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GLUCOCORTICOID INDUCTION OF RAT ANGIOTENSIN II TYPE 1A RECEPTOR GENE PROMOTER

Shuhsei Uno, Deng-Fu Guo*, Masahide Nakajima, Hideyuki Ohi, Teruaki Imada, Ryuji Hiramatsu, Hiroshi Nakakubo*, Norifumi Nakamura and Tadashi Inagami*#

Research Div., The Green Cross Corp., 2-25-1, Shodai-Ohtani, Hirakata, Osaka, Japan

*** Dept. of Biochem., Vanderbilt Univ., School of Medicine, Nashville, TN 37232**

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Summary. Promoter/luciferase reporter analysis indicated that the 5'-flanking region of rat angiotensin II type 1A receptor gene was functional, both in the rat aortic smooth muscle cells (RASMCs) and in Y1 cells derived from mouse adrenal cortex. In response to dexamethasone (Dex), transcriptional activity of promoter increased in transfected RASMCs, suggesting the functional cis-action of a glucocorticoid responsive elements (GRE). Furthermore, the expression of the rat AT_{1A} gene was also up-regulated by Dex at the levels of both mRNA and protein in RASMCs. © 1994 Academic Press, Inc.

Angiotensin II (Ang II) has multiple functions in the cardiovascular system, it causes vasoconstriction, stimulates aldosterone production and causes vascular smooth muscle hypertrophy and hyperplasia (1). Cellular responses induced by Ang II are mediated through its binding to the G protein-coupled, Ang II type 1 receptor (AT_1), in target tissues. Human (2, 3), rat (4, 5) and bovine (6) AT_1 have been cloned and sequenced. Recent studies on the genomic organization of the rat Ang II type 1A receptor (AT_{1A}) indicate that it consists of at least four exons and three introns. The 5'-flanking region of the rat AT_{1A} gene has been identified as a functional promoter in cultured rat vascular smooth muscle cells (RASMCs) (7, 8).

Glucocorticoid induces hypertension in animals and humans (9). It has been reported that glucocorticoid potentiated the vasoconstrictor responses to catecholamine (10), Ang II and vasopressin (11), suggesting that increased pressor responsiveness may be an important contributor to the rise in blood pressure. Promoter regions of the rat AT_{1A} gene contain putative glucocorticoid responsive elements (GREs). Furthermore, the presence of a

#To whom correspondence should be addressed.

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functional glucocorticoid receptor has been known in both RASMCs (12) and Y1 cells (13). We report herein that the 5'-flanking region (promoter) of the first exon is functional as a promoter in RASMCs and Y1 cells, and the promoter has a functional glucocorticoid-inducible enhancer element in RASMCs.

MATERIALS AND METHODS

Cell culture Rat aortic smooth muscle cells (RASMCs) were isolated from adult male Spraugue-Dawley rats and cultured in Dulbecco's modified Eagle's medium (DMEM) by the method of Gunther (14). Y1 cells, derived from a mouse adrenal cortical tumor, were obtained from the American Type Culture Collection and were grown in Ham F10 medium (Gibco Laboratories, Grand Island, NY), supplemented with 15% horse serum (Hyclone Laboratories, Logan, UT) and 2.5% fetal calf serum (Filtron, Inter Med., Tokyo Japan) at 37 °C under 5 % CO_2 and 95% air, in a humidified incubator.

Construction of AT_{1A} -luciferase expression vectors A 2.9 kb-long fragment in the 5'-flanking region from a *Not* I site, -2887 upstream of the 5'-end of exon 1, to immediately 5'-to exon 1 of the rat AT_{1A} gene was amplified and *Bgl* II sites were added to both ends by a polymerase chain reaction (PCR) technique. The amplified fragment was inserted into the *Bgl* II site of a PicaGene basic vector (Toyo Inki, Tokyo, Japan), and named pATLu(-2887/-1). The pATLu(-2887/-1) contained 2887 bp upstream from exon 1 of the rat AT_{1A} gene. The region from the *Not* I site to the *Xho* I site, 487 bp from the 5'-end of exon 1, was deleted from pATLu(-2887/-1) and this plasmid was named pATLu(-487/-1). An additional deletion up to a *Pvu* II site (-199) was constructed and named pATLu(-199/-1).

Cell transfection and luciferase assays Cells were seeded into 10-cm culture dishes at 1 x 10^6 cells and were cultured for 24 h. They were transfected with a luciferase fusion DNA plasmid (2 μg/dish) using DEAE-dextran. The luciferase assay was performed according to the instruction of the manufacture (PicaGene, Toyo Inki Tokyo, Japan) and the luciferase activity was measured on a luminometer (L-5300027, Luminoscan, Dainippon Seiyaku, Osaka, Japan).

Quantitation of the rat AT_{1A} mRNA levels in RASMCs The total cellular RNA was isolated from cultured RASMCs according to Chomczynski et al. (15). Northern blot analyses were carried out as described by Sambrook et al.(16). To distinguish the rat AT_{1A} mRNA and AT_{1B} mRNA, we used 3' untranslated regions as hybridization probes. The rat AT_{1A} specific probe that consists of the region spanning 11 bp to 699 bp downstream from the stop codon of the rat AT_{1A} gene was isolated from plasmid pI2E3 (5) by PCR. The region from 12 bp to 698 bp downstream from the stop codon of the rat AT_{1B} gene was amplified from plasmid pAT1BR (17) by PCR and used as an AT_{1B} specific probe. Quantitation was achieved by measuring the density of bands on exposed film by an image analyzer system (The microcomputer Imaging Device System, Imaging Research, Tokyo, Japan). Total RNA from cells with no stimulus was applied to each membrane and used of correction for RNA between each blotting.

Binding assay to RASMCs Cells in confluency in 24-well plates were maintained for 2 days without serum, they were then treated for another 16 h with Dex (1 μM), and the Ang II binding assay was performed as follows. Cells were washed twice with the assay medium (DMEM + 25 mM N-2-hydroxyethlpiperazine-N'-2-ethanosulfonic acid + 0.25 % bovine serum albumin) followed by incubation with an incubation medium [0.4 ml assay medium + 0.05 ml ^{125}I-Ang II (0.25 μCi, 2200 Ci/mmol, New England Nuclear) ± 0.05 ml 10^{-5} M saralasin (Peptide Institute)] for 45 min at room temperature. After the incubation, they were washed three times with ice cold phosphate buffered saline, followed by lysis with 0.5 ml of 0.5 N NaOH. Cell-bound ^{125}I-Ang II were counted on a gamma counter (1282 Compugamma; LKB). The specific binding of ^{125}I-Ang II was obtained by subtracting

nonspecific binding, which was determined in the presence of 1 μM saralasin. Protein concentration was determined using a protein assay reagent (Bio-Rad, SanDiego, CA). Results were expressed as cpm/mg protein.

RESULTS AND DISCUSSION

The promoter activity was examined by a luciferase assay. Various promoter regions were inserted upstream of the promoterless luciferase gene, and the fusion constructs were introduced into RASMCs and Y1 cells. The luciferase activity of the constructs in transient transfection is shown in Fig. 1. Plasmid pATLu(-2887/-1) containing a long promoter region conferred a high level of expression of luciferase activity on both RASMCs and Y1 cells. One of the possible mechanism of glucocorticoid-induced hypertension has been thought to be the enhancement of vascular sensitivity to Ang II (18). Nucleotide sequence analysis revealed three glucocorticoid responsive element sequences in the promoter region (-870 to -856 bp (GRE1), -770 to -756 bp (GRE2) and -407 to -378 bp (GRE3) from the 5'-end of exon 1) one of them overlaps an AP-1 recognition sequence (-387 to -381). To test whether these sequences are sufficient to induce a glucocorticoid response, RASMCs were transfected with the plasmids and incubated with 1μM dexamethasone (Dex) for 16 h, and then the luciferase activity was determined (Fig. 2). The pATLu(-2887/-1) showed a significant response to Dex. The pATLu(-2887/-1) induced a 30% increase by Dex treatment, plasmids lacking sequence between -2887 to -487 (pATLu(-487/-1) and pATLu(-199/-1)) did not response to Dex. These results suggest that GRE1 and/or GRE2, located in the promoter of the rat AT_{1A} gene, function positively in response to Dex in RASMCs. Similar results were obtained with Y1 cells transfected with these constructs. The effect of Dex on the rat AT_{1A} mRNA and protein levels were also investigated in RASMCs (Fig. 3).

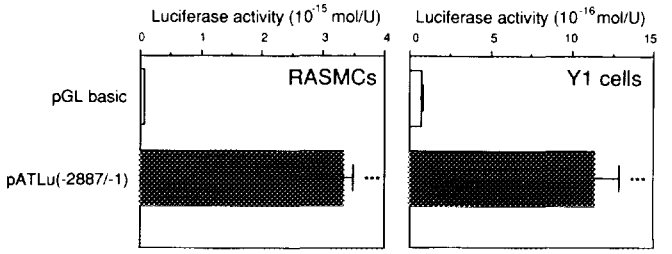

Figure 1. The luciferase activity of the luciferase-promoter fusion construct containing a long (-2887 to -1) region of the rat AT_{1A} promoter in RASMCs and in Y1 cells. The construct was cotransfected with the ß-galactosidase reporter gene (ß-galactosidase control vector) into both RASMCs and Y1 cells. Values are expressed as luciferase / ß-galactosidase ratio mean±SEM (n=4). ***$P < 0.001$ vs. the basic vector transfected.

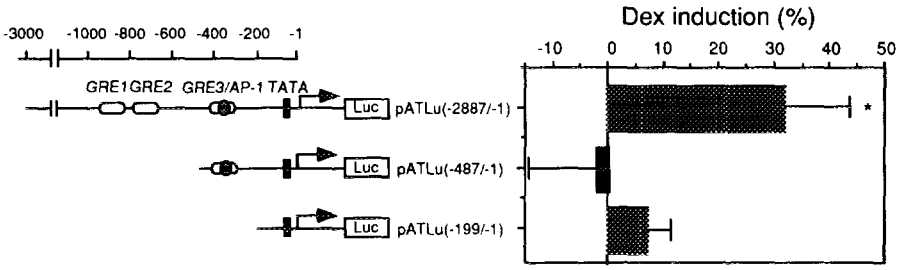

Figure 2. Effects of Dex on the luciferase activity of deletion mutants of the promoter of the rat AT_{1A} gene fused to the luciferase reporter gene in RASMCs. Constructs were cotransfected with the ß-galactosidase reporter gene (ß-galactosidase control vector) into RASMCs. The transfected cells were treated with Dex (1 μM) for 16 hr and harvested. Values demonstrate relative increases in the luciferase / ß-galactosidase ratio of each construct caused by Dex as calculated by comparing the ratios of the Dex-treated sample to untreated controls and are expressed as mean±SE (n=3). * $P < 0.05$ vs. Dex-untreated transfectants.

Northern hybridization of the rat AT_{1A} and AT_{1B} transcripts was performed using isoform specific probes. As shown in Fig. 3A, the rat AT_{1A} mRNA level was increased 2.5-fold in Dex treated cells. No transcript hybridizing with the rat AT_{1B}-specific probe was observed (data not shown). The Dex treatment of RASMCs for 16 hr induced a 30% increase in ^{125}I-Ang II binding (Fig. 3B), suggesting that both GRE1 and/or GRE2 may be involved in the regulation of mRNA and the receptor number.

In Cushing's syndrome, glucocorticoids increase the circulating level of Ang II due to an increase in the hepatic production of angiotensinogen (9). Since the AT_{1A} subtype is the

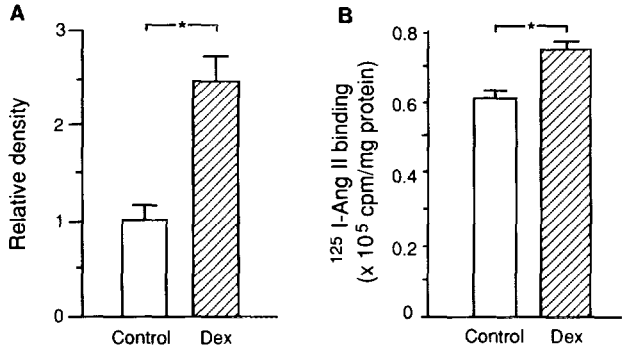

Figure 3. Changes of the rat AT_{1A} in (*A*) mRNA and (*B*) protein levels in RASMCs as results of the Dex treatment. (*A*) Total RNAs (10 μg) from control cells and Dex (1 μM)-treated cells (16 h) were subjected to Northern blot analysis. After hybridization, filters were stained using methylene blue as described by Sambrook et al.(17), and 28 S and 18 S were observed equally in each lane. Results are expressed as relative densitometric ratio vs. Dex-untreated cells (n=3). * $P < 0.05$. (*B*) ^{125}I Ang II binding assays were performed for control cells and Dex (1 μM)-treated cells (16 h). Results are expressed as cpm / mg protein mean±SEM (n=3). * $P < 0.05$.

major form of the Ang II receptor in rat vascular smooth muscle cells (19), we may predict that in the pathogenesis of glucocorticoid-induced hypertension; 1) glucocorticoid itself increases vascular sensitivity to Ang II by enhancing the promoter activity of the vascular AT_{1A} gene and increasing the receptor number; 2) an elevated concentration of circulating Ang II, due to elevated angiotensinogen, enhances steroidogenesis in the adrenal glands. In glucocorticoid-induced hypertension, pressor response to norepinephrine is also enhanced (20). An increased pressor response to both norepinephrine and Ang II has been reported in patients with Cushing's syndrome (21). Although the relative significance of the two mechanisms mentioned above, or other mechanisms in glucocorticoid-induced hypertension await further studies and clarification, the present studies demonstrate a positive contribution of glucocorticoids to AT_{1A} expression in vascular smooth muscle cells and adrenal cortical cells by enhancing the promoter function of the receptor gene.

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