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Inhibition and down-regulation of gene transcription and guanylyl cyclase activity of NPRA by angiotensin II involving protein kinase C

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Abstract

The objective of this study was to investigate the role of protein kinase C (PKC) in the angiotensin II (Ang II)-dependent repression of *Npr1* (coding for natriuretic peptide receptor-A, NPRA) gene transcription. Mouse mesangial cells (MMCs) were transfected with *Npr1* gene promoter-luciferase construct and treated with Ang II and PKC agonist or antagonist. The results showed that the treatment of MMCs with 10 nM Ang II produced a 60% reduction in the promoter activity of *Npr1* gene. MMCs treated with 10 nM Ang II exhibited 55% reduction in NPRA mRNA levels, and subsequent stimulation with 100 nM ANP resulted in 50% reduction in guanylyl cyclase (GC) activity. Furthermore, the treatment of MMCs with Ang II in the presence of PKC agonist phorbol ester (100 nM) produced an almost 75% reduction in NPRA mRNA and 70% reduction in the intracellular accumulation of cGMP levels. PKC antagonist staurosporine completely reversed the effect of Ang II and phorbol ester. This is the first report to demonstrate that ANG II-dependent transcriptional repression of *Npr1* gene promoter activity and down-regulation of GC activity of translated protein, NPRA is regulated by PKC pathways.

Keywords: Natriuretic peptide receptor; *Npr1* promoter; Gene transcription; Protein kinase C; Mesangial cells

The natriuretic peptide family consists of peptide hormones atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), and C-type natriuretic peptide (CNP), each being derived from a separate gene [1]. ANP exerts its effects at a number of sites and mediates several physiological responses, such as producing natriuresis and diuresis in the kidney [2], inhibiting aldosterone synthesis and secretion in the adrenal gland [3], and producing vasorelaxation in vascular smooth muscle cells (VSMC) [4]. Three subtypes of natriuretic peptide receptors have been cloned and include natriuretic peptide receptor-A (NPRA), receptor-B (NPRB), and receptor-C (NPRC) [5,6]. NPRA is considered to be the primary biological receptor of both ANP and BNP because most of the physiological effects of these hormones are triggered by generation of its second messenger cGMP [7,8].

The expression and activation of NPRA is regulated by a number of factors including auto-regulation by the natriuretic peptides themselves [9–11], as well as other hormones such as endothelin [12], angiotensin II (Ang II) [13–16], and vasopressin [17]. The two vasoactive peptide hormones Ang II (vasoconstrictive) and ANP (vasodilatory) interact and antagonize the biological effects of each other at various levels [9]. ANP has been shown to inhibit Ang II-induced contraction of isolated glomeruli and cultured mesangial cells [18]. Additionally, ANP also inhibits Ang II-stimulated activation of protein kinase C (PKC) and mitogen activated protein kinase (MAPK) in VSMC and mouse mesangial cells (MMCs) in a cGMP-dependent manner [19,20]. Furthermore, ANP has been shown to decrease Ang II-evoked secretion and steroidogenesis in cultured glomerulosa

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cells [21]. Conversely, Ang II is also known to reduce ANP-dependent cGMP levels by stimulating cGMP hydrolysis, presumably via a calcium-dependent cGMP phophodiesterase [14,22].

In addition to the hormonal factors, it has been shown that PKC negatively regulates the NPRA protein [23] and this negative regulation is mediated by dephosphorylation of NPRA [24]. Although, PKC has been shown to dephosphorylate and desensitize the NPRA receptor, there has been no report about PKC being involved in the transcriptional regulation of *Npr1* gene, which encodes for NPRA. Interestingly, it has been shown that several protein kinases regulate the transcription of other receptor proteins by activating transcription factors [25–27]. In the present study, we have investigated the role of PKC in the Ang II-dependent repression of *Npr1* gene promoter.

Materials and methods

Materials. The pGL3-basic vector, pRL-TK, pGL3-control plasmids, and dual luciferase assay system were purchased from Promega (Madison, WI). Plasmid isolation kit was obtained from Qiagen Inc. (Valencia, CA). Sequence-specific oligonucleotides were purchased from Midland Certified Reagent Company Inc. (Midland, TX). The cell culture media, fetal calf serum, insulin, transferrin, and sodium selenite (ITS), as well as Lipofectamine-2000 were purchased from Invitrogen (Carlsbad, CA). Ang II was purchased from Peninsula Laboratories Inc. (King of Prussia, PA).

Plasmid construction. All the promoter-luciferase reporter constructs were made by cloning the DNA fragments from −1182 to −914 of *Npr1* gene promoter region, upstream of the SV-40 promoter firefly luciferase gene in the pGL3-promoter vector. All of the forward primers (F1, F2, F3, F4, and F5) contained a *Mlu*I restriction site, whereas the reverse primer contained a *Bgl*II restriction site at the 5′ end. The PCR primers used in this study were as follows: F1 (−1182 to −1127 bp), 5′-ATC GGA ACG CGT ACT GGC ACT TGA CAC AGC TGG TCC-3′; F2 (−1128 to −1072 bp), 5′-TAC GGA ACG CGT CTG GCT CGC CTC TAC TTG ATT GCC-3′; F3 (−1071 to −1028 bp), 5′-ACT GGA ACG CGT CAT ACT CCT GGG GCA AGC GCG AGC G-3′; F4 (−1026 to −986 bp), 5′-ATC GGA ACG CGT GGC ACT TGA CAC AGC TGG TCC ACT-3′; F5 (−984 - to −914 bp), 5′-ATC GGA ACG CGT TCC TGG ACT GGC ACT TGA CAC AGC-3′ and the reverse primer was R2 (55–33 bp), 5′-TAC GGA AGA TCT GCG GGT GCG CCA GCG AGG AAA GG-3′. These restriction sites were used for cloning of the amplified fragments into *Mlu*I–*Bgl*II-restricted pGL3-promoter vector. All plasmid constructs were sequenced across both junctions to confirm the nucleotide sequence and the predicted orientation.

Transient transfection and luciferase assay. The MMCs were cultured and seeded in 12-well plates at a density producing approximately 80% confluence on the next day as described previously [20]. The cells were then transfected by using Lipofectamine-2000 reagent according to the manufacturer's instructions, with the test plasmid and pRL-TK utilized as internal transfection control. The luciferase activities were measured by TD 20/20 luminometer (Turner Designs, Sunnyvale, CA) with 20 μl cell extract using dual luciferase reporter assay system. In the transfection experiments, a pGL3-control vector containing both the SV40 promoter and enhancers was used as a positive control, and the empty pGL3-basic vector was used as a negative control. The results were normalized for the transfection efficiency as relative light units per *Renilla* luciferase activity.

Cell culture and hormonal treatment. To study the effect of Ang II, MMCs were seeded in 24-well plates at 80–90% confluence and were transfected with 1 μg test plasmid and 0.3 μg pRL-TK using Lipofectamine-2000 reagent according to manufacturer's instructions. After 24 h, the cells were washed twice with serum-free medium containing 0.1% BSA (assay medium) which were treated with Ang II in fresh assay medium. The cells were harvested after 16 h, and dual luciferase assay was performed as described above. The results were normalized for the transfection efficiency as relative light units per *Renilla* luciferase activity.

Preparation of plasma membranes. The plasma membranes were prepared by suspending the MMCs in 5 volumes of sodium phosphate buffer (10 mM, pH 7.4) containing 250 mM sucrose, 150 mM NaCl, 1 mM PMSF, 5 mM benzamidine, 5 mM EDTA, and 10 μg/ml each of leupeptine and aprotinine, as described previously [28]. The cells were homogenized and centrifuged at 2000 rpm for 10 min at 4 °C and the supernatant collected was re-centrifuged at 30,000 rpm for 1 h at 4 °C. The resultant supernatant was discarded and the pellet was resuspended in 1 ml of Hepes buffer (50 mM, pH 7.4) containing 150 mM NaCl, 1 mM PMSF, 5 mM benzamidine, 5 mM EDTA, and 10 μg/ml each of leupeptine and aprotinine and recentrifuged at 30,000 rpm for 1 h at 4 °C. The final pellet was suspended in 200 μl of Hepes buffer (pH 7.4). The protein concentration was estimated by using Bio-Rad protein assay kit, based on Bradford method.

Guanylyl cyclase (GC) activity assay. The GC activity was assayed as described by Leitman et al., [29], with some modifications. A 50 μg aliquot of plasma membranes was added to 100 ml GC assay buffer containing Tris–HCl buffer (50 mM, pH 7.6), 4 mM $MnCl_2$, 2 mM IBMX, 1 mM BSA, 5 units of creatinine phosphokinase, 7.5 mM creatine phosphate, 0.5 mM GTP, and 1 μM ANP. The samples were incubated in a water bath at 37 ° C for 10 min. The reaction was stopped by adding 900 μl of sodium acetate (55 mM, pH 6.2) to each sample tube. The sample tubes were placed initially in hot water bath for 3 min and then on ice for 15 min to deactivate the proteins. The samples were then centrifuged at 12,000 rpm for 5 min. The supernatant was collected and the cGMP assay was performed.

cGMP assay. The MMCs grown in the assay medium were treated with varying concentrations of Ang II (0.001 nM to1000 nM) in a time-dependent manner. The cells were then treated with (0.1 M) HCl for 30 min, scraped and centrifuged at 600*g* at 4 °C. The supernatant thus collected was used for the cGMP assay. The assay was performed using the direct cyclic GMP correlate-EIA kit (Assay Designs, Inc., Ann Arbor, MI.) according to the manufacturer's instructions.

Reverse transcriptase-polymerase chain reaction (RT-PCR) assay. Confluent MMCs were treated with or without Ang II in differential concentrations in assay medium. After harvesting the cells, total RNA was extracted by micro-to-midi (Invitrogen, Carlsbad, CA) RNA purification system. One microgram of total RNA was reverse transcribed by using the Superscript one-step RT-PCR with platinum *Taq* system. The amplification was carried out using 35 cycles with denaturing at 94 °C for 15 s, annealing at 60 °C for 30 s, and extension at 72 °C for 1 min. The upstream primer (5′-ATC TAT TTC AGT GAT ATC GTG GGC-3′; 2964 to 2987 bp) and downstream primer (5′-CAT CGA ACT CTT CCA GCA CAG-3′; 3420 to 3400 bp) were designed from published mouse cDNA sequence [30]. Control experiments were performed with RNA samples but without reverse transcriptase. The PCR product of 456 bp was electrophoresed through 1.5% agarose gel and stained with ethidium bromide. The gel was digitized, and signal intensities of the corresponding bands were quantified using Alpha Imager (Alpha Innotech Corp, San Leandro, CA). The specific primers for β-actin gene were included in the PCR as an internal control.

Statistical analysis. The results were expressed as mean ± SE. The statistical significance was evaluated by a 1-way ANOVA or with Student's *t* test, and differences were considered significant if the probability value was <0.05.

Results

The results of the present study demonstrate that the treatment of *Npr1* ΔR1 (−1182/−918 bp)-transfected MMCs with 10 nM Ang II produced a 60% reduction in

luciferase activity as compared to PGL3-transfected control cell (Fig. 1A and B). Furthermore, treatment with 10 nM Ang II elicited a 55% reduction in NPRA mRNA levels (Fig. 2A and B). Additionally, administering both Ang II and 100 nM phorbol ester to MMCs induced a greater inhibitory response, producing an almost 75% reduction in NPRA mRNA levels (Fig. 2B). Treatment of MMCs with Ang II drastically reduced GC activity by 50% (as measured by cGMP production) and the combination of Ang II and phorbol ester inhibited GC activity by almost 70% (Fig. 3). Interestingly, treatment of cells with PKC inhibitor staurosporine reversed the Ang II- and phorbol ester -induced *Npr1* promoter repression by 93–95% (Fig. 4A and B). Incubation of MMCs with staurosporine (100 nM) further reversed the inhibitory effects of Ang II and phorbol ester on GC activity of NPRA by 95% (Table 1).

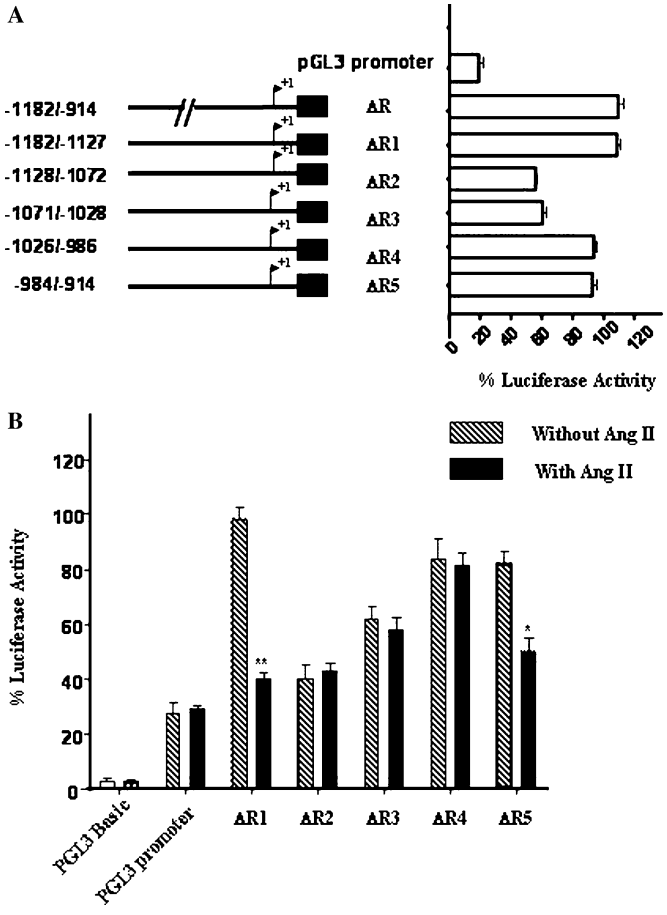

Fig. 1. (A) Deletion analysis of *Npr1* gene promoter in the region −1182/−914 upstream of transcription start site 1 (tss1): A diagramatic representation of different lengths of the 5′-flanking region of mouse *Npr1* gene, inserted upstream of firefly luciferase gene. The numbers shown next to the schematics indicate the nucleotide positions for the 5′ and 3′ ends of the constructs, respectively. The arrows indicate the tss designated as 1. (B) MMCs were transiently transfected with 1 μg of either ΔR1, ΔR2, ΔR3, ΔR4 or ΔR5 deletion constructs and 300 ng of pRL-TK. After 24 h, cells were treated with or without Ang II for 16 h. The results are expressed as means ± SE from six independent experiments. $^{*}P < 0.05$, $^{**}P < 0.001$ vs control.

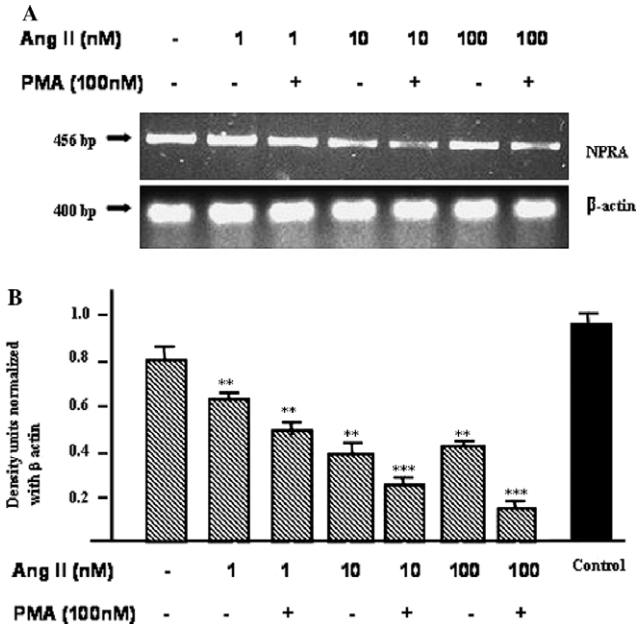

Fig. 2. Effect of Ang II and phorbol ester on NPRA mRNA levels in MMCs: (A) Representative example of reverse transcription–PCR experiment evaluating the transcript level of NPRA and β-actin in MMCs after treatment with 100 nM phorbol ester for 2 h and then with 1–100 nM Ang II for 16 h. (B) Results are expressed as percentage of untreated control of the ratio of optical densities of NPRA RT-PCR product versus β-actin product. Vertical bars represent means ± SE from four independent experiments. $^{**}P < 0.05$, $^{***}P < 0.001$ vs control.

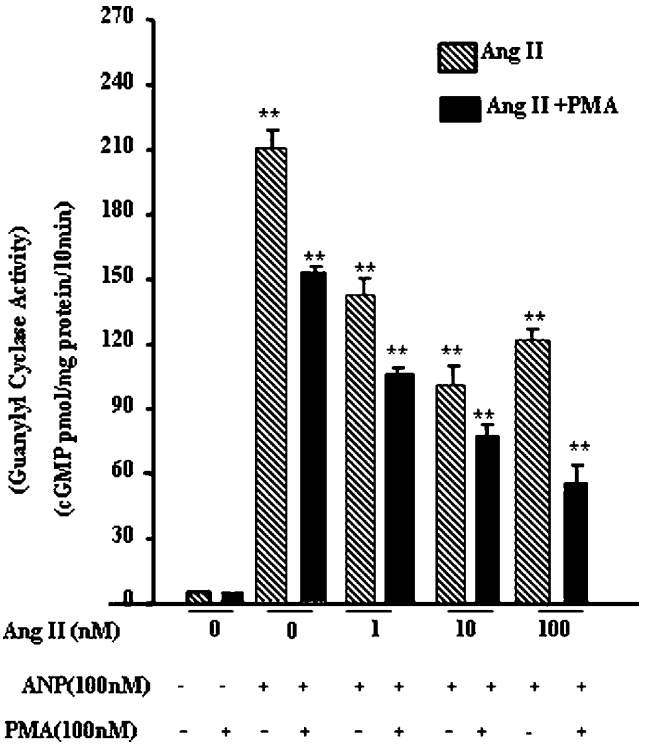

Fig. 3. Effect of Ang II and phorbol ester on guanylyl cyclase activity and cGMP levels in MMC cells: MMCs were initially treated with 100 nM phorbol ester for 1 h and then with 0.1 nM to 1000 nM Ang II for 16 h. The MMCs were then stimulated with ANP and guanylyl cyclase activity and cGMP production were measured. The results are expressed as means ± SE from four independent experiments. $^{**}P < 0.01$, $^{***}P < 0.001$.

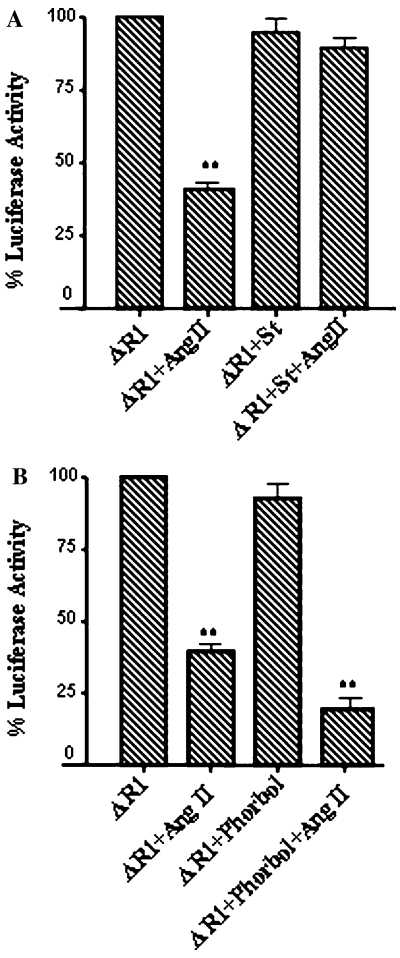

Fig. 4. Effect of Ang II, phorbol ester, and/or staurosoporine on *Npr1* gene transcription and luciferase activity in MMCs: Cells were transiently transfected with 1000 ng of ΔR1 deletion construct and 300 ng of pRL-TK. After 24 h, cells were treated either with 100 nM phorbol ester (A) or 100 nM staurosporine (B) for 1 h, and then incubated with 10 nM Ang II for 16 h. Normalized luciferase activity is shown as a percentage of the activity of untreated group. The results are expressed as means ± SE from four independent experiments. $^{**}P < 0.001$ vs control.

Table 1
Relative luciferase and GC activities in cells treated with Ang II, phorbol ester and/or staurosporine

Treatment	Assay			
	Luciferase activity	% Inhibition	GC activity	% Inhibition
Control	100	0	100	0
Ang II	$40 \pm 3^{*}$	$60 \pm 3^{*}$	$50 \pm 3^{*}$	$50 \pm 3^{*}$
Phorbol ester	$65 \pm 4^{*}$	$35 \pm 4^{*}$	$55 \pm 4^{*}$	$45 \pm 4^{*}$
Ang II + phorbol ester	$18 \pm 2^{**}$	$82 \pm 2^{**}$	$27 \pm 3^{**}$	$73 \pm 3^{**}$
Ang II + staurosporine	92 ± 3	8 ± 3	93 ± 2	7 ± 3

MMCs were transiently transfected with 1000 ng of ΔR1 deletion construct and 300 ng of pRL-TK. After 24 h, cells were either treated with 100 nM phorbol ester or 100 nM stauosporine for 1 h, and were then incubated with 10 nM Ang II for 16 h. Normalized luciferase activity is shown as a percentage of the activity of untreated group. To determine guanylyl cyclase (GC) activity, MMCs were then stimulated with ANP and the cGMP was measured. The results are expressed as means ± SE from four independent experiments.

* $P < 0.01$.

** $P < 0.001$.

Discussion

The results of this study demonstrate that Ang II and phorbol ester dramatically suppress *Npr1* gene transcription and down-regulate GC activity of NPRA receptor. These results clearly show that Ang II-induced transcriptional repression of *Npr1* gene is mediated through PKC. Previous studies have shown that Ang II inhibits ANP-stimulated cGMP accumulation by activating protein tyrosine phosphatase activity [15] and by enhancing cGMP hydrolysis, apparently via a calcium-dependent cGMP phophodiesterase [14,22]. It has also been reported that pretreatment with phorbol ester attenuated cGMP formation in adrenocortical carcinoma cells exhibiting a high density of NPRA. However, in the presence of a PKC inhibitor, the phorbol ester-mediated attenuation of ANP-stimulated cGMP formation was blocked [23]. To demonstrate the PKC mediation in the Ang II-dependent transcriptional repression, we have used PKC agonist phorbol ester and PKC inhibitor staurosporine and measured the *Npr1* promoter activity and GC activity of the translated protein NPRA. The results demonstrate that treatment of MMCs with Ang II and phorbol ester reduced NPRA mRNA levels and GC activity by 75% and 70%, respectively. Furthermore, the treatment of MMCs with combination of Ang II and phorbol ester, resulted in an 80% reduction in promoter activity and treatment with staurosporine, caused a complete reversal of the Ang II-mediated transcriptional repression, suggesting the PKC mediation of Ang II-dependent *Npr1* gene repression.

This is the first report to demonstrate that the transcriptional repression of *Npr1* gene promoter and down-regulation of GC activity of the translated protein NPRA are regulated by Ang II involving PKC. Another intriguing aspect of this study is that Ang II acts as a repressor instead of an activator of gene transcription. This novel regulatory mechanism of PKC mediating the transcriptional repression of *Npr1* gene and down-regulation of translated protein by Ang II will help in further understanding of role of Ang II in the ANP/NPRA signaling pathway regulating the physiology and pathophysiology of hypertension and cardiovascular homeostasis.

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