Vol. 185, No. 1, 1992 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 29, 1992 Pages 142-146

TWO DISTINCT PATHWAYS IN THE DOWN-REGULATION OF TYPE-1 ANGIOTENSIN II RECEPTOR GENE IN RAT GLOMERULAR MESANGIAL CELLS

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Received April 13, 1992

Summary: The mRNA level of the type-1 angiotensin II receptor (AT_1) was down-regulated by angiotensin II in cultured rat glomerular mesangial cells. The effect was maximum with 1 μM AII at 6 h, sensitive to cycloheximide, and specific to AT_1 since this phenomenon was blocked by DuP753, an AT_1 antagonist, but not by type-2 antagonist PD123319. Dibutyryl cAMP, forskolin, and cholera toxin also caused AT_1 down-regulation. These effects were not altered by either the protein kinase A inhibitor H-8 or cycloheximide. Calcium ionophore A23187, pertussis toxin, protein kinase C inhibitor staurosporine, or prolonged incubation with phorbol ester were without effect. These results suggest that there are at least two pathways to down-regulate AT_1 mRNA; one way is an angiotensin II-induced, protein kinase C-independent, and cycloheximide-sensitive pathway and the other is an angiotensin II-independent, cAMP-induced, and cycloheximide-insensitive pathway.

The process by which cellular responses to hormonal stimulation diminish over time despite its continuous presence is referred to as desensitization. The underlying mechanisms have been investigated extensively especially for the β-adrenergic receptor (review 1). However, there have been few studies concerning angiotensin II (AII) receptor regulation (2), and the molecular mechanism is poorly understood. The recent cloning of the AII receptor cDNA (3-6) provided us with the opportunity to explore the control of AII receptor gene expression .

The present studies were designed to determine the molecular mechanisms of AII receptor regulation at the level of mRNA, using quantitative polymerase chain reaction (PCR) techniques .

MATERIALS AND METHODS

Materials: AII, cycloheximide, dibutyryl cAMP (Bt_2cAMP), phorbol 12-myristate 13-acetate (PMA), pertussis toxin, cholera toxin, A23187, forskolin, and staurosporine were purchased from Sigma. N-[2-(methylamino)ethyl]-5-isoquinolinesulphonamide (H-8) was obtained from Seikagaku America Inc., and DuP753 and PD123319 were from Du Pont-Merk (Wilminton, DE), and Warner Lambert Co. (Ann Arbor, MI). Oligonucleotides were

0006-291X/92 $4.00

synthesized by Midland Certified Reagent (Midland, TX). [^{32}P]dCTP (3,000 Ci/mmol) was purchased from Amersham.

Cell culture: Rat glomerular mesangial cells were cultured in RPMI1640 medium containing 10% fetal bovine serum supplemented with antibiotics, as previously described (7). Serum was deprived 24-48 h before each experiment.

Measurement of type-1 AII receptor mRNA level: Total RNA was extracted as described (8), and quantitative PCR amplification techniques with an internal standard were employed for type-1 AII receptor (AT_1) mRNA as described previously (5, 9) with some modifications. Total RNA (500 ng) and deletion-mutated cRNA (see ref. 5) were reverse-transcribed using random hexamers as primers. PCR was performed by two-step amplification; 25 cycles of 94°C for 15 sec and 72°C for 30 sec using GeneAmp PCR System 9600 (Perkin-Elmer Cetus). The sequences of sense and antisense primers for AT_1 receptor were: 5'-ACCCCTCTACAGCATCTTTGTGGTGGGGA-3' and 5'-GGGAGCGTCGAATTCCGAGACTCATAATGA-3', respectively. In some experiments, [^{32}P]dCTP was included in the PCR reaction mixture. Amplification of the same sample with β-actin primers confirmed that equal amounts of RNA were reverse-transcribed. The sequences of sense and antisense primers for β-actin were: 5'-GTTCCGATGCCCCGAGGATCT-3' and 5'-GCATTTGCGGTGCACGATGGA-3', respectively, and the PCR schedule was 20 cycles of 94°C for 15 sec, 50°c for 20 sec, and 72°C for 20 sec. PCR amplification gave 479 bp, 191 bp, and 361 bp of fragments originated from AT_1, deletion mutated AT_1 and β-actin mRNA, respectively.

RESULTS AND DISCUSSION

To optimize the quantitative PCR, the range of concentrations of sample RNA and internal control cRNA as well as the number of amplification cycles were chosen within the exponential phase. (Fig.1A and B). Linear relationship in the ratio of sample RNA to internal control cRNA was maintained throughout the range we observed (Fig. 1B).

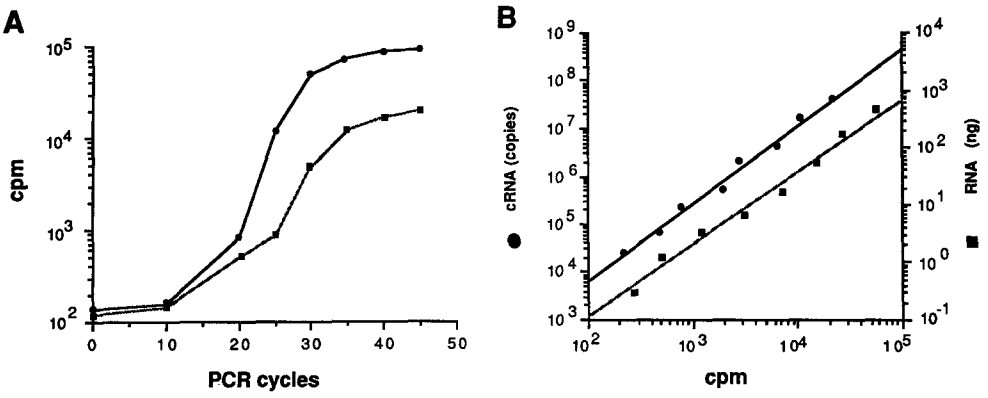

Fig.1. PCR quantification of AT_1 receptor mRNA in rat mesangial cells.
(A) Total RNA from rat mesangial cells (500 ng, ■) and $2x10^7$ cRNA molecules of internal standard (●) were co-amplified for various number of PCR cycles. (B) Serial 1:3 dilution from 500 ng of rat mesangial cells total RNA (■) and $2x10^7$ cRNA molecules of internal standard (●) were co-amplified for 25 cycles. Five micro Ci of [^{32}P]dCTP was included in each PCR reaction mixture. Samples were loaded on agarose gel, and bands were excised to measure their radioactivities.

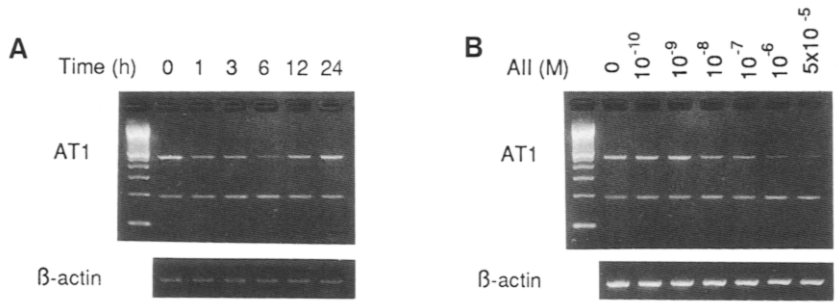

Fig.2. Time course and dose dependence of AII-induced AT_1 receptor down-regulation. Confluent mesangial cells were stimulated with (A) 1 μM AII for various periods of time or (B) various concentrations of AII for 6 h. Total RNA (500 ng) from each sample was amplified with $2x10^7$ cRNA molecules of internal standard for 25 cycles. The left lane in each panel shows 100 bp ladder (BRL) as a molecular size marker.

Recently, we have cloned highly homologous cDNAs encoding rat AT_1 receptor subtypes (AT_{1A} and AT_{1B}) (5,6). In order to quantify the amount of AT_{1A} mRNA specifically, we performed two-step amplification. Under this condition, co-amplification of AT_{1B} was avoided, since the digestion pattern by Hae III of the PCR fragment (6) was identical to that of AT_{1A}, but different from that of AT_{1B} (data not shown). Furthermore, amplification was not observed when cRNA transcribed from AT_{1B} cDNA was used as a template. (data not shown).

Up to 70% of the receptor message was lost by 6 h of treatment with AII in a dose dependent manner, followed by a gradual return toward control levels by 24 h (Fig 2A and B). This effect was blocked by DuP753, an AT1 antagonist, but not by type-2 AII receptor antagonist PD123319, suggesting that AII-induced AT_1 mRNA down-regulation is mediated specifically through AT_1 receptor (Fig 3A).

The membrane permeable cAMP analogue dibutyryl cAMP (Bt_2cAMP) also caused AT_1 receptor mRNA down-regulation. Interestingly, cycloheximide completely abolished the effect of AII, but not that of Bt_2cAMP, suggesting that there are two distinct pathways for AT_1 receptor down-regulation, with only one requiring *de novo* protein synthesis.(Fig. 3B). In order to clarify the molecular mechanisms for AT_1 mRNA down-regulation induced by AII and cAMP, their signal transduction systems were investigated using various activators and/or inhibitors (Fig. 4). Unexpectedly, protein kinase C (PKC) inhibitor staurosporine or prolonged preincubation with PMA to deplete cellular PKC activity did not affect AII-induced AT_1 receptor down-regulation (Fig 4; lane 1,2,4,5), as well as its control level (data not shown). Furthermore, calcium ionophore A23187 did not mimic the effect of AII (Fig 4; lane 6 and 14). Therefore, the regulation of AT_1 mRNA in the AII-induced down-regulation is possibly determined by some unknown signalling systems rather than PKC-Ca^{2+} pathway. These data are also intriguing in light of the previous findings that AII receptor is internalized without activating phosphoinositide (PI)

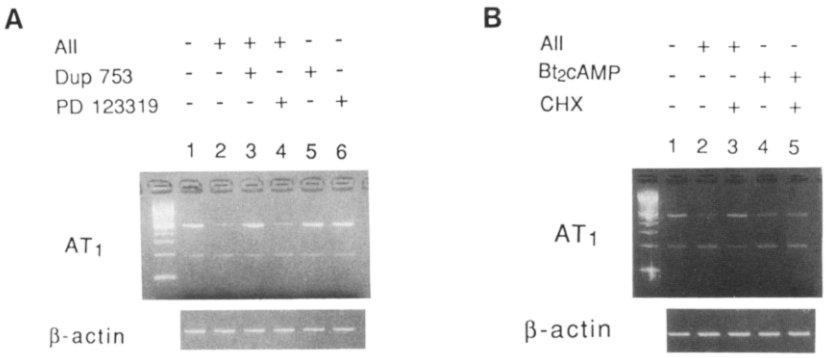

Fig.3. Effect of AII receptor antagonists, Bt_2cAMP, and cycloheximide on receptor message.
(A) Mesangial cells were stimulated with 0.1 μM AII in the presence or absence of DuP753 (1 μM), or PD 123319 (1 μM) for 6 h. DuP753 and PD 123319 were added 30 minutes prior to agonist stimulation. (B) Cells were incubated with AII (1 μM), Bt_2cAMP (1 mM), or vehicle in the presence or absence of cycloheximide (CHX, 10 μg/ml) for 6 h.

turnover (10), and that AII receptor down-regulation does not involve G_i, Ca^{2+}, or PKC (2).

When cells were treated with forskolin or cholera toxin to stimulate adenylate cyclase, reduction in mRNA levels were comparable with those observed with Bt_2cAMP (Fig 4; lane 6,8-10). These changes did not appear to involve protein kinase A (PKA) activation, since they were not blocked by PKA inhibitor H-8 at a concentration far above the ID_{50} for PKA (10 μM) (Fig.4; lane 6-8, 11-13). Bouvier et. al demonstrated that there are at least two pathways by which cAMP may decrease the number of β_2-adrenergic receptors (β_2AR); in addition to the observation that phosphorylation of β_2AR by PKA increases the rate of down-regulation, cAMP reduces steady state levels of β_2AR mRNA and thus

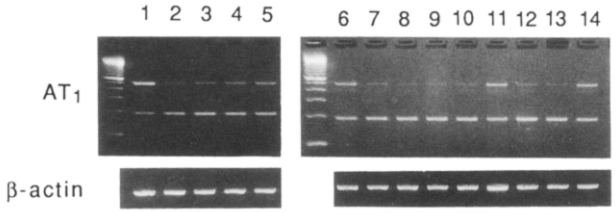

Fig.4. Regulation of AT_1 receptor expression: Effect of activation/inhibition of signalling pathways.
Cells were stimulated with various reagents. The stimulation corresponding to each lane is as follows: lane 1 and 6; control, lane 2 and 7; 1 μM AII, lane 3; preincubation with pertussis toxin (20 ng/ml) for 12 h then AII (1 μM), lane 4; preincubation with PMA (100 ng/ml) for 12 h then AII (1 μM), lane 5; preincubation with staurosporine (10 nM) for 1 h then AII (1 μM), lane 8; Bt_2cAMP (1 mM), lane 9; forskolin (25 μM), lane 10; cholera toxin (10 ng/ml), lane 11; H-8 (10 μM), lane 12; preincubation with H-8 (10 μM) for 1 h then AII (1 μM), lane 13; preincubation with H-8 (10 μM) for 1 h then Bt_2cAMP (1 mM), and lane 14; A23187 (1 μM). RNA was extracted 6 h after each stimulation.

lowers receptor density (11). The precise mechanism for the cAMP-induced AT_1 mRNA down-regulation is currently unknown. However, an effect of cAMP on post transcriptional events such as AII receptor mRNA destabilization must be considered, since recognizable cAMP responsive element sequences are not found in the AT_1 promoter region (Guo and Inagami, unpublished data). This idea is supported by the finding that, for a number of other cellular genes, cAMP has effects on both transcription rate and mRNA stability (12,13).

AII-induced down-regulation was not dependent upon active G_i, since its inactivation with pertussis toxin did not affect the down-regulation (Fig. 4; lane1 and 3), an observation consistent with a previous report that G_i is not required for AII receptor desensitization or that ADP-ribosylation of G_i does not alter down-regulation. In mesangial cells, AII stimulates PI turnover by activating the pertussis toxin-insensitive G_q protein like in many cell types (14,15), but does not affect adenylate cyclase activity (16).

Taken together, while both AII and cAMP induce AT_1 receptor mRNA down-regulation, completely different effect of cycloheximide (abolition of AII-induced down-regulation but no effect on cAMP-induced desensitization) indicate that they control the level of AT_1 mRNA levels by distinctly different signalling pathways.

Acknowledgments: We thank Dr. T. Homma for providing us with rat mesangial cells, Du Pont-Merk for DuP 753 and Warner Lambert Co. for PD123319. This work was supported by the Research Grants DK 43883 and DK 39261 (KFB), and HL 14192 and HL 35323 (TI) from the National Institute of Health, and the Kidney Disease Center at Vanderbilt University.

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