ANG II stimulation of neuritogenesis involves protein kinase B in brain neurons

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ANG II is a potent neuromodulatory hormone in the brain. It stimulates sympathetic pathways and vasopressin release, two key cellular actions involved in the central control of blood pressure (19, 22). Activation of sympathetic pathways by ANG II is associated with increased turnover and release of catecholamines in hypothalamic-brain stem nuclei (22, 23, 25). Neuronal cells in primary cocultures from these brain areas have been used to elucidate the cellular and molecular mechanisms of neuromodulatory actions of ANG II. These studies have established that interaction of ANG II with the neuronal ANG II type 1 (AT1) receptor stimulates turnover, synthesis, and release of catecholamines in a manner similar to that observed in vivo (8). Activation of the AT1 receptor initiates two distinct signaling pathways: one involving Ras-Raf-mitogen-activated protein (MAP) kinase and the other involving phosphatidylinositol 3-kinase (PI3K) and protein kinase B (PKB; see Refs. 13, 27, 29). Ras-Raf-MAP kinase signaling is a key pathway in ANG II stimulation of neuro-modulation in both normotensive Wistar-Kyoto (WKY) rat (13, 27) and spontaneously hypertensive (SH) rat neurons. In addition, the PI3K-PKB signaling pathway is crucial exclusively in ANG II-stimulated neuro-modulation of SH rat brain neurons (29). However, its role in the neurons from normotensive rat brain remains to be elucidated. This study was conducted to test the hypothesis that the PI3K-PKB signaling cascade regulates neuritogenesis in normotensive rat brain neurons. The rationale for this hypothesis was based on observations that the PI3K-PKB cascade is important in neuronal survival and their protection from apoptosis (1). We present data in support of this hypothesis.

MATERIALS AND METHODS

Materials

One-day-old normotensive WKY rats were obtained from our breeding colony, which originated from Harlan Sprague-Dawley (Indianapolis, IN). DMEM, plasma-derived horse serum (PDHS), and 1× crystalline trypsin (10,000 units/mg) were obtained from Central Biomedia (Irwin, MO). ANG II and monoclonal antibodies to growth-associated protein (GAP)-43 and tubulin were purchased from Sigma (St. Louis, MO). Losartan was a gift from DuPont/Merck (Wilmington, DE), and PD-123319 was purchased from RBI (Natick, MA). [γ-32P]ATP (3,000 Ci/mmol) and chemiluminescence assay reagents were obtained from DuPont/NEN (Boston, MA). A polyclonal antibody to PKB was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Specific monoclonal anti-neurofilament antibody has been used as previously described (30). All other reagents were purchased from Fisher Scientific (Pittsburgh, PA) and were of the highest quality. Finally, sense (SON) and antisense (AON) oligonucleotides to PKB with the following sequences were synthesized by Gemini Biotech (Alachua, FL): PKB SON sequence, 5′-CTTCA-CATGCGTACGTCGTT-3′ and PKB SON sequence, 5′-AAGCAGTGAGCAGCTTGTGAG-3′. The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
Methods

Preparation of neuronal cells in primary culture from the WKY rat brain. The protocol to establish hypothalamic brain stem neuronal cells in primary culture has been well established and has been previously published from our group (16, 18). These cultures have been successfully used as an in vitro model to elucidate the cellular and molecular mechanisms of ANG II actions in the brain (16, 18). Briefly, anatomical blocks containing hypothalamic and brain stem areas of 1-day-old WKY rats were dissected. Cells were dissociated and plated on poly-L-lysine-precocated 35-mm culture dishes (3 × 10⁶ cells/dish) in DMEM containing 10% PDHS. Cultures were established essentially as described elsewhere (16, 18). They contain 85–95% neuronal cells and 5–15% astrocytic glial cells. Our previous studies have established that ANG II regulation of cellular actions involving various signaling kinases is exclusively a neuronal event, and there is little participation of contaminating glial cells in this process (12, 29). This observation is consistent with an exclusive neuronal effect of ANG II in the brain (17).

Measurement of PKB activity. PKB activity was measured by an established method (7). Briefly, neuronal cells were suspended in a lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM NaF, 1 mM Na₃VO₄, 1 mM phenylmethylsulfonyl fluoride, 10% glycerol, 1% Nonidet P-40, 2 μM leupeptin, and 2 μM aprotinin). Lysates were centrifuged at 12,000 g for 10 min at 4°C, and supernatants (200 μg protein) were used to immunoprecipitate PKB using a PKB-specific polyclonal antibody (27). Immune complexes were collected with protein A/G plus agarose and rinsed three times with the lysis buffer and one time with the kinase assay buffer (20 mM HEPES, pH 7.4, 1 mM dithiothreitol, 10 mM MnCl₂, and 10 mM MgCl₂) before being used for the in vitro kinase assay. The reaction mixture contained 20 μl of the immune complex [5 μM ATP containing 10 μCi [γ-³²P]ATP and 5 μg histone 2B (H2B) in a final reaction volume of 50 μl in the kinase buffer]. The reaction was run for 10 min at 30°C and stopped by the addition of 5× SDS-PAGE sample buffer, and the reaction products were separated by SDS-PAGE. Phosphorylated H2B was quantitated by a UVP Imagestore 5000 system as described elsewhere (13, 27).

AON depletion of neuronal PKB. Neuronal cultures, established in 35-mm-diameter tissue culture dishes, were transfected with 2 μM AON or SON for PKB using Lipfectin Reagent for 48 h at 37°C (28). Cells were collected and analyzed for PKB immunoreactivity to establish the extent of PKB depletion. Western blots for GAP-43 and PKB. Neuronal cells were incubated in the lysis buffer, and lysates were centrifuged at 6,000 g for 10 min at 4°C. The resulting supernatants (10 μg protein) were subjected to SDS-PAGE, and proteins were transferred to a nitrocellulose membrane (27). After blocking the nonspecific binding by incubation in 5% nonfat dry milk in 20 mM Tris- HCl, pH 8.0, 150 mM NaCl, and 0.05% Tween 20 for 1 h, membranes were incubated with either GAP-43 (1:2,000 dilution) or PKB (1:2,000 dilution) antibodies for 1 h at room temperature. This was followed by an incubation of membranes with anti-mouse IgG (Amersham International) or anti-rabbit IgG Fab fragment (Santa Cruz Biotechnology) conjugated to horseradish peroxidase. Protein bands were detected by chemiluminescence and quantitated essentially as described previously (27).

Immunofluorescence staining of neurofilaments. Neuronal cells, grown for 5 days in 35-mm tissue culture dishes, were treated with ANG II for 3 days. Cultures were rinsed with PBS, pH 7.4, fixed in 4% paraformaldehyde-PBS, pH 7.4, for 1 h at room temperature, and incubated with 5% normal goat serum, containing 1% BSA and 0.3% Triton X-100 in PBS, pH 7.4 (PBST). They were then incubated with monoclonal neurofilament antibody (1:100 dilution in PBST-1% BSA) for 1 h at room temperature. After removal of excess antibody by rinsing the cells with PBST four to five times, cultures were stained with fluorescein isothiocyanate-conjugated anti-mouse IgG Fab fragment, and images were captured by a fluorescent microscope with the use of a digital camera and analyzed as described elsewhere (30).

Neurons were randomly selected and drawn using Scion Image software for the measurements of neurite lengths. The length of the entire neurite arborization was measured, and the number of primary neurites per neuron was determined and expressed as a percentage of the control. A total of 150 neurons was counted for each experimental condition. For neuronal survival, the number of phase-bright neurons was counted within a defined area of the culture dish before ANG II treatment. This estimate was used as an initial number of neurons. After ANG II treatment for 3 days, cells were stained with 4’,6-diamidino-2-phenylindole (DAPI), and the number of stained nuclei were counted in the same defined area. Survival of neurons was expressed as a percentage of DAPI-stained cells over the initial number of neurons.

Transfection of neurons and expression of green fluorescent protein-PKB. cDNA encoding the full length-coding region of PKB with green fluorescent protein (GFP) was cloned in the mammalian expression vector pCI-Neo (Promega) under control of the cytomegalovirus promoter. The entire humanized GFP sequence, including a unique 5' XbaI site followed by a Kozak sequence and translation start site was directionally cloned into NheI and EcoRI cleaved modified pCI-Neo vector.
called pCI-Neo-GFP, as described previously (24). This vector allows expression of cDNA with and an appropriate 5' EcoRI and 3' SalI site as a fusion protein containing an NH2-terminal GFP. The full-length coding region of PKB was subcloned into the pCI-Neo-GFP and produced the pCI-GFP-PKB plasmid. Neurons were plated on poly-l-lysine-precoated 35-mm-diameter tissue culture dishes and grown for 1 day before transfection with either the control plasmid (pCI-GFP) or the experimental plasmid (pCI-GFP-PKB), essentially as detailed elsewhere (26). DNA (6 μg) in 60 μl containing 25 mM CaCl2 was mixed with 60 μl of 2× 274 mM NaCl, 10 mM KCl, 1.4 mM Na2HPO4, 15 mM D-glucose, and 42 mM HEPES, pH 7.07 (HBS), and the precipitate was allowed to be formed for 30 min at room temperature. The conditioned medium was removed from neuronal cultures and saved. The cells were incubated with 2 ml of DMEM for 60 min at 37°C followed by drop-wise addition of DNA/calcium phosphate precipitate with constant but gentle mixing. Cultures were incubated for 8 h at 37°C followed by “shocking” the cells for 1–2 min with 1× HBS containing 10 mM MgCl2 and 5% glycerol in 4 mM HEPES, pH 7.5. Transfection media was replaced with the saved conditioned medium, and cells were returned to the incubator at 37°C for an additional 2 days. Fluorescent images representing the expression of GFP or GFP-PKB in live neurons were captured and analyzed (12). This protocol resulted in a transfection efficiency of 2–3% neurons, which is considered very good for neuronal cells in primary culture. These transfection conditions have been found to be an appropriate compromise between efficiency of transfection and suitable preservation of neuronal morphology.

Experimental group and data analysis. Each data point in the measurement of PKB activity and GAP-43 immunoreactivity was obtained from three culture dishes, the cells for Fig. 2. Effect of ANG II on neurite extension. Neuronal cultures were prepared from 1-day-old rats. On day 5, cultures were incubated with or without 100 nM ANG II at 37°C for 3 days. Cultures were fixed with 4% paraformaldehyde and subjected to immunofluorescence analysis with a neurofilament specific antibody as described in Methods. A: bright-field and fluorescence images of neuronal cells. Bar represents 20 μm. B: quantification of neurite length and branching as described in Methods. Values represent the degree of increase in the length and branching in ANG II-treated cells over control and was calculated by counting 150 neurons. *P < 0.05. C: quantification of neuronal survival was carried out as described in Methods.
which were derived from multiple brains of 1-day-old rats. Each experiment was repeated at least three times, unless stated otherwise. Images from autoradiographs were captured with the UVP Image store 5000 system; and bands were quantitated and corrected for equal loading by normalizing with tubulin; and data were presented as means ± SE.

Comparisons between the control and experimental groups were made using Student’s t-test and ANOVA with Statistica Software (Tulsa, OK). All immunoﬂuorescence experiments were repeated at least four times with ﬁve culture dishes, and a representative is presented.

RESULTS

GAP-43 plays a key role in guiding the growth of axons and development of new neuronal connections (2). Thus the expression of this protein and its cellular levels have been used as a marker for neuritogenesis (2). In the present study, we have used both the levels of GAP-43 immunoreactivity and immunoﬂuorescent visualization of neuroﬁlaments to assess neuritogenic activity of ANG II in the brain neurons. Treatment of neurons with ANG II for 3 days resulted in a dose-dependent increase in the levels of GAP-43 immunoreactivity. A threefold increase was observed with 100 nM ANG II (Fig. 1). This stimulation was completely blocked by coincubation of ANG II with 10 μM losartan, an AT1 receptor subtype-specific antagonist, but not by 10 μM PD-123319, an AT2 receptor subtype-specific antagonist (Fig. 1). The increase in GAP-43 was associated with an increase in the network of neurites presented by each neuronal cell soma (Fig. 2A). This included an increase in neurite length and branching of neurites (Fig. 2B). An average of 2.3- to 2.8-fold more neurites and branching were observed with ANG II treatment for 3 days. In contrast, ANG II had no significant effect on the total number of neurons, as judged by DAPI-stained nuclei in the absence and presence of ANG II. These observations established that ANG II, via its action on the AT1 receptor, is a potent neuritogenic hormone for WKY rat brain neurons.

The effect of ANG II on PKB activity was also determined. ANG II caused a time-dependent stimulation of PKB activity, and maximal stimulation of 3.5-fold was observed in 10 min (Fig. 3). This increase was blocked by 10 μM losartan but not by 10 μM PD-123319 (Fig. 4). We used AON to PKB to further confirm the involvement of PKB. Treatment of neurons for 48 h with 2 μM AON speciﬁc to PKB caused a 90% decrease in the neuronal PKB immunoreactivity (Fig. 5). The depletion was speciﬁc, since PKB SON had no effect (Fig. 5). PKB-depleted neurons exhibited an ~95% decrease in the ability of ANG II to stimulate GAP-43 (Fig. 6A). Similarly, neurite growth was signiﬁcantly blocked by this treatment (Fig. 6B). PKB-depleted neurons showed a 100% decrease in the ability of ANG II to induce neurite lengths and branching (Fig. 6C). Further conﬁrmation of the involvement of the PI3K-PKB pathway was provided by the use of wortmannin, a speciﬁc inhibitor of PI3K. Incubation of WKY rat brain neurons with 100 nM wortmannin for 3 days resulted in a 95% decreases in ANG II stimulation of GAP-43 levels (Fig. 7). In contrast, inhibition of the MAP kinase signaling pathway by PD-98059 had little effect on ANG II stimulation of GAP-43 (Fig. 7).

Neurons were transfected with a plasmid containing GFP-PKB to observe the effect of PKB overexpression on
neurite growth in an attempt to further establish the role of PKB. A representative neuron overexpressing PKB, as evident by the expression of GFP representing GFP-PKB, showed well-developed neurites (Fig. 8B). The fluorescence was found throughout the neuronal soma. Its distribution in the neurites, however, displayed a nodular appearance and was concentrated in varicose-like structures (Fig. 8B). In contrast, a representative control neuron transfected with GFP alone exhibited very few neurites, and the distribution of fluorescence was not nodular.

![Fig. 5. Effect of PKB-antisense oligonucleotide (AON) on neuronal PKB immunoreactivity. Neuronal cultures were transfected with 2 μM PKB-specific AON or sense oligonucleotide (SON) as described in Methods. After transfection (48 h), cellular proteins were subjected to Western blot analysis for quantitation of PKB immunoreactivity.](image1)

![Fig. 6. Effect of PKB-AON on ANG II-stimulated neuritogenesis. A: effect on GAP-43 immunoreactivity. Neurons were subjected to PKB-AON treatment as described in Fig. 5. After AON treatment (48 h), cultures were treated with 100 nM ANG II and incubated for an additional 3 days. Protein extracts were subjected to Western blot analysis for GAP-43. Data are means ± SE (n = 3). *P < 0.05 vs. control. †P < 0.01 vs. ANG II treatment. B: effect of PKB-AON treatment on ANG II-induced neurite extension. Neurons were subjected to PKB depletion essentially as described in Figs. 5 and 6A. They were incubated with 100 nM ANG II for 3 days. Cells were fixed and used for neurofilament immunofluorescence. Bar is 20 μm. C: effect on neurite length and branching. Data from A and B were analyzed as described in Methods. Significant differences (P < 0.01) were observed between control and ANG II treatment (†) and between ANG II treatment alone and PKB-depleted plus ANG II-treated cells (#).](image2)

![Fig. 7. Effects of wortmannin and PD-98059 on ANG II stimulation of GAP-43 immunoreactivity. Neuronal cultures were incubated with or without 100 nM ANG II for 3 days at 37°C in the presence of 100 nM wortmannin or 50 μM PD-98059. GAP-43 immunoreactivity was determined, normalized with tubulin, and presented as the degree of increase as described in Methods. Data are means ± SE (n = 3). *P < 0.05 vs. control. †P < 0.01 vs. ANG II treatment.](image3)
In addition, the fluorescence was significantly increased in growth cone-like structures, as confirmed by costaining with a tubulin antibody (Fig. 8, D and E). These observations indicate that PKB overexpression induces neurite growth and that ANG II causes translocation of PKB into a growth cone-like structure.

**DISCUSSION**

We demonstrate that ANG II is a potent neuritogenic hormone in WKY rat brain neurons and that PKB is a central kinase in this action. Evidence for this is threefold: 1) ANG II stimulates PKB activity, 2) depletion of PKB attenuates ANG II-induced neuritogenic action, and 3) overexpression of PKB induces neurite growth in the absence of ANG II.

Previous studies have indicated a link between neuritogenic and apoptotic pathways. For example, nerve growth factor (NGF) stimulation of neurotrophic/neuritogenic actions, which involves PI3K, adversely affects apoptosis (6). Such a relationship between the two pathways does not exist in the brain neurons. Evidence for this conclusion include the following: 1) inhibition of PI3K does not stimulate apoptosis by ANG II, and 2) AT1 receptor stimulation that regulates the PI3K-PKB cascade does not affect apoptosis. In fact, neuronal apoptosis is regulated by the AT2 receptor subtype (21). Additionally, the neuritogenic action of ANG II is direct and is not a result of its effect on the survival of neurons, as evidenced by our study and those of others (6, 10).

The mechanism by which ANG II stimulates neuritogenesis remains unclear. However, it is likely that ANG II stimulates translocation of PKB to the nucleus (3, 14, 29). Once in the nucleus, PKB phosphorylates transcription factors relevant to the promotion of neuritogenic activity. Support for this view is based on the
PKB regulates neuritogenesis

observations that PKB regulates phosphorylation of such factors as FKHRL-1, a Forkhead/dauer formation (DAF) family transcription factor (4, 11), and insulin response sequences (IRS; see Ref. 9). The effect of PKB on IRS, which can influence insulin and insulin like growth factor (IGF)-I, is of great relevance in this context. For example, one possible scenario could be that activation of the AT1 receptor regulates PKB, which stimulates insulin, and neuritogenic actions of IGF-I. This hypothesis is supported by the observation that insulin is a potent neuritogenic hormone and stimulates PI3K in these neurons (15). In addition, cross-talk between the AT1 receptor, a G protein-coupled receptor, and the tyrosine kinase receptor (epidermal growth factor, IGF-I, insulin, and platelet-derived growth factor) has been demonstrated (20). However, further experiments will be needed to provide conclusive evidence if the effects of PKB are direct or via tyrosine kinase receptor activation.

This study also demonstrates that ANG II stimulates translocation of PKB into growth cones. It is tempting to speculate that PKB translocation may be key in regulating the molecular trafficking and be part of the cytoplasmic signaling system in promoting neurite extension. It is likely that PKB may phosphorylate one or more of the cytoskeletal proteins to stimulate the guidance of growth cones for neurite extension. Evidence that the PI3K-PKB pathway is involved in phosphorylation of focal adhesion kinase and paxillin, two proteins associated with growth cone function and cytoplasmic trafficking of cytoskeletal molecule (5), supports this view. Additionally, it is quite possible that both nuclear and cytoplasmic signaling, initiated by the AT1 receptor activation of PKB, are involved in a coordinated fashion to stimulate the neuritogenic action of ANG II. Our ability to transfect and visualize live brain neurons in primary culture would be an important asset in delineating the precise subcellular signaling mechanism.

Finally, in vivo relevance of these observations remains speculative at the present time. It is quite possible that ANG II may be involved in the development of appropriate neuronal connections and neurite regeneration in the cardioregulatory areas of the brain during development. Thus alterations in the AT1 receptor expression and/or its signaling may result in abnormal functional connections in the SH rat brain. This may be of critical consequence in the pathophysiology of centrally mediated hypertension. Thus the hypothesis is, in part, supported by the evidence that the renin-angiotensin system is altered and that AT1 receptor signaling is distinct in the neurons of SH rats (8, 17).

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