

Angiotensin II Regulation of Neuromodulation: Downstream Signaling Mechanism from Activation of Mitogen-activated Protein Kinase

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Abstract. Angiotensin II (Ang II) stimulates expression of tyrosine hydroxylase and norepinephrine transporter genes in brain neurons; however, the signal-transduction mechanism is not clearly defined. This study was conducted to determine the involvement of the mitogen-activated protein (MAP) kinase signaling pathway in Ang II stimulation of these genes. MAP kinase was localized in the perinuclear region of the neuronal soma. Ang II caused activation of MAP kinase

and its subsequent translocation from the cytoplasmic to nuclear compartment, both effects being mediated by AT₁ receptor subtype. Ang II also stimulated SRE- and AP₁-binding activities and fos gene expression and its translocation in a MAP kinase-dependent process. These observations are the first demonstration of a downstream signaling pathway involving MAP kinase in Ang II-mediated neuromodulation in noradrenergic neurons.

BRAIN angiotensin II (Ang II)¹ plays a key role in the central control of blood pressure (BP). This action is initiated by the interaction of Ang II with the AT₁ receptor subtypes that are localized on the neurons of the cardioregulation-relevant areas of the brain (Saavedra, 1992; Steckelings et al., 1992; Timmermans et al., 1993; Raizada et al., 1994; Wright et al., 1994). Physiological mechanisms of Ang II-mediated BP control involve stimulation of vasopressin release, stimulation of sympathetic pathways involving catecholamines (CA), and dampening of the baroreceptor reflex (Saavedra, 1992; Steckelings et al., 1992; Timmermans et al., 1993; Raizada et al., 1994; Wright and Harding, 1994). The importance of the brain Ang II and neuronal AT₁ receptors in the control of BP is further supported by studies with the spontaneously hypertensive (SH) rat, a genetic model for human essential hypertension. They demonstrate that the SH rat expresses a hyperactive brain Ang II system as a result of increased levels of Ang II and AT₁ receptors (Raizada et al., 1993a,b, 1994). Interruption in this system's hyperactivity

results in the normalization of BP, further confirming the involvement of this hormonal system in the development and establishment of hypertension and high BP in the SH rat model (Raizada et al., 1994).

In spite of excellent physiological studies defining the involvement of CA and vasopressin in Ang II-mediated control of BP, relatively little is known about the cellular and molecular bases of these actions of Ang II in the brain. For example, it has been shown that Ang II stimulates turnover of brain CA by interacting with AT₁ receptor subtypes (Raizada et al., 1994; Sumners et al., 1985, 1987; Sumners and Raizada, 1986); however, the signal transduction events associated with this turnover, synthesis, and uptake of CA remain to be defined (Sumners et al., 1985, 1987; Sumners and Raizada, 1986). This lack of information could be due in part to the inherent complexity of the brain, which contains multiple cell types, each with its own unique properties, and in part to the presence of discrete anatomical and physiological aspects of the brain. We have developed neuronal cells in primary cultures from the hypothalamus-brainstem areas of 1-d-old rat brain to circumvent some of these problems and have used this *in vitro* system as a model to investigate the cellular and molecular mechanisms of Ang II actions (Raizada et al., 1994; Sumners and Raizada, 1993; Sumners et al., 1995). These studies have demonstrated that Ang II stimulation of neuromodulation observed *in vivo* could be equated to its *in vitro* stimulation of norepinephrine transporter (NET), tyrosine hydroxylase (TH), and dopamine β -hydroxylase at both the transcriptional and posttranscriptional levels (Lu et al., 1996; Yu et al., 1996). The regulation of

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1. *Abbreviations used in this paper.* Ang II, angiotensin II; AON and SON, antisense and sense oligonucleotide; BP, blood pressure; CA, catecholamines; MAP, mitogen-activated protein; NET, norepinephrine transporter; RT-PCR, reverse transcriptase-polymerase chain reaction; SH, spontaneously hypertensive; SRE, serum response element; TH, tyrosine hydroxylase.

this neuromodulation in neurons involves an interaction of the AT₁ receptor with Ras that initiates a cascade of signaling events involving the activation of Raf-1 and MAP kinase (Yang et al., 1996). Thus, our studies have indicated that the activation of MAP kinase appears to be a key event in the signaling mechanism of AT₁ receptor-mediated neuromodulation.

The objective in this study was to further our understanding of the sequence of cellular events leading to neuromodulation by characterizing the signal transduction steps downstream of the activation of MAP kinase. This information would be crucial in elucidating how MAP kinase is involved in the transfer of neuromodulatory signals, initiated at the plasma membrane by AT₁ receptors from cytoplasmic compartment to the nucleus. The observations demonstrate that MAP kinase is primarily localized around the nuclear membrane and that activation by Ang II results in its translocation into the nucleus. This translocation is associated with increases in SRE-binding activities, c-fos gene expression, and in AP₁-binding activity. These increases would potentially stimulate the transcription of the NET and TH genes.

Materials and Methods

Materials

1-d-old WKY rats were obtained from our breeding colony, which originated from Harlan Sprague-Dawley (Indianapolis, IN). DME, plasma-derived horse serum, and 1× crystalline trypsin (10,000 BAEE/mg) were obtained from Central Biomedica (Irwin, MO). Ang II and monoclonal anti-TH antibody were purchased from Sigma Chemical Co. (St. Louis, MO). Losartan potassium was a gift from DuPont/Merck (Wilmington, DE), and PD123319 was from Res. Biochems., Inc. (Natick, MA). [γ -³²P]ATP (3,000 Ci/mmol) and chemiluminescence assay reagents were from DuPont/NEN (Boston, MA). Reverse transcriptase-polymerase chain reaction (RT-PCR) Kit was purchased from Perkin Elmer Cetus (Norwalk, CT), and DYNAL beads and other reagents for poly(A⁺)RNA isolation were from DYNAL, Inc. (Lake Success, NY). Polyclonal anti-Fos and anti-AT₁ receptor antibodies, horseradish peroxidase-conjugated anti-rabbit antibody, and oligonucleotides of SRE and AP₁ consensus sequences (described below) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal anti-MAP kinase rabbit antibody and its agarose conjugates were from Upstate Biotechnology Inc. (Lake Placid, NY). This antibody recognized both nonphosphorylated and phosphorylated p42 and p44 MAP kinase subtypes. Monoclonal anti-AT₁ receptor antibody was a gift from Dr. G.P. Vinson (Queen Mary and Westfield College, London, UK) (Vinson et al., 1995), and monoclonal anti-neurofilament antibody was provided by Dr. J. Shaw (Department of Neuroscience University of Florida, Gainesville, FL). Polyclonal rhodamine-conjugated anti-rabbit IgG Fab fragment and polyclonal FITC-conjugated anti-mouse IgG Fab fragment were purchased from Boehringer Mannheim (Indianapolis, IN). Gel-shift Assay Kit was purchased from Promega Corp. (Madison, WI). All other biochemicals were from Fisher Scientific (Pittsburgh, PA) and were of analytical and molecular biology grades. Primers for c-fos, TH, NET, and MAP kinase sense and antisense oligonucleotide (SON and AON) were synthesized in the DNA synthesis facility of the Interdisciplinary Center for Biotechnology Research, University of Florida. The sequences of these primers are as follows: (TH) sense: 5'-GGGATGGGAATGCTGTTCTCAAC-3'; antisense: 5'-CGAGAGGCATAGTTCCTGAGCTT-3'; (NET) sense: 5'-CCGCATCCATGCTTCTGGCGGGATGAA-3'; antisense: 5'-GGGCAGGCTCAGATGGCCAGCCAGTGT-3'; (MAP kinase) sense oligonucleotide: 5'-CGACTTGCTATCAAGAAAATC-3'; antisense oligonucleotide: 5'-GATTTTCTTGATGCAACTCG-3'; (SRE consensus) 5'-GGATGTCATATTAGGACATCT-3' (AP₁ consensus) 5'-CGCTTGATGAGTCAGCCGGAA-3' GCGAACTACTCAGTCGCCTT-3'.

Methods

Preparation of Neuronal Cultures from WKY Rat Brains. Neuronal cultures were prepared essentially as described previously (Raizada et al., 1984, 1993a). Briefly, hypothalamus-brainstem of 1-d-old Wistar-Kyoto normotensive rat brains, which contained the paraventricular nucleus, the supraoptic, anterior, lateral, posterior, dorsomedial and ventromedial nuclei, the medullar oblongata, and the pons, were dissected and brain cells were dissociated by trypsin. Cells were plated on poly-L-lysine precoated tissue culture dishes in DME containing 10% plasma-derived horse serum. Culture dishes of 35-mm diam (3 × 10⁶ cells) or 100-mm diam (2 × 10⁷ cells) were prepared for experiments. Cultures were treated with 1% cytosine arabinoside for 3 d followed by establishment of cultures for 10 d before their use in the experiments. These cultures have been shown to contain 90–95% neuronal cells and 5–10% astroglial cells (Raizada et al., 1984, 1993a, 1994).

Measurement of MAP Kinase Activity by In-Gel Assay. MAP kinase activity was measured essentially as described elsewhere (Sadoshima et al., 1995; Yang et al., 1996). Briefly, neuronal cells grown in 35-mm culture dishes were rinsed three times with ice-cold PBS, pH 7.4, and lysed by incubation with 0.5 ml of lysis buffer (25 mM Tris-HCl, 25 mM NaCl, 1% Triton X-100, 1% deoxycholic acid, 0.1% SDS, 1 mM sodium orthovanadate, 10 mM sodium fluoride, 10 mM sodium pyrophosphate, 0.5 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 0.8 μg/ml leupeptin) for 10 min at 4°C. Lysates from triplicate culture dishes were pooled and incubated with 10 μg of agarose-conjugated anti-MAP kinase antibody at 4°C overnight. Immunoprecipitates were collected by centrifugation and electrophoresed on 10% SDS-PAGE containing 0.5 mg/ml myelin basic protein. The gel was then washed twice in 20% 2-propanol, 50 mM Tris-HCl, pH 8.0, and twice in 50 mM Tris-HCl, pH 8.0, containing 5 mM 2-mercaptoethanol. Each wash lasted for 1 h at room temperature with gentle shaking. MAP kinase activity was measured by incubating the gel with 40 mM Hepes, pH 8.0, 2 mM DTT, 10 mM MgCl₂, 0.5 mM EGTA, 40 μM ATP, and 10 μCi [γ -³²P]ATP (3,000 Ci/mM) for 30 min at room temperature, followed by autoradiography as described previously (Yang et al., 1996).

Immunofluorescence Staining of MAP Kinase. Neuronal, astroglial, and vascular smooth muscle cells established in 35-mm culture dishes were washed twice in PBS, pH 7.4, and fixed in neutral-buffered 1% formalin for 1 h at 4°C. After three rinses in PBS, pH 7.4, cells were permeabilized by incubation with methanol at -20°C for 2 min, incubated with 10% FBS for 1 h at 37°C to suppress nonspecific binding, followed by incubation with anti-MAP kinase antibody at 5 μg/ml in a PBS-BSA solution (1× PBS, 0.8% BSA), for 16 h at 4°C. Excess primary antibody was removed by washing with PBS, pH 7.4, followed by incubation with 10 μg/ml rhodamine-conjugated anti-rabbit IgG Fab-fragments prepared in PBS-BSA solution for 1 h at 37°C. Cells were washed free of unbound second antibody by incubating them with PBS, pH 7.4, for 5 min with gentle shaking. They were coverslipped and subjected to confocal microscopic analysis (Lu et al., 1996).

Double Immunofluorescence Staining for MAP Kinase and Neurofilaments. Neuronal cells were stained first with anti-MAP kinase antibody as described above. This was followed by staining the cultures for neurofilaments by incubation with mouse monoclonal antineurofilament antibody at a concentration of 5 μg protein/ml for 1 h at 37°C as described previously (Lu et al., 1996). FITC-conjugated anti-mouse IgG Fab-fragment in PBS-BSA solution was used as second antibody. Cells were washed six times each with PBS, pH 7.4, coverslipped, and analyzed by confocal microscopy essentially as described previously (Lu et al., 1996).

Preparation of Cytoplasmic and Nuclear Extracts from Neuronal Cultures. Neuronal cultures that had been established in 100-mm-diam culture dishes were rinsed twice with PBS, pH 7.4, and cells were collected by scraping the monolayer with the help of a teflon scraper. Nuclear and cytoplasmic extracts were prepared essentially as described elsewhere with minor modification (Abmayr and Workman, 1992). Briefly, the cell pellet was incubated in a solution containing 10 mM KCl, 1.5 mM MgCl₂, 10 mM Hepes, 0.5 mM DTT, and 0.2 mM PMSF, pH 7.0, for 10 min at 4°C, followed by homogenization with 15 gentle strokes using a type-B pestle in a Dounce homogenizer. The lysis of >90% neurons was determined by microscopic examination. The cytoplasmic extract was collected by centrifugation of the homogenate at 3,300 g for 15 min, and the remaining nuclear pellet was lysed by nuclear lysis buffer (20 mM Hepes, pH 7.9, 25% glycerol, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.2 mM PMSF, 0.5 mM DTT, and 30 mM KCl). The nuclear extract was collected by centrifugation at 25,000 g for 30 min at 4°C. Both cytoplasmic and nuclear extracts were dialyzed overnight against 1× gel-shift assay buffer (20% glycerol, 5 mM MgCl₂,

2.5 mM EDTA, 2.5 mM DTT, 250 mM NaCl, 50 mM Tris-HCl, pH 7.5). Protein concentrations of the extracts were determined using a Bradford protein assay (BioRad Labs, Hercules, CA).

Measurement of SRE and AP₁-binding Activities by Gel-shift Assay.

SRE and AP₁ nucleotide consensus sequences were used in a gel-shift assay essentially as described elsewhere (Bhat et al., 1994). 3 pmol of either SRE or AP₁ oligonucleotides were first labeled with [γ -³²P]ATP at 5' end by 10-min incubation at 37°C with 4 U of T4-polynucleotide kinase in 10 μ l solution containing 70 mM Tris-HCl, pH 7.6, 10 mM MgCl₂, 5 mM DTT, and 10 μ Ci [γ -³²P]ATP. The reaction was stopped by the addition of 1 μ l 0.5 M EDTA, and the volume was expanded to 100 μ l with 1 \times gel-shift assay buffer. ³²P-labeled oligonucleotides (0.1 μ Ci) were mixed with either 5 μ g nuclear extract protein (for both SRE and AP₁ analysis) or 25 μ g cytoplasmic extract protein (for AP₁ analysis) containing 1 \times gel-shift assay buffer, 100 ng salmon sperm DNA, 2 μ g DNA duplex poly(dI-dC), and 10 μ g BSA in a final volume of 10 μ l. The mixture was incubated at room temperature for 15 min and electrophoresed on a 5% PAGE in 20 mM Tris-acetate buffer containing 5 mM EDTA, pH 8.0, for 90 min at a constant 200 V. Gel was decasted, wrapped in a plastic bag, and exposed to x-ray film overnight at -70°C (Lu et al., 1996). Films were processed and analyzed by an Imagestore System (Ultra Violet Products, Ltd., San Gabriel, CA). The densities of shifted bands on the gel representing the oligonucleotide-protein complexes were quantitated by the SW5000 Gel Analysis program and the data are presented as absorbance derived from the image analysis program (Lu et al., 1996).

Double Fluorescence Staining of AT₁ Receptor with NET mRNA and TH mRNA and TH with NET mRNA in Neuronal Cultures. RT in situ PCR was combined with immunocytochemistry to determine the colocalization of the AT₁ receptor with NET and TH mRNAs and TH with NET mRNA essentially as described previously (Lu and Raizada, 1995; Lu et al., 1996). Briefly, neuronal cells were fixed in neutral-buffered 1% formalin, digested with trypsin and RNase-free DNase, and subjected to reverse transcriptase reactions with the use of specific primers for either NET or TH mRNA essentially as described previously (Lu et al., 1996). Cells were washed, ethanol-dehydrated, and an eight-cycle in situ PCR was performed at 94°C/45 s, 60°C/45 s, and 73°C/1 min for each cycle. Cells were rinsed with PBS, pH 7.4, and incubated for 1 h with either anti-AT₁ receptor or anti-TH monoclonal mouse antibodies, followed by incubation with 10 μ g/ml FITC-conjugated anti-mouse IgG Fab fragments for 1 h at 37°C. Cells were washed in PBS, and the dual stainings of rhodamine-labeled NET mRNA or TH mRNA and FITC-labeled AT₁ receptor or TH were analyzed and collected by confocal microscope as described above.

Quantitation of c-fos mRNA by RT-PCR. The protocol for quantitation of c-fos mRNA by RT-PCR was essentially as described previously (Lu and Raizada, 1995; Lu et al., 1995).

Confocal Microscopic Analysis of Fluorescent Images of Neuronal Cells. Fluorescent-stained cells were dehydrated by ethanol and mounted in methylsalicylate and coverslipped. Samples were then examined under an inverted microscope (BH-2; Olympus Corp., Lake Success, NY) that was interfaced to a laser-scanning confocal system (MRC 600; BioRad Labs) including a krypton/argon laser. Optical sections were collected at 1- μ m intervals and the images were Kalman-averaged. To present double staining, the images were pseudocolored by the XV program (Silicon Graphics Incorporated, Sunnyvale, CA) with green for FITC and red for rhodamine (Sumners et al., 1991).

Experimental Groups and Data Analysis. Each data point in the measurements of MAP kinase activity, nuclear MAP kinase contents, nuclear and cytoplasmic SRE or AP₁ gel-shift activities, and c-fos mRNA was obtained from three culture dishes, and each experiment was repeated three times. Data presented are mean \pm SE. Comparisons between data points were made by using one-way analysis of variance and Dunnett's test from the Statistica software (Statistica Inc. Orlando, FL). All immunofluorescent experiments were repeated at least six times; 100-250 cells were examined and representative images were collected.

Results

Effects of Ang II on MAP Kinase in Neuronal Cultures

Incubation of neuronal cultures with 100 nM Ang II caused a time-dependent stimulation of MAP kinase activity. The increase was significant as early as 5 min, reached maximal levels of approximately fourfold in 15 min, fol-

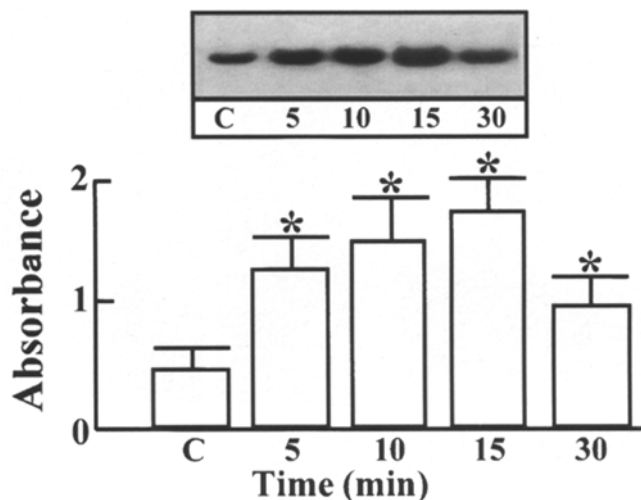


Figure 1. Effect of Ang II on MAP kinase activity in neuronal cultures. Neuronal cultures were incubated without (C) or with 100 nM Ang II for the indicated time periods. This was followed by the measurement of MAP kinase activity by in-gel assay essentially as described in Methods. (Top) A representative autoradiogram. (Bottom) Mean \pm SE ($n = 3$). (*) Significantly different from control ($P < 0.05$).

lowed by a significant decline in 30 min (Fig. 1). The stimulation was mediated by the AT₁ receptor subtype since it was completely blocked by 10 μ M losartan but not by PD123319, an AT₂ receptor subtype antagonist. A representative double-staining pattern depicting the distribution of MAP kinase immunoreactivity in relation to neurofilament immunoreactivity in the neuron is shown in Fig. 2. Staining for neurofilaments was diffused and distributed throughout the cytoplasmic compartment of the neuronal soma (green). Significant staining was also seen in the neurites. In contrast, the same neuron, when stained for MAP kinase immunoreactivity, depicted a discrete distribution of this enzyme (red). MAP kinase immunoreactivity was predominantly localized around the nucleus, although a little staining was seen distributed throughout the cytoplasmic compartment of the cell soma. A combined image for the same cell depicting green, red, and yellow confirms the perinuclear stainings of MAP kinase in the neuron. The unique staining of MAP kinase around the nucleus was characteristic for neuronal cells in primary culture, since astroglial cells prepared from the same brains did not show such staining. In these cells, MAP kinase immunoreactivity was diffused throughout the cytoplasmic compartment of the cell. In addition, vascular smooth muscle cells in culture showed diffused staining of this enzyme (data not shown). The perinuclear staining of MAP kinase was not an artifact of experimental protocol since many other combinations of fixation and staining showed similar distribution. Finally, this pattern of staining in neurons was further confirmed by the use of another MAP kinase antibody (Santa Cruz, Inc., Santa Cruz, CA). Treatment of neuronal cultures with Ang II caused a dramatic redistribution of MAP kinase immunoreactivity (Fig. 3). After 5 min of incubation with Ang II, the MAP kinase immunoreactivity that was distributed around the nucleus began to show a patchy appearance. In addition, the staining also

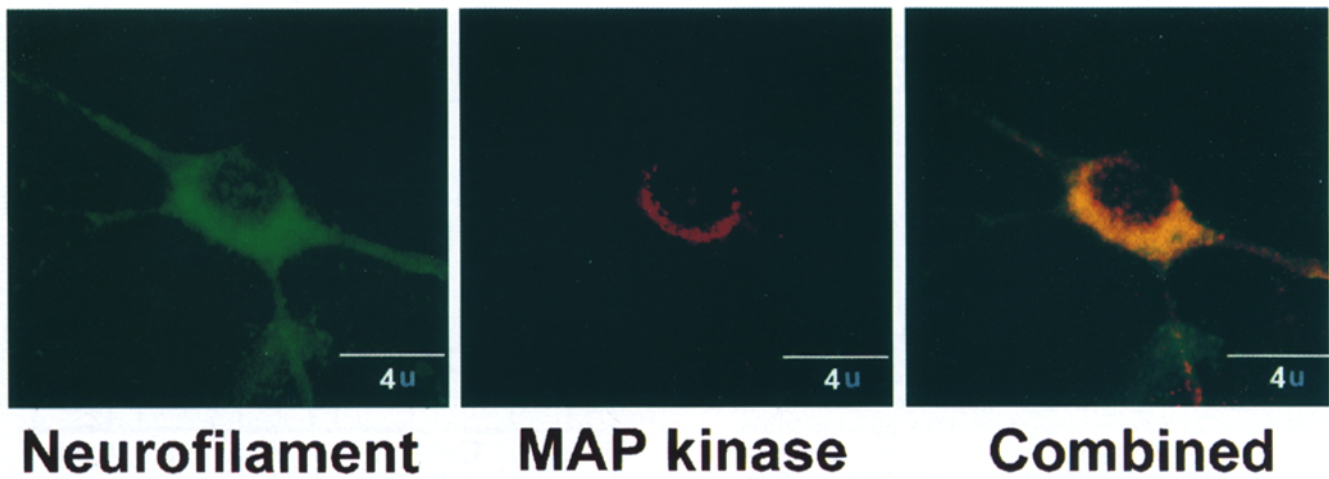


Figure 2. Colocalization of neurofilaments and MAP kinase in neuronal cultures with the use of double immunofluorescent staining. Neuronal cultures were fixed and subjected to double staining with the use of specific antibodies to neurofilaments and MAP kinase essentially as described in Methods. Fluorescent images containing neurofilaments (*green*), MAP kinase (*red*), and a combination of neurofilaments and MAP kinase (*yellow*) were analyzed with the use of a confocal microscope.

began to appear inside the nucleus. By 15 min, the majority of the immunoreactivity that had been present in the cytoplasmic compartment was translocated into the nucleus. The translocated staining for MAP kinase in the nucleus displayed a patchy and aggregate appearance. These aggregates began to disappear by 30 min of incubation with Ang II at which time a uniform, even distribution of MAP kinase immunoreactivity was observed in the nucleus (Fig. 3). These immunocytochemical observations suggested that Ang II stimulates the translocation of MAP kinase from the perinuclear region to within the nucleus. This translocation was completely blocked by 10 μ M losartan but not by PD123319, indicating that AT₁ receptor subtypes are involved in Ang II stimulation of MAP kinase translocation.

Immunoblot analysis was carried out essentially as described previously (Yang et al., 1996) to confirm such a nu-

clear translocation of MAP kinase by Ang II. Neuronal cultures were treated with 100 nM Ang II in the presence or absence of either losartan or PD123319 for 30 min, the nuclei were isolated, and the nuclear extracts were used to immunoprecipitate MAP kinase, followed by immunoblotting. Fig. 4 A shows that very little phosphorylated p42 or p44 protein (two major forms of phosphorylated MAP kinase) was present in the nuclear fraction of control neuronal cultures. Ang II caused an approximately fourfold increase in both phosphorylated p42 and p44. Losartan treatment (but not PD123319) blocked Ang II stimulation of the levels of both phosphorylated p42 and p44. MAP kinase translocated into the nucleus showed enzyme activity as determined by an in-gel assay. Fig. 4 B shows that the levels of MAP kinase activity increased as a function of time in the nuclear fraction with a maximal increase of six-fold in 15 min.

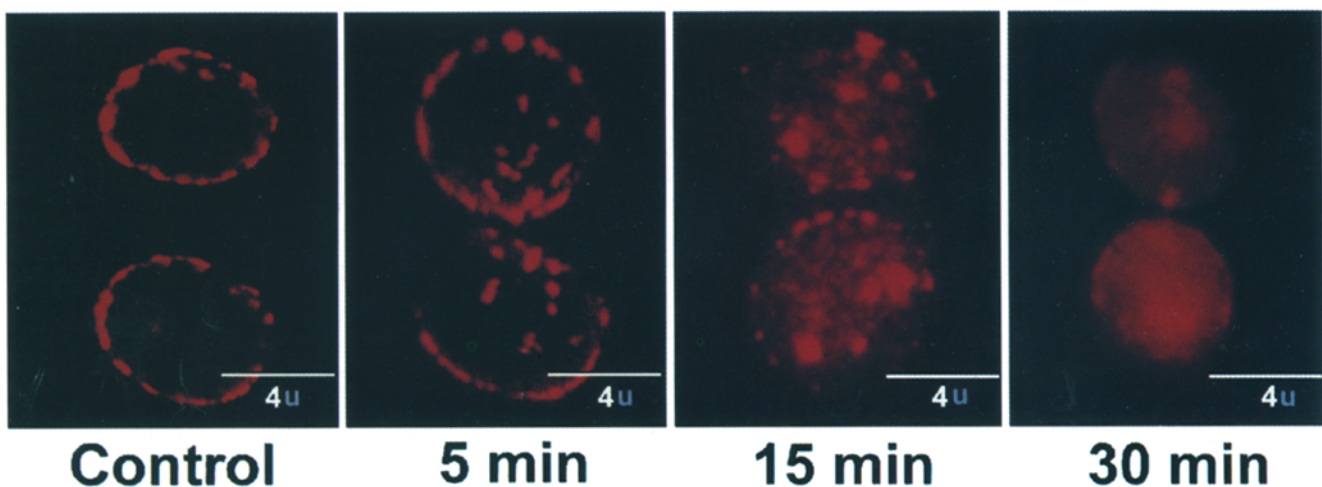


Figure 3. Effect of Ang II on redistribution of MAP kinase immunoreactivity in neuronal cultures. Neuronal cultures were incubated with 100 nM Ang II for the indicated time periods. Images represent two cells at each time point and are representative of six separate experiments in which a total of \sim 100 cells were observed.

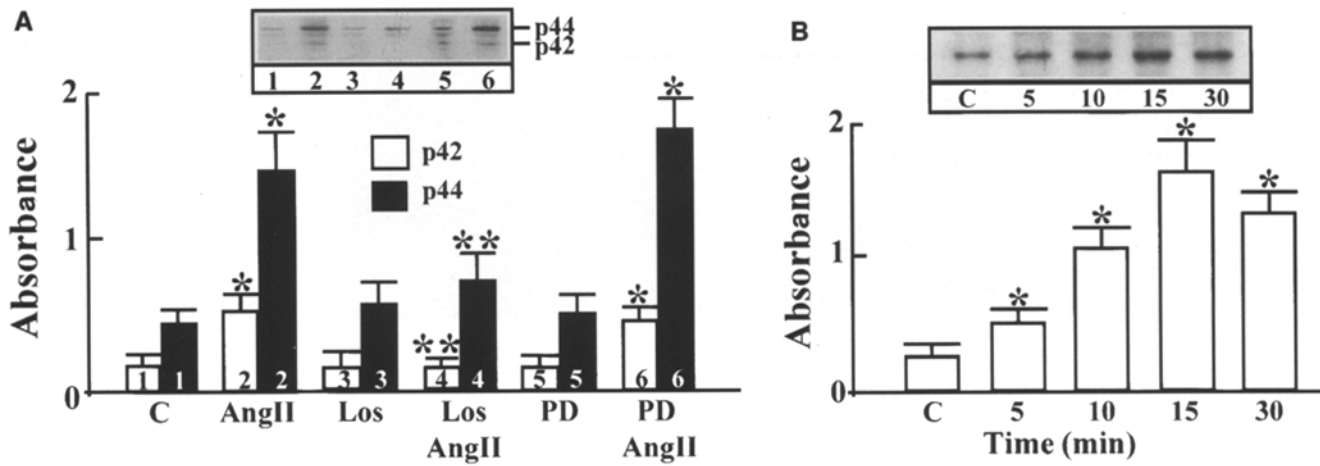


Figure 4. (A) Effects of losartan and PD123319 on Ang II-stimulated translocation of MAP kinase into the nucleus. Neuronal cultures were incubated without (C) or with 100 nM Ang II for 30 min in the absence or presence of 10 μ M losartan (Los) or PD123319 (PD). Nuclear extracts were prepared and MAP kinase was precipitated by incubating the extracts with anti-MAP kinase antibody conjugated to agarose followed by immunoblotting as described in Methods. (Top) A representative autoradiogram. (Bottom) Quantitation of bands corresponding to phosphorylated p42 and p44. (two major forms of phosphorylated MAP kinases) was carried out essentially as described previously (Yang et al., 1996). Data are presented as mean \pm SE ($n = 3$) for phosphorylated p42 (\square) and p44 (\blacksquare). (*) Significantly ($P < 0.05$) different from control (C). (**) Significantly ($P < 0.05$) different from Ang II-treated cells. (B) Effect of Ang II on MAP kinase activity in the nuclear fractions of neuronal cultures. Nuclear fractions were isolated from Ang II-treated neuronal cultures. MAP kinase activity was determined by in-gel assay essentially as described in Methods. (Top) Representative autoradiogram. (Bottom) Quantitation of a representative band corresponding to MAP kinase activity. Data are mean \pm SE ($n = 3$). (*) Significantly different ($P < 0.05$) from zero time.

Mechanism of Ang II Stimulation of Neuromodulation: Involvement of MAP Kinase

Our previous studies have demonstrated that chronic incubation of neurons by Ang II causes a persistent increase in tyrosine hydroxylase activity and norepinephrine uptake, effects associated with increased gene expression for TH and NET (Lu et al., 1996; Yu et al., 1996). We have used Ang II's ability to stimulate both these activities as a measure of the neuromodulatory actions of this hormone in the neurons. Based on these (Lu et al., 1996; Yu et al., 1996) and other observations (Yang et al., 1996), we had hypothesized that the activation of MAP kinase by Ang II results in its translocation into the nucleus, where it mediates the activation of transcription factor(s) relevant to stimulation of c-fos transcription. This involves activation of SRE in the c-fos promoter. It leads to the activation of AP₁ sites on the promoter region of the TH and NET genes to stimulate the transcriptions of these genes. Thus, our next objective was to test this hypothesis by studying the effects of Ang II on SRE-binding activity and on Fos, two key players in this pathway.

Incubation of neuronal cultures with Ang II resulted in a time-dependent stimulation of SRE-binding activity as measured by the gel-shift assay (Fig. 5). A stimulation of approximately twofold was observed as early as 5 min and reached maximal levels of eightfold in 15 min. The effect of MAP kinase AON on Ang II stimulation of SRE-binding activity was determined to confirm the role of MAP kinase in this process. Neuronal cultures were preincubated with 1 μ M AON or SON for MAP kinase for 24 h as described previously (Yang et al., 1996). This treatment causes a \sim 70% decrease in both p42 and p44 isoforms of MAP kinase and causes a significant attenuation of Ang II

stimulation of TH and NET mRNA levels (Yang et al., 1996). In addition, MAP kinase AON caused a significant decrease in MAP kinase immunostaining (Fig. 6). Fig. 7 shows that pretreatment of neuronal cultures with MAP kinase AON resulted in a significant attenuation of Ang II

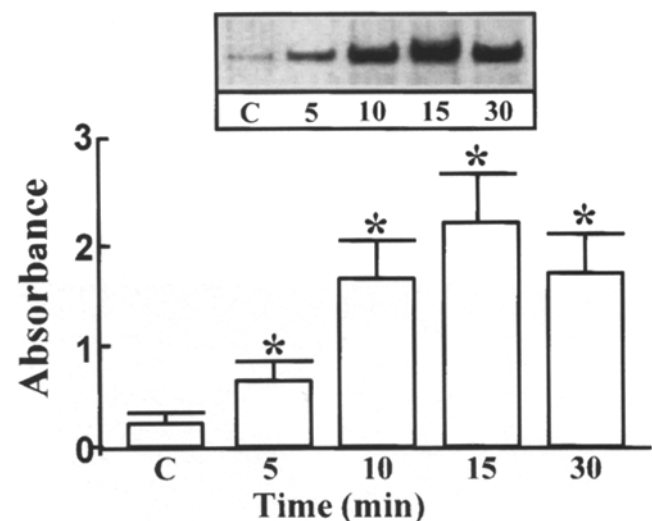


Figure 5. Effect of Ang II on SRE-binding activity in nuclear extracts of neurons. Neuronal cultures were incubated with 100 nM Ang II for the indicated time periods. Nuclear extracts were isolated and incubated with the SRE-consensus oligonucleotide sequence. Binding of the oligonucleotides was determined by gel-shift assay essentially as described in Methods. (Top) A representative autoradiogram. (Bottom) A band corresponding to SRE oligonucleotide-protein complex was quantitated from three separate experiments and the data are presented as mean \pm SE ($n = 3$). (*) Significantly different ($P < 0.05$) from zero time.

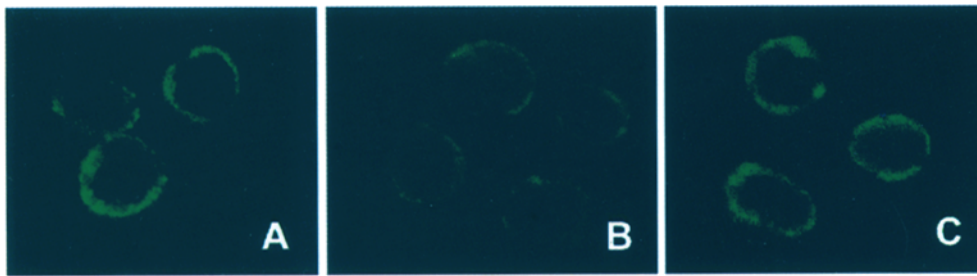


Figure 6. Depletion of immunostaining of MAP kinase by MAP kinase antisense oligonucleotide. Neuronal cultures were incubated without (A) or with 1.0 μ M MAP kinase AON (B) or with MAP kinase SON (C) for 24 h at 37°C. Cells were fixed and subjected to immunostaining for MAP kinase as described in Methods.

stimulation of SRE-binding activity. In contrast, SON for the enzyme did not inhibit Ang II stimulation of SRE-binding activity.

Next, we studied the effect of Ang II on AP₁-binding activity, fos gene expression, and the involvement of MAP kinase in this process. The rationale for this was based on our previous observations that have established that Ang II stimulates *c-fos* gene expression in neurons and that intervention in this stimulation by *c-fos* antisense oligonucleotide attenuates Ang II's stimulatory actions on NET and TH genes (Lu et al., 1996; Yu et al., 1996). Cytosolic and nuclear fractions from control and Ang II-treated neuronal cultures were prepared and the levels of AP₁-binding activity were quantitated. Fig. 8 shows a transient time-dependent increase in AP₁-binding activity in the cytosolic fraction. AP₁-binding activity was low in the cytosol of control neuronal cells. It increased with time, reaching a maximal level of 11-fold in 15 min followed by a significant decrease in 30 min. In contrast to the cytosolic fraction, the increase of AP₁-binding activity in the nuclear fraction showed a lag of \sim 5 min, followed by its time-dependent increase, reaching a maximal stimulation of 10-fold in 30 min. At this point, the level of nuclear AP₁-binding activity was approximately twofold higher than the cytoplasmic level. MAP kinase AON was used to determine the involvement of this enzyme in Ang II stimulation of AP₁-binding activity. After preincubation of neuronal cultures for 24 h with MAP kinase AON or SON, cells were incubated with 100 nM Ang II and whole-cell extracts were

used for the determination of AP₁-binding activity and Fos-mRNA levels. Ang II stimulation of AP₁-binding activity was inhibited by 80% by MAP kinase AON and not by SON (Fig. 9). In addition, treatment of neuronal cultures with MAP kinase AON resulted in the attenuation of Ang II stimulation of *c-fos* mRNA levels (Fig. 10).

Finally, we addressed the issue of the relative localization of various components of the Ang II effector system in our neuronal cultures. Our hypothesis that Ang II stimulation of NET and TH genes involves MAP kinase, SRE, and Fos assumes that these events are occurring in a single neuron and that there is no interneuronal flow of signaling molecules. Since neuronal cultures are prepared from the hypothalamic-brainstem areas and contain functionally diverse neurons, it was imperative to demonstrate colocalization of these components to validate this hypothesis. Fig. 11 shows a series of images from immunocytochemical staining in combination with *in situ* RT-PCR that suggests that the components of the Ang II effector system are localized in one neuron. With the use of two separate AT₁ receptor antibodies we found that AT₁ receptor immunoreactivity (*green*) was colocalized with neurons containing Fos, NET mRNA, and TH mRNA. In addition, the TH immunoreactivity was colocalized with the NET mRNA in Ang II-treated cultures. These data indicate that AT₁ receptors are localized on noradrenergic neurons that contain essential components of the Ang II signaling pathways responsible for this hormone's neuromodulatory actions.

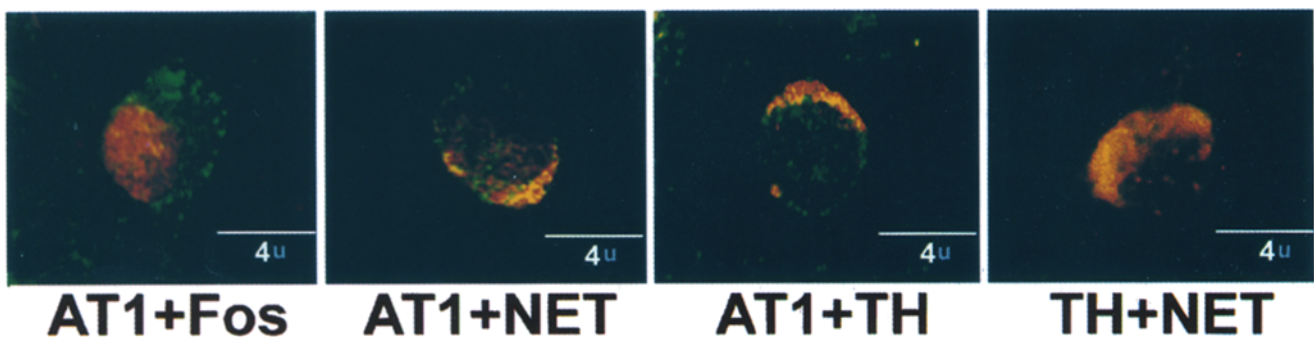


Figure 11. Colocalization of AT₁ receptors with Fos immunoreactivity, NET and TH messenger RNAs, and TH immunoreactivity with NET mRNA in Ang II-treated neurons. Neuronal cultures were treated with 100 nM Ang II for 20 min at 37°C for colocalization of AT₁ receptor with Fos immunoreactivity (*ATI+Fos*), and 2 h for AT₁ receptor and NET mRNA (*ATI+NET*), AT₁ receptor and TH mRNA (*ATI+TH*), and TH immunoreactivity and NET mRNA (*TH+NET*). Cultures were fixed and used for colocalization essentially as described in Methods. Red represented Fos immunoreactivity, NET mRNA, and TH mRNA, and green represented AT₁ receptor and TH immunoreactivities. Yellow represented colocalization of the two stainings. Images are representative from six separate experiments where \sim 80 cells were observed for each colocalization analysis.

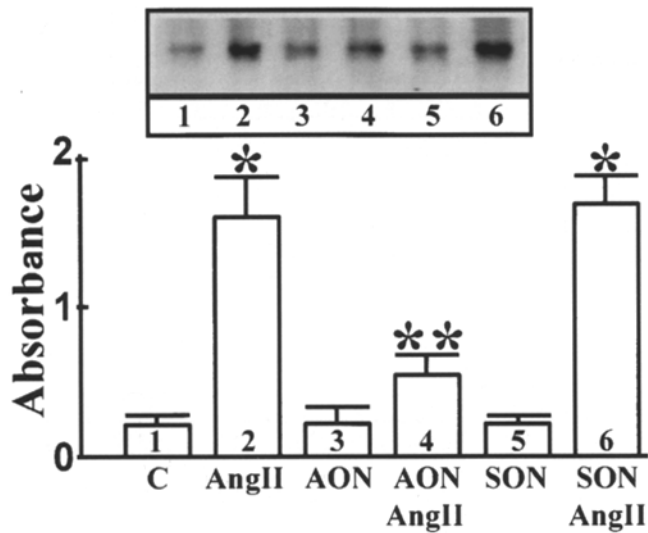


Figure 7. Effect of MAP kinase antisense oligonucleotide on Ang II stimulation of SRE-binding activity in neurons. Neuronal cultures were preincubated with 1 μ M AON or SON for MAP kinase for 24 h at 37°C. This was followed by incubation with 100 nM Ang II for 30 min at 37°C. Nuclei were isolated and lysed, and nuclear extracts were used to carry out gel-shift analysis for the determination of SRE-binding activity essentially as described in Methods. (Top) A representative autoradiogram. (Bottom) Mean \pm SE ($n = 3$). (*) Significantly different ($P < 0.05$) from control (C). (**) Significantly different ($P < 0.05$) from Ang II-treated cells.

Discussion

The observations presented in this study are the first demonstration that MAP kinase immunoreactivity is primarily localized around the nucleus and that its activation and translocation into the nucleus by Ang II are key regulatory events in this hormone's neuromodulatory actions in neurons. In addition, a sequence of signaling events involved in the Ang II stimulation of TH and NET genes has been described based on experimental data presented in this study and elsewhere (Yang et al., 1996).

AT₁ receptors are distributed rather uniformly on the neuronal plasma membrane. Within 5 min of incubation with Ang II, they undergo redistribution and become associated with Ras (Yang et al., 1996). This interaction is transient and results in the activation of Ras followed by the interaction of Ras with Raf-1 that ultimately leads to the activation of Raf-1 (Yang et al., 1996). These events involving the AT₁ receptor, Ras, and Raf-1 have been suggested to occur on the plasma membrane of the neurons, and are important in the Ang II-mediated activation of MAP kinase. Activation of MAP kinase is accompanied by its translocation into the nucleus in 15 min. The translocated enzyme is the active form of the MAP kinase as evidenced by the presence of the phosphorylated p42 and p44 isoforms in the nuclear extracts of Ang II-treated neurons. In addition, nuclear enzymes possess kinase activity as shown by the in-gel assay (Fig. 4 B). The activation and translocation of MAP kinase subsequently participate in the Ang II-mediated activation of SRE-binding activity and c-fos gene expression, and AP₁-binding activity. Evidence for this conclusion is derived from the observations

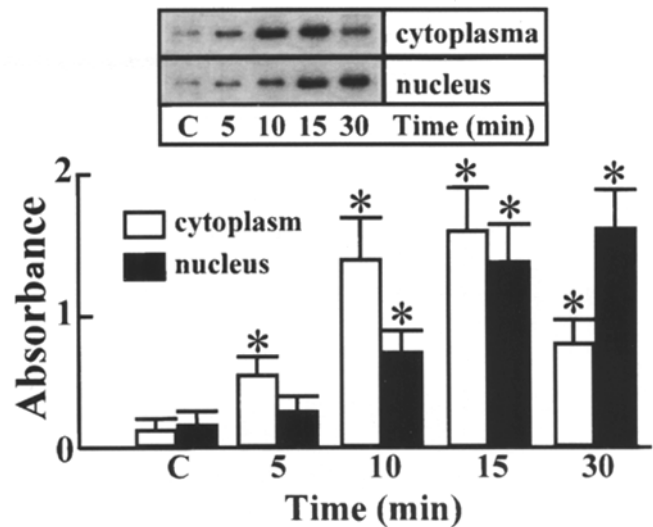


Figure 8. Distribution of AP₁-binding activity in cytoplasmic and nuclear fractions of Ang II-treated neurons. Neuronal cultures were treated with 100 nM Ang II for the indicated time periods. Cytoplasmic (□) and nuclear (■) extracts were prepared and subjected to gel-shift analysis to determine the levels of AP₁-binding activity essentially as described in Methods. (Top) Representative autoradiograms for cytoplasmic and nuclear fractions. (Bottom) Mean \pm SE ($n = 3$) of the band corresponding to AP₁ oligonucleotide-protein complex. (*) Significantly different ($P < 0.05$) from zero time.

that show that the Ang II-mediated stimulation of SRE-binding activity, c-fos gene expression, and AP₁-binding activity are attenuated by the preincubation of neuronal cells with AON for MAP kinase. These observations demonstrate that translocation of MAP kinase into the nucleus may be required for the Ang II-mediated activation of SRE-binding activity, c-fos gene expression, and AP₁-binding activity in the neurons. This is consistent with the proposed role of this enzyme in other systems (Gonzalez et al., 1993; Khalil and Morgan, 1993; Ward et al., 1994). Our data also suggest the hypothesis that activation of these transcription factors by Ang II results in their binding to the AP₁-binding site on the promoter regions of TH and NET genes to stimulate their transcription. There is sufficient evidence to support this hypothesis: (a) AP₁-binding activity is increased in neurons treated with Ang II. (b) AP₁-binding sites, which are Ang II responsive, are demonstrated in the promoter region of at least one of the genes (TH) (Goc and Stachowiak, 1994). (c) MAP kinase stimulates activation of various transcription factors, including Fos (this study) and Jun, which have been shown to interact with the AP₁-binding site (Beato, 1991; Puri et al., 1995). The fact that Ang II stimulates c-fos and that c-fos stimulation is important for Ang II stimulation of NET and TH genes further supports this hypothesis (Lu et al., 1996; Yu et al., 1996).

There are two unique features of these observations. First, MAP kinase immunoreactivity is primarily localized around the nucleus with little distribution in the cytoplasmic compartment in the neurons. This distribution is unique to neurons since other studies have shown that MAP kinase is diffusely distributed throughout the cyto-

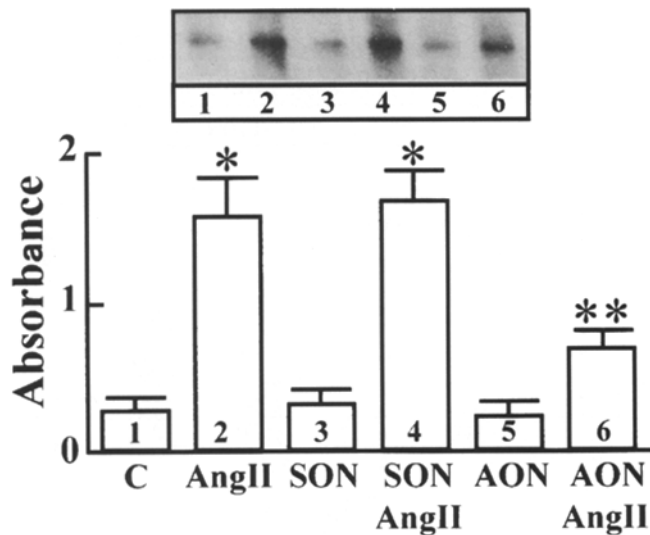


Figure 9. Effect of MAP kinase antisense oligonucleotide on Ang II stimulation of AP₁-binding activity in the neurons. Neuronal cultures were preincubated with 1.0 μ M MAP kinase AON or SON for 24 h at 37°C. Whole cell lysates were prepared and used for gel-shift analysis for the quantitation of AP₁-binding activity essentially as described in Methods. (Top) A representative autoradiogram. (Bottom) Mean \pm SE ($n = 3$). (*) Significantly different ($P < 0.05$) from control (C). (**) Significantly different ($P < 0.05$) from Ang II-treated cells.

plasmic compartment in other cell types (Khalil and Morgan, 1993; Lamy et al., 1993; Zhao et al., 1995). Thus, it is tempting to suggest that MAP kinase may be anchored to the nuclear membrane in neurons. This view is supported by studies demonstrating the anchoring of other protein kinases to the cytoskeletal and other membranes by anchoring proteins (Mochly-Rosen, 1994). Additional experiments would be needed to confirm this conclusion. The relevance of such a localization of MAP kinase and its role in AT₁ receptor-mediated signaling mechanism is not evident at the present time. However, it may be possible that the enzyme could be responsible for the phosphorylation of specific protein(s) that may participate in the translocation of MAP kinase and other proteins into the nucleus. This view is supported by many observations in the literature demonstrating the phosphorylation of proteins located on the nuclear membrane by other kinases that are involved in their transport across the nuclear membrane (Hennekes et al., 1993; Murray et al., 1994). For example, protein kinase C-mediated phosphorylation of lamin B is associated with its transport across the nuclear membrane (Hennekes et al., 1993; Murray et al., 1994). Consistent with this is our data showing that MAP kinase AON blocks transport of STAT-3 into the nucleus (Raizada, K., H. Yang, and D. Lu, unpublished observation).

The second unique feature of these findings is the demonstration that MAP kinase is involved in the regulation of TH and NET, two genes whose products are key players in the synthesis, turnover, and metabolism of CA. Thus, those neuromodulatory actions of Ang II that involve norepinephrine turnover, synthesis, and release (Raizada et al., 1994; Sumners et al., 1985, 1987; Sumners and Raizada, 1986) are mediated by the activation of MAP ki-

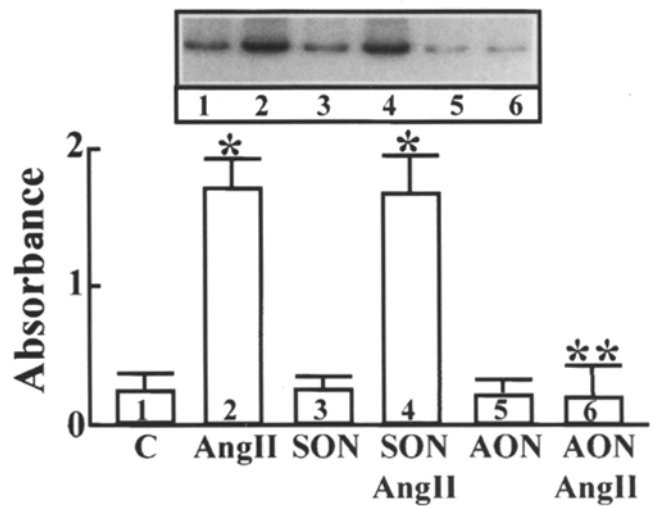


Figure 10. Effect of MAP kinase antisense oligonucleotide on Ang II stimulation of c-fos mRNA in the neurons. Neuronal cultures were pretreated with 1.0 μ M MAP kinase AON or SON for 24 h at 37°C. Poly(A⁺)RNA was isolated after treatment of cultures with 100 nM Ang II for 20 min at 37°C. RT-PCR was carried out with the use of specific c-fos primers essentially as described in Methods. (Top) A representative autoradiogram. (Bottom) Mean \pm SE of the band in the PCR representing c-fos mRNA. (*) Significantly different ($P < 0.05$) from control (C). (**) Significantly different ($P < 0.05$) from Ang II-treated cells.

nase. Although the involvement of this enzyme in the growth promoting and other actions of various hormones, including Ang II, has been well documented (Sadoshima et al., 1995; Yamazaki et al., 1995), its role in neuromodulation in terminally differentiated neurons in primary culture is a new observation. In fact, the participation of MAP kinase in the neuromodulatory actions of Ang II is highly specific since the neurotrophic actions that involve plasminogen activator inhibitor-1 do not involve this enzyme. This conclusion is based on our previous observations indicating that MAP kinase AON failed to block Ang II stimulation of PAI-1 mRNA in neuronal cultures, whereas it abolished the stimulation of TH and NET mRNA levels by Ang II (Wright and Harding, 1994).

In summary, these observations provide evidence for a sequence of signal transduction events initiated by the interaction of Ang II with the AT₁ receptor and involving the activation and translocation of MAP kinase into the nucleus. These events are key in transcriptional control of CA-relevant genes and thus in chronic stimulation of CA turnover in neurons. However, many questions remain, including: (a) How does activation of MAP kinase participate in the transport of signaling molecules across nuclear membrane activated; (b) how does Raf-1 participate in the activation of MAP kinase since their distribution is far apart in the cells; and (c) what is the nature of the anchor protein(s) for MAP kinase in the neurons? Future studies will attempt to answer these issues.

We wish to thank Elizabeth Brown (University of Florida) for neuronal cultures, and Jennifer Brock for typing and preparation of the manuscript. The Confocal Microscopy Core of the Structural Biology Center and the DNA Synthesis Core of the ICBR provided valuable assistance during the completion of this investigation.

The research was supported by the National Institutes of Health grant 33610. Di Lu was post-doctoral fellow of the American Heart Association, Florida Affiliate.

Received for publication 14 May 1996 and in revised form 12 July 1996.

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