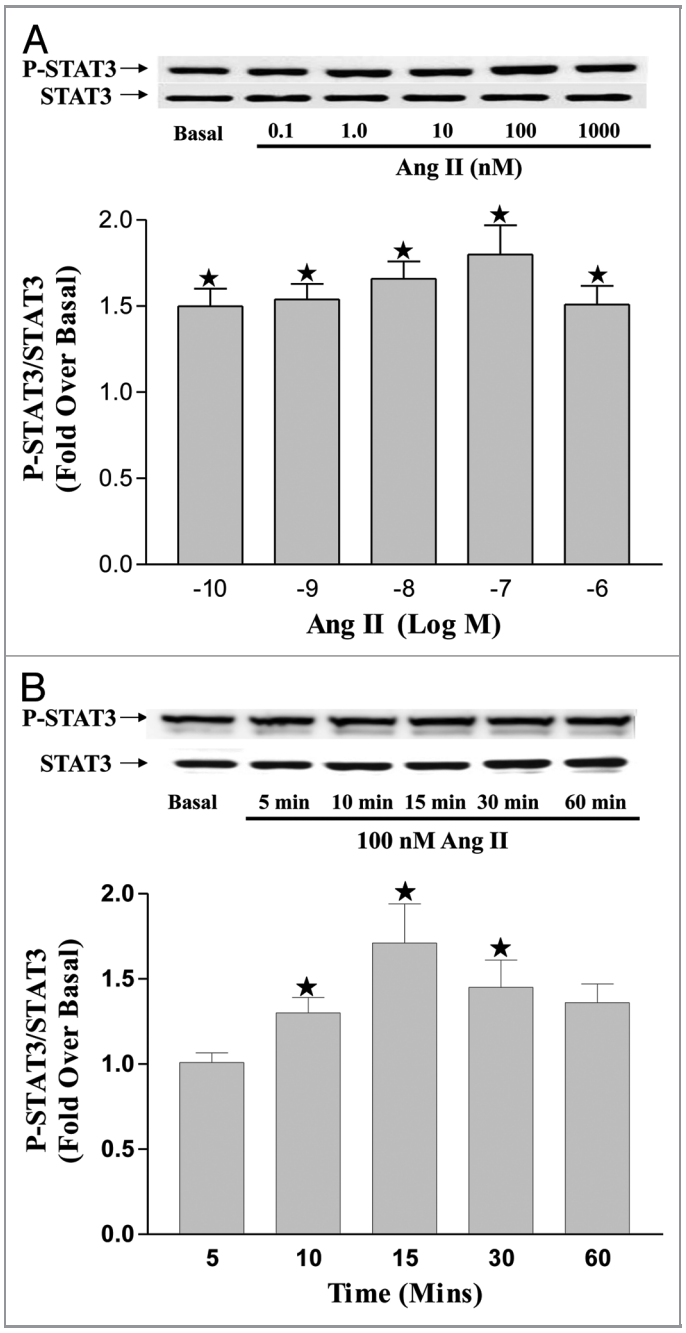

Figure 2. Effect of concentration and time on Ang II-induced STAT3 protein phosphorylation. Quiescent cerebellar astrocytes were incubated with increasing concentrations of Ang II for 15 min (A) or with 100 nM Ang II for 5 min to 60 min (B). STAT3 protein phosphorylation was measured by western analysis using an antibody specific for the phosphorylated form of STAT3. Protein loading was visualized using a non-phosphorylated STAT3 antibody. The data were analyzed by densitometry and the amount of stimulation was calculated as the fold-increase over basal in the presence of vehicle. Each value represents the mean ± SEM of preparations of cerebellum astrocytes isolated from 6 or more litters of neonatal rat pups. *denotes $p < 0.05$ as compared with basal levels for STAT3 phosphorylation in astrocytes prepared from the cerebellum.

Table 2. Effects of AG490, Losartan and PD123319 on Ang II-induced STAT3 phosphorylation

Treatment	Fold over basal
100 nM Ang II	1.66 ± 0.2
10 μM Losartan	0.91 ± 0.1
10 μM Losartan + 100 nM Ang II	1.16 ± 0.3*
10 μM PD123319	0.9 ± 0.1
10 μM PD123319 + 100 nM Ang II	1.41 ± 0.2
50 μM AG490	0.84 ± 0.13
50 μM AG490 + 100 nM Ang II	0.80 ± 0.2*

*Denotes $p < 0.05$ as compared with Ang II stimulation of STAT3 phosphorylation. Basal and stimulated astrocytes were treated with DMSO, the vehicle for the inhibitors. Data were quantified by densitometric analysis and are the results of individual experiments of six or more preparation of astrocytes.

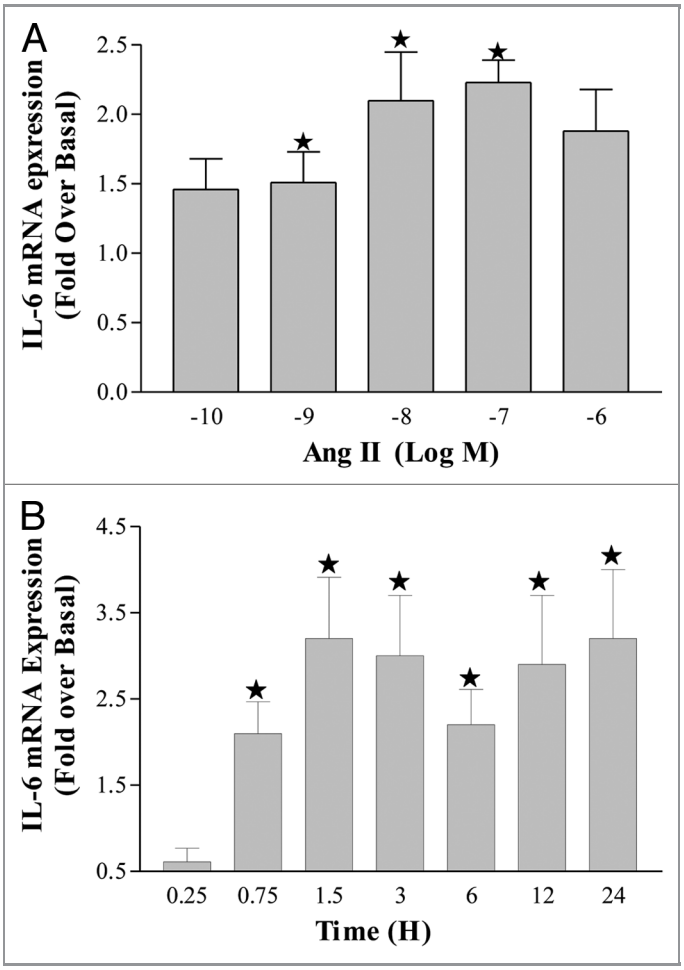

Figure 3. Effect of Ang II on IL-6 gene expression. Quiescent monolayers of cerebellar astrocytes were incubated with increasing concentrations of Ang II (A) or with 100 nM Ang II for 6 h to 48 h (B). IL-6 mRNA expression was analyzed by quantitative PCR. The amount of Ang II-stimulated IL-6 secretion was calculated as the fold-increase over basal. Each value represents the mean ± SEM of preparations of cerebellar astrocytes isolated from six or more litters of neonatal rat pups. *denotes $p < 0.05$ as compared with basal levels for IL-6 mRNA expression in astrocytes prepared from the cerebellum.

Table 3. Effects of AG490, Losartan and PD123319 on Ang II-induced IL-6 mRNA

Treatment	Fold over basal
100 nM Ang II	2.60 ± 0.07
50 μM AG490	0.76 ± 0.06
50 μM AG490 + 100 nM Ang II	0.76 ± 0.09*

*Denotes $p < 0.05$ as compared with Ang II-mediated IL-6 mRNA expression. Basal and stimulated astrocytes were treated with DMSO, the vehicle for the inhibitor. Values are calculated based on individual experiments of six or more preparation of astrocytes.

mRNA expression of 2.2 ± 0.2 fold over basal was observed with 100 nM Ang II. Quiescent cerebellar astrocytes were also pretreated with 100 nM Ang II for time periods ranging from 15 min to 24 h. Ang II increased IL-6 mRNA expression in a biphasic manner with peak effects occurring at 3 h, and over a 12 to 24 h time period (**Fig. 3B**). These findings suggest that Ang II may act in a proinflammatory manner to induce IL-6 mRNA expression in these cells.

To determine whether the JAK-STAT pathway is involved in Ang II-induced IL-6 mRNA expression, quiescent astrocytes were also pretreated for 15 min with 50 μM AG490, followed by stimulation with 100 nM Ang II. The JAK2 inhibitor completely abolished Ang II-induced IL-6 mRNA expression (**Table 3**). These findings suggest that the JAK-STAT pathway is involved in Ang II induction of IL-6 mRNA expression in these cells.

JAK2 mediates Ang II-induced astrocyte growth. To determine whether JAK2 mediates Ang II astrocyte growth, subconfluent quiescent astrocytes were pretreated for 15 min with 10 μM AG490, followed by a 48 h treatment with 100 nM Ang II. As shown previously,[29] it is necessary to use a lower concentration of AG490, since higher levels of the inhibitor significantly reduced basal ^{3}H-thymidine incorporation. ^{3}H-thymidine was added during the last 24 h of treatment to determine DNA synthesis as an index of astrocyte growth. As we have shown previously, Ang II significantly stimulated growth of cerebellar astrocyte (**Table 4**). The JAK2 inhibitor, AG490, significantly prevented (by 86%) Ang II-mediated astrocyte growth. These findings suggest a role for JAK2 in Ang II growth effects.

Discussion

Our results demonstrated that Ang II induced JAK2 phosphorylation in a time- and concentration-dependent manner in rat cerebellar astrocytes. Our study also revealed that Ang II induces

Table 4. Effects of AG490 on Ang II-induced astrocyte DNA synthesis

Treatment	Fold Over Basal
100 nM Ang II	2.14 ± 0.3
10 μM AG490	0 0.72 ± 0.4
10 μM AG490 + 100 nM Ang II	1.16 ± 0.2*

*Denotes $p < 0.05$ as compared with Ang II-stimulated thymidine incorporation. Basal and stimulated astrocytes were treated with DMSO, the vehicle for the inhibitor. Values are calculated based on individual experiments of five or more preparation of astrocytes.

JAK2-STAT3 phosphorylation in cerebellar astrocytes via the AT1 receptor. Furthermore, pretreatment of astrocytes with the selective JAK2 antagonist, AG 490, not only prevented the Ang II-induced JAK2 phosphorylation, but also the phosphorylation of its downstream transcription factor STAT3 (**Tables 1 and 2**). The JAK-STAT signaling pathway is considered as a stress-responsive signaling cascade that transduces signals from cell surface receptors to the nucleus, thereby modulating gene expression. Ang II interacts with Ang AT1 receptors leading to activation of the JAK-STAT signaling pathway, to cause various cellular responses such as proliferation, differentiation, migration and apoptosis.[16-18] This is the first study to show that both JAK2 and STAT3 are activated by Ang II in cerebellar astrocytes. We have previously shown a similar effect of the peptide in astrocytes isolated from the brainstem.[29] Astrocytes, isolated from the brainstem and cerebellum, are responsive to Ang II. Although our studies showed that brainstem astrocytes were more responsive to Ang II, in terms of JAK2 phosphorylation, than cerebellar astrocytes, this was a small effect which was not significant. These findings suggest that Ang II has similar effects in different areas of the brain.

There are three STAT isoforms, STAT1, STAT2 and STAT3 that are known to mediate Ang II effects.[18,19] While STAT1 and STAT2 promote growth arrest and apoptosis, STAT3 protects against apoptosis and enhances cell proliferation,[31] and thus we targeted this isoform in our studies. Our study showed that 100 nM Ang II induced cell proliferation in cerebellar astrocytes (**Table 4**). We further showed that inhibition of JAK2 prevented Ang II-mediated astrocyte proliferation. In terms of cellular proliferation, astrocytes isolated from the brainstem[29] and the cerebellum (**Table 4**) reacted similarly to Ang II. Ang II stimulated the proliferation of rat astrocytes through the Ang AT1 receptor, an effect mediated by MAP kinase pathways, the endogenous tyrosine kinase Src, and membrane bound tyrosine kinases.[8,9] These findings suggest that along with other intracellular pathways that the JAK2-STAT3 pathway is also involved in Ang II growth promoting effects and identifies this pathway as a mediator of Ang II proinflammatory effects.

IL-6 is an important cytokine involved in Ang II effects.[32] This cytokine has multiple biological activities such as induction of cell proliferation, inflammation and differentiation.[21] Ang II regulates IL-6 secretion and gene expression in many cell types,[17,23-27] and we have recently shown in brainstem astrocytes that Ang II induces IL-6 secretion.[29] In this study, we investigated whether Ang II increased IL-6 mRNA expression in rat cerebellar astrocytes. Our study revealed that IL-6 mRNA expression was upregulated by Ang II in a concentration- and time-dependent manner in these cells. Maximal IL-6 mRNA expression was observed with 100 nM Ang II (**Fig. 3A**). Ang II-induced IL-6 mRNA expression was biphasic with two peak effects observed at 3 h and between 12 and 24 h (**Fig. 3B**). Similar to our findings, a biphasic effect of Ang II to induce IL-6 secretion and mRNA levels was observed in vascular smooth muscle cells.[33] Since Ang II induces several known growth factors, it was suggested that this may be the cause of the biphasic effect in vascular smooth muscle cells. Nevertheless, the reason(s) for this biphasic effect in astrocytes is uncertain but suggests that Ang II has proinflammatory effects in these cells and may regulate short-term (the initial increase) and long-term (24 h increase) release of IL-6.

Ang II is a multifunctional peptide with a myriad of effects in the body, including proinflammatory effects.[14,34-36] IL-6 is a potent inflammatory cytokine which has been shown by us,[29] and others[33,37] to be induced by Ang II through actions at the AT1 receptor. These findings suggest that IL-6 may be an important cytokine involved in Ang II effects. In this study, we showed that Ang II induces IL-6 mRNA expression suggesting that this maybe one mechanism involved in Ang II proinflammatory effects. The JAK-STAT pathway mediates Ang II-induced IL-6 production.[38] Previously in brainstem astrocytes, we showed that inhibition of the JAK-STAT pathway with AG490 inhibited IL-6 secretion.[29] In the current study, we showed for the first time in cerebellar astrocytes that Ang II induces IL-6 mRNA expression, an effect mediated by the JAK-STAT pathway. Here, we have demonstrated in a different area of the brain which also exhibits a high concentration of Ang II receptors and distinct Ang II signal transduction pathways, that Ang II has proinflammatory effects mediated by the Ang AT1 receptor and the JAK-STAT pathway.

Activated astrocytes contribute to central inflammatory effects and tissue repair[39]; thus, it is important to study the pathways that lead to local immune responses (here, IL-6 production and astrocyte proliferation) in the brain. In addition, centrally produced Ang II may be involved in inflammation. There is a significant increase in the production of brain angiotensinogen, the precursor molecule for Ang II, during an inflammatory response.[40] Moreover, it has been shown in angiotensinogen-deficient mice that during cold injury, there is attenuation of GFAP expression and decreased laminin levels in astrocytes, resulting in an incomplete reconstitution of the blood brain barrier.[41] These findings suggest that during inflammation or physical trauma to the brain, there is an increase in Ang II, which may act in a paracrine fashion to stimulate growth and proliferation of astrocytes triggering astrocyte activation. Our studies have shown that Ang II increases the mRNA expression and release of the proinflammatory cytokine IL-6 from astrocytes, effects that are mediated by the JAK-STAT pathway. Thus, studies such as these and our previous studies,[29] are important in delineating the non-classical Ang II pathways leading to the activation of inflammatory responses in areas of the central nervous system known to express Ang receptors and associated signaling pathways. Finally, increases in central IL-6 and/or peptides of the RAS are associated with diseases such as stroke, Alzheimer disease, heart failure and hypertension. Thus, our findings support the relevance of the brain RAS, in particular the role of astrocytes.

Materials and Methods

Ang II was obtained from Bachem. The JAK2 inhibitor AG490 was purchased from Cal Biochem. PD123319, the selective AT2 receptor inhibitor was obtained from Sigma, and Losartan (AT1 receptor inhibitor) was kindly provided by DuPont Merck. The PhosphoSTAT3, STAT3, PhosphoJAK2 and JAK2 antibodies

were purchased from Cell Signaling Technology. Western blotting supplies were purchased from either GE Health Care or Biorad Laboratories or from Pierce Biotechnology. The BCA protein kit was obtained from Pierce Biotechnology. Quantitative PCR products including the primer sets for IL-6 were obtained from Applied Biosystems. All other chemicals were purchased from either VWR International or Sigma.

Astrocyte preparation. Timed pregnant Sprague-Dawley rats were obtained from Charles River Laboratories and maintained in the ALAAC-accredited animal facility of Nova Southeastern University. During the astrocyte isolation procedure, care was taken to minimize pain and discomfort to the animals. Primary cultures of astrocytes were prepared from the cerebellum of 2–3 d old neonatal rat pups by physical dissociation as previously described.[12] Cells were maintained in DMEM/F12 with 10% FBS, 100 mg/mL penicillin and 100 units/mL streptomycin at 37°C in a humidified incubator (5% CO_2 and 95% air). Cultures were fed every 3 to 4 d until confluent. Confluent monolayers were placed in DMEM/F12 containing 10 mM Hepes, pH 7.5, 10% FBS and antibiotics and shaken overnight to remove oligodendrocytes. Astrocytes were detached with trypsin/EDTA (0.05% trypsin, 0.53 mM EDTA), replated at a ratio of 1 to 10, and grown to approximately 80% to 90% confluency prior to use. Isolated cells were about 95% pure astrocytes showing a positive immunoreactivity with an antibody against glial fibrillary acidic protein (GFAP) and negative immunoreactivity with markers for neurons, or oligodendrites.

Cell lysate preparation. Cultured astrocytes were made quiescent by a 48 h treatment with serum-free media and all treatments were conducted in serum-free media. Immediately following treatments, cell lysates were prepared by washing cells with phosphate-buffered saline containing 0.01 mM $NaVO_4$ followed by solubilization in supplemented lysis buffer (100 mM NaCl, 50 mM NaF, 5 mM EDTA, 1% Triton X-100, 50 mM TRIS-HCl, 0.01 mM $NaVO_4$, 0.1 mM PMSF and 0.6 μM leupeptin, pH 7.4). The supernatant was clarified by centrifugation (12,000x g for 10 min, 4°C) and the protein concentrations of the cell lysates were measured by the BCA method.

Western blot analysis. Solubilized proteins were separated in 10% polyacrylamide gels and transferred to nitrocellulose membranes. Nonspecific binding to the membranes was prevented by incubation with 5% Blotto (5% evaporated milk, 1% Tween-20 in Tris-buffered saline). Subsequently, membranes were probed with the following antibodies that specifically recognized the activated phosphorylated form of the proteins: JAK2 (1:1000 in Tris-buffered saline containing 1% BSA); STAT3 (1:1000 in Tris-buffered saline containing 5% BSA). After incubating with primary antibodies, the membranes were probed with goat anti-rabbit antibody coupled to horseradish peroxidase. The immunoreactive bands were visualized using ECL reagents and the data quantified by densitometry.

To visualize non-phosphorylated forms of JAK2 and STAT3, solubilized proteins were separated by electrophoresis in 10% polyacrylamide gels. The proteins were subsequently transferred to nitrocellulose membranes and then nonspecific binding was minimized by blocking with 5% Blotto. The membranes were then incubated with an anti-STAT3 antibody or with an anti-JAK2 antibody. The membranes were subsequently probed with goat anti-rabbit antibody coupled to horseradish peroxidase. The immunoreactive bands were visualized using ECL reagents and quantified by densitometry.

Total RNA extraction and IL-6 mRNA expression. Total RNA was extracted from astrocytes using the trizol method. Total RNA concentrations were determined using Biorad SmartSpec™ spectrophotometer. Two micrograms of total RNA from each sample were reverse transcribed into the complementary strand DNA using a high capacity reverse transcription reagent kit (Applied Biosystems). Quantitative PCR (qPCR) was performed using TaqMan Universal master mix, and the TaqMan gene expression assay for rat IL-6 all supplied by Applied Biosystems. Samples were assayed in triplicates in 96-well plates using the StepOne™ plus Real time PCR system from Applied Biosystems. The widely accepted comparative Ct (threshold cycle) method was used to perform relative quantification of qPCR results.[42] An arithmetic formula (fold difference = $2^{-\Delta\Delta Ct}$) was used to calculate the relative IL-6 mRNA expression in Ang II-stimulated astrocyte cultures as compared with the unstimulated controls, after normalization to levels of the housekeeping control gene, β-actin. Data are thus expressed as fold change in IL-6 mRNA expression as compared with basal IL-6 mRNA expression in unstimulated cells.

Measurement of DNA synthesis. Astrocytes growing in 24-well plates were made quiescent by a 48 h treatment with serum-free media. Individual wells were then treated for 48 h with 100 nM Ang II in the presence and absence of 10 μM AG490. ^{3}H-thymidine (0.25 Ci/mL culture medium) was added during the last 24 h of treatment. Basal and Ang II-induced DNA synthesis was measured in the presence of DMSO which was used to dissolve AG490. Newly synthesized DNA was precipitated with 5% TCA, dissolved in 0.25 N NaOH, and quantified by liquid scintillation spectrometry as previously described.[43]

Statistical analysis. All data are expressed as the mean ± SEM of 4 or more experiments, as indicated. T-tests or repeated measures one-way analysis of variance (ANOVA) with Dunnett's post test was used to compare treatment groups with control, using PRISM (GraphPad). The criterion for statistical significance was $p < 0.05$.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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