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Angiotensin II activates JAK2/STAT3 pathway and induces interleukin-6 production in cultured rat brainstem astrocytes

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ABSTRACT

We have shown that tyrosine kinases and mitogen-activated protein kinases mediate angiotensin II (Ang II) effects in cultured rat astrocytes. In this study, we investigated whether Ang II induces Janus kinase (JAK) 2, signal transducer and activators of transcription (STAT) 3 phosphorylation, and interleukin-6 (IL-6) secretion in cultured brainstem rat astrocytes. Ang II increased JAK2 phosphorylation in a time- and dose-dependent manner. Maximal phosphorylation of 1.7 ± 0.4 fold above basal was observed at 15 min with 100 nM Ang II. Losartan (10 μM), an AT_1 receptor blocker, inhibited Ang II-mediated JAK2 phosphorylation, while 10 μM PD123319, an AT_2 receptor blocker, was ineffective. The JAK2 inhibitor, AG490 (50 μM), prevented Ang II JAK2 phosphorylation. Ang II also stimulated STAT3 in a concentration- and time-dependent manner. Maximal phosphorylation of 0.8 ± 0.11 above basal was observed at 15 min with 100 nM Ang II. Treatment with AG490 reduced Ang II phosphorylation of STAT3 and Ang II-induced astrocyte growth suggesting that JAK2 is an upstream signal in these Ang II effects. Ang II also stimulated IL-6 secretion from brainstem astrocytes in a concentration- and time-dependent manner. Maximal IL-6 secretion of 0.7 ± 0.2 above basal was observed with 100 nM Ang II after 48 h of treatment. Losartan decreased Ang II-induced IL-6 secretion while PD123319 was ineffective. Interestingly, AG490 reduced Ang II-stimulated IL-6 secretion. Our study showed for the first time that Ang II induced JAK2/STAT3 phosphorylation and IL-6 secretion through activation of the Ang II AT_1 receptor in brainstem astrocytes. In addition, Ang II stimulated IL-6 secretion and astrocyte growth through the JAK2 pathway in brainstem astrocytes. These results provide new insights into pro-inflammatory and mitogenic signaling mechanisms of Ang II in astrocytes.

1. Introduction

Angiotensin II (Ang II) is a biologically active octapeptide, and a primary effector of the renin angiotensin system (RAS). Dysregulation of Ang II effects is tied to numerous cardiovascular diseases including hypertension, congestive heart failure, and others [1,2]. In the brain Ang II elicits dipsogenic responses, increased salt appetite, vasopressin release, and increased sympathetic out flow through receptors found on neurons and astrocytes. In the central nervous system, astrocytes are a major source of angiotensinogen, the precursor molecule for Ang II [3–5]. In addition, both type 1 and type 2 Ang II receptors as well as both forms of angiotensin converting enzymes are also found in astrocytes [6–8]. These findings suggest an important role for astrocytes in central effects of Ang II.

Almost all of the actions of Ang II are mediated by a seven transmembrane-spanning high affinity receptor termed the AT_1 receptor [2]. AT_1 receptor activation leads to cell growth, vasoconstriction, salt, and water retention [9]. In astrocytes, it has been shown that Ang II interaction with the AT_1 receptor causes activation of several intracellular signals including mitogen activated protein kinases, tyrosine kinases, protein kinase C (PKC), immediate early response genes, and others [10–15]. These Ang II-mediated AT_1 receptor effects lead to astrocyte growth and other effects.

The JAK/STAT pathway has been shown to convey the Ang II signal from the plasma membrane to the nucleus via stimulation of the Ang II AT_1 receptor on vascular smooth muscle cells [16]. In addition, the JAK/STAT signaling cascade was shown to be an important link between activation of the AT_1 receptor and nuclear transcriptional changes leading to cell growth [16]. Astrocytes express JAK/STAT proteins [17,18]; however, it is unknown whether Ang II signals through the JAK/STAT pathway in these cells.

Interleukin-6 (IL-6) is a pleiotropic pro-inflammatory cytokine that participates in multiple biological processes including proliferation, differentiation, survival, and apoptosis [19,20]. In the central nervous system, evidence suggests that astrocytes contribute to inflammation and tissue repair by providing a local innate immune response [21]. Activated astrocytes secrete key soluble mediators, (CXCL10, CCL2, IL-6 and BAFF) that are involved with both innate and adaptive immune responses [22]. Previous studies have shown that Ang II induces IL-6 production through the AT_1 receptor in vascular

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smooth muscle cells [23,24]. Moreover, the JAK/STAT signaling pathway is involved with Ang II-mediated IL-6 production in smooth muscle cells [25]. In astrocytes, it is unknown whether Ang II elicits IL-6 production.

In the present study, we investigated whether Ang II activates the JAK/STAT pathway in cultured brainstem astrocytes. In addition, we determined whether Ang II induced IL-6 secretion and astrocyte growth via the JAK/STAT pathway in these cells. Brainstem astrocytes were chosen since previous studies suggest that this area of the brain had the highest expression of the Ang II AT_1 receptor subtype [15].

2. Materials and methods

2.1. Materials

Ang II was obtained from Bachem (Torrance, CA). AG490, a selective JAK2 and JAK3 inhibitor, was purchased from Cal Biochem (La Jolla, CA). PD123319, the selective AT_2 receptor antagonist was obtained from Sigma (St. Louis, MO) and losartan (AT_1 receptor antagonist) was kindly provided by Du Pont Merck (Wilmington, DE). The PhosphoStat3, Stat3, PhosphoJAK2, and JAK2 antibodies were purchased from Cell Signaling Technology (Beverly, MA). ECL reagent and Western blotting supplies were purchased from GE health care (Piscataway, NJ) or Biorad Laboratories (Hercules, CA). IL-6 ELISA kits were purchased from R&D Systems (Minneapolis, MN). The BCA protein kit was obtained for Pierce Biotechnology (Rockford, IL). All other chemicals were purchased from either VWR International (Suwannee, GA) or Sigma (St. Louis, MO).

2.2. Astrocyte preparation

Timed pregnant Sprague–Dawley rats were obtained from Charles River Laboratories (Wilmington, MA) 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 brainstem of 2–3 days old neonatal rat pups by physical dissociation as previously described [15]. Cells were maintained in DMEM/F12 with 10% FBS, 100 µg/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 days 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 and negative immunoreactivity with markers for neurons (enolase), or oligodendrites (cyclic nucleotide phosphohydrolase).

2.3. 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 (12,000 × *g* for 10 min, 4 °C) and the protein concentration was measured by the Lowry method [26].

2.4. Western blot analysis

Solubilized proteins were separated in 10% polyacrylamide gels and transferred to nitrocellulose membranes. Nonspecific binding to the membranes was blocked 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: STAT3 (1:1000 in Tris-buffered saline containing 5% BSA); JAK2 (1:1000 in Tris-buffered saline containing 1% BSA). After incubating with primary antibodies, the membranes were probed with goat anti-rabbit antibody coupled to horseradish peroxidase (1:2000 dilution in 5% Blotto). The immunoreactive bands were visualized using ECL reagents and the data quantified by densitometry.

To visualize non-phosphorylated forms of STAT3 and JAK2, 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 (1:1000 in Tris-buffered saline containing 5% BSA) or with an anti-JAK2 antibody (1:1000 in Tris-buffered saline containing 5% BSA). The membranes were subsequently probed with goat anti-rabbit antibody coupled to horseradish peroxidase (1:5000 dilution in 5% Blotto). The immunoreactive bands were visualized using ECL reagents and quantified by densitometry.

2.5. ELISA for IL-6 measurement

Subconfluent astrocytes growing in 6-well plates were washed with phosphate buffered saline and made quiescent by treating for 24 h with serum-free media. Cells were subsequently treated with Ang II or the various inhibitors, the medium collected and centrifuged at 12,000 rpm for 1 min. The supernatant was stored at −70 °C prior to analysis. Proteins were estimated based on the BCA method according to the manufacturer's instructions (Pierce Biotechnology, Rockford, IL). ELISAs for IL-6 were performed according to the manufacture's protocol (R&D Systems, Minneapolis, MN).

2.6. 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 [27].

2.7. Statistics

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 $p < 0.05$.

3. Results

3.1. JAK2 activation by Ang II

To determine whether Ang II activated JAK2 in cultured astrocytes from neonatal rat brain stem, cells were incubated with increasing concentrations of Ang II (0.1 nM to 1 µM) for 15 min. Ang II increased the phosphorylation of JAK2 protein in a concentration-dependent