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Steroid Hormones Upregulate Rat Angiotensin II Type 1A Receptor Gene: Role of Glucocorticoid Responsive Elements in Rat Angiotensin II Type 1A Promoter

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The transcription of the rat angiotensin II type 1A receptor gene is stimulated by glucocorticoids. To clarify the molecular mechanism for glucocorticoid action in rat vascular smooth muscle cells, we investigated the effects of dexamethasone on the promoter activity of the angiotensin II type 1A receptor by using promoter/luciferase reporter gene constructs and heterologous context constructs (containing the thymidine kinase promoter) in transfected vascular smooth muscle cells. There are three putative glucocorticoid responsive elements in the promoter. However, only one glucocorticoid responsive element was found to respond to dexamethasone (1 μM). The region was located at positions, −756 to −770 bp upstream of the transcription initiation site. A glucocorticoid antagonist, RU38486, completely blocked the induction by dexamethasone, suggesting that the glucocorticoid responsive element was functional through a specific glucocorticoid receptor. Compared with the angiotension II type 1A receptor promoter, no effect by dexamethasone was observed in vascular smooth muscle cells transfected with the angiotensin II type 1B receptor promoter/luciferase reporter gene constructs. We concluded that the dexamethasone-induced increase in the transcription of the angiotensin II type 1A receptor gene occurred through the binding to GRE up the glucocorticoid-specific receptor.

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INTRODUCTION

Angiotensin II (Ang II) has multiple functions in the cardiovascular system [1]. It includes vasoconstriction, stimulation of aldosterone production, vascular smooth muscle hypertrophy and hyperplasia and facilitation of adrenergic release [2]. At least two major isoforms of Ang II receptor, AT_1 and AT_2, have been identified by non-peptidic isoform-specific antagonists [3] and molecular cloning [4–8]. Among these, the AT_1 receptor mediates many of the classic functions assigned to Ang II to date, whereas the functions of the AT_2 receptor have yet to be established.

Glucocorticoid induces hypertension in animals and human. It has been reported that glucocorticoid potentiated the vasoconstrictor responses to Ang II [9], suggesting that the increased pressor responsiveness may be an important contributor to the rise in blood pressure. In rat vascular smooth muscle cells (VSMC), in which the AT_{1A} is the major Ang II receptor, the transcription of this gene is induced by glucocorticoid hormones [10–13] and aldosterone [14, 15]. However, the mechanism of glucocorticoid-induced increase in the mRNA level of AT_{1A} gene has not been determined. In order to clarify the molecular basis for response to glucocorticoids of VSMC, we generated a series of the promoter/luciferase reporter gene and heterologous context constructs, and used these to identify a functional regulatory site in the rat AT_{1A} receptor promoter.

EXPERIMENTAL

Materials

Dexamethasone and bovine serum albumin (BSA) were obtained from Sigma Chemical Co. (St Louis, MO, U.S.A.), Dulbecco's modified Eagle medium (DMEM), phosphate-buffered saline (PBS) and fetal

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calf serum (FCS) were from GIBCO/BRL (Gaithersburg, MD, U.S.A.). RU38486, a glucocorticoid antagonist, was a gift from Roussel-UCLAF (Romainville, France).

Cell culture

VSMC were isolated from adult male of spontaneously hypertensive rats (SHR) purchased from Charles River Laboratories, MA by the method of Gunther *et al.* [16]. The medium was changed every 3 or 4 days. Cells below 6th passages were used.

Plasmid constructs

A 980 bp EcoRI–BstXI fragment in the 5′-flanking region of the rat AT_{1A} receptor gene was blunted and inserted into the pBluescript KS (+) vector. It was then cleaved by SacI–XhoI digestion, and inserted into the same sites of the pGL2 basic vector (Promega, Madison, WI). This plasmid was named pLuc1. From plasmid pLuc 1, two additional constructs were generated by using NsiI and XhoI restriction enzymes, respectively. Resultant constructs with 799 and 560 bp sequences upstream from the transcription initiation site of the AT_{1A} promoter were named pLuc2 and pLuc3.

For heterologous context experiments, two fragments digested with either EcoRI–NsiI (123 bp) or NsiI–XhoI (230 bp) were blunted and inserted into the EcoRV site of the pBluescript KS (+) vector, then the SacI–XhoI fragments were recloned into the same restriction enzyme sites of pTKLuc basic vector (which contains a segment of the herpes simplex virus thymidine kinase promoter between −105 to +51 bp), and named pTKLucFl and pTKLucF2, respectively. Four 25-mers (sense and antisense) of oligonucleotides (corresponding to −751 to −775 bp and −851 to −875 bp) prepared in an Applied Biosystems 380A DNA synthesizer (Foster City, CA), were gel-purified. The annealed double-strand oligomers were inserted into the pBluescript KS (+) vector, then cleaved by SacI–XhoI digestion, and inserted into the pTKLuc basic vector and named pTKLucG1 and pTKlucG2, respectively.

To test the effect of dexamethasone on the rat AT_{1B} promoter, three AT_{1B} promoter/luciferase reporter gene constructs were generated. A 1514 bp EcoRI–BstXI fragment in the 5′-flanking region of the rat AT_{1B} receptor gene was blunted and inserted into the Bluescripte KS (+) vector. It was cleaved by KpnI–ScaI digestion, and inserted into the same sites of the pGL2 basic vector. This plasmid was named pLucB1. From plasmid pLucB1, two additional constructs were generated by using AccI and NheI restriction enzymes, respectively. Resultant constructs with 700 and 381 bp sequence upstream from the transcription initiation site of the rat AT_{1B} promoter. They were named pLucB2 and pLucB3.

Transient transfections and luciferase assays

VSMC were seeded at a density of 5×10^6 cells/60 mm dish, and grown in DMEM containing 10% FCS overnight. Transient transfections were performed by using the DEAE-dextran method as recommended by the manufacturer (Promega, Madison, WI). For luciferase assays, cells were cotransfected with 6 μg of plasmid DNA and 2 μg of a pSV-β-galactosidase vector (Promega, Madison, WI) to allow for normalization for transfection efficiency. After the transfection, cells were grown in DMEM containing 10% FCS for 2 days, washed, then exposed to DMEM containing 0.2% BSA with or without dexamethasone (1 μM) for 16 h. The luciferase activity was measured in a luminometer (Model of Optocomp I, MGM Instruments, Hamden, CT). The β-galactosidase activity was determined by absorbance at 405 nm in a spectrophotometer.

To test aldosterone affects the AT_{1A} promoter function, VSMC transfected with 6 μg of pTKLucG2 and 2 μg of pSV-β-galactosidase vector were treated with 100 nM of aldosterone for 16 h.

RESULTS

Three putative GREs were found by homology search [Fig. 1(A)]. The first two elements were located at positions −856 to −870 bp and −756 to −770 bp upstream of the transcription initiation site of the rat AT_{1A} receptor gene [17]. The third putative GRE overlaps with a putative AP-1 recognition sequence, and is located at positions −393 to −407 bp upstream of the transcription initiation site. To clarify whether these three putative glucocorticoid responsive elements are functional, we generated three deletion mutants of the AT_{1A} promoter/luciferase constructs. To test the effect of dexamethasone on the transcription of the rat AT_{1A} receptor gene, rat VSMC transfected with each of three constructs were incubated with dexamethasone (1 μM) for 16 h, then the luciferase activity was determined. As shown in Fig. 1(B), pLuc1 and pLuc2 showed significant induction by dexamethasone. As discussed below, it was noted that pLuc2 produced a greater induction than pLuc1. pLuc3 did not show a recognizable response to dexamethasone. Experiments with shorter constructs further confirmed the absence of response to dexamethasone of GRE3. Thus, neither a 489 bp promoter/luciferase (containing GRE3) nor a 331 bp construct without any GRE responded to dexamethasone. These results suggest that either GRE1 or GRE2 or both are functionally active but GRE3 is not. To identify the active GRE(s) heterologous context constructs were generated.

As shown in Fig. 2(B), fragments containing GREl or GRE2 were constructed into a heterologous context construct. VSMC transfected with pTKLucF1 or pTKLucF2 were stimulated by dexamethasone (1 μM)

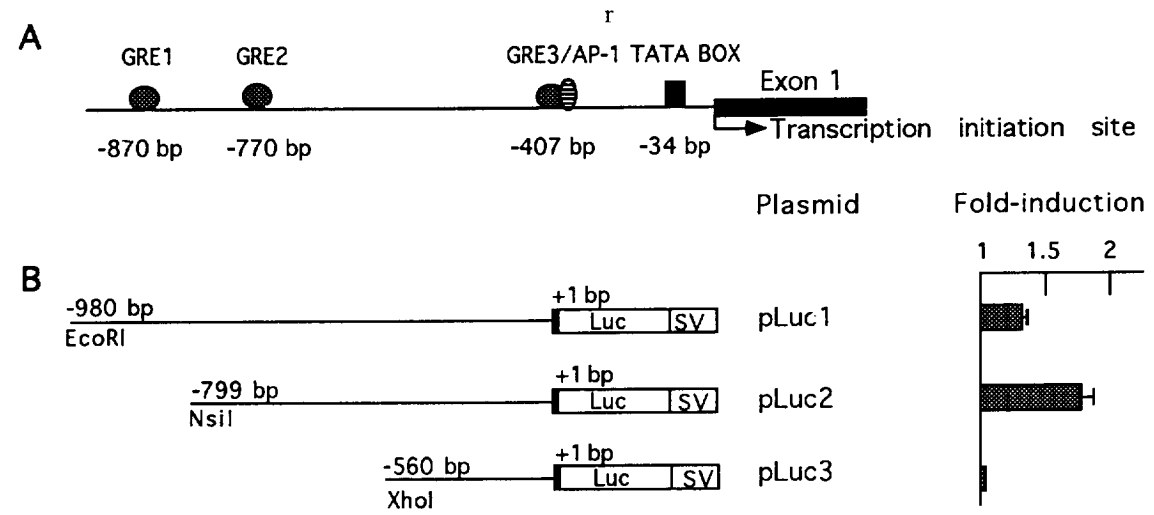

Fig. 1. Chimeric constructs generated from 5′-deleted mutants of the rat AT_{1A} receptor promoter and the luciferase reporter gene and their promoter activity in VSMC. The glucocorticoid responsive elements (GRE) in the rat AT_{1A} promoter are illustrated in (A). The chimeric plasmid (pLucl) consists of the rat AT_{1A} promoter region between −980 bp to +1 bp relative to the transcription initiation site [17], the luciferase reporter gene, and the SV40 splice and polyadenylation signals (SV40). The structure of the constructs and the fold-induction of luciferase activity by dexamethasone are shown in (B). Data are presented as fold-induction (mean ± SEM) of 4 independent transfections.

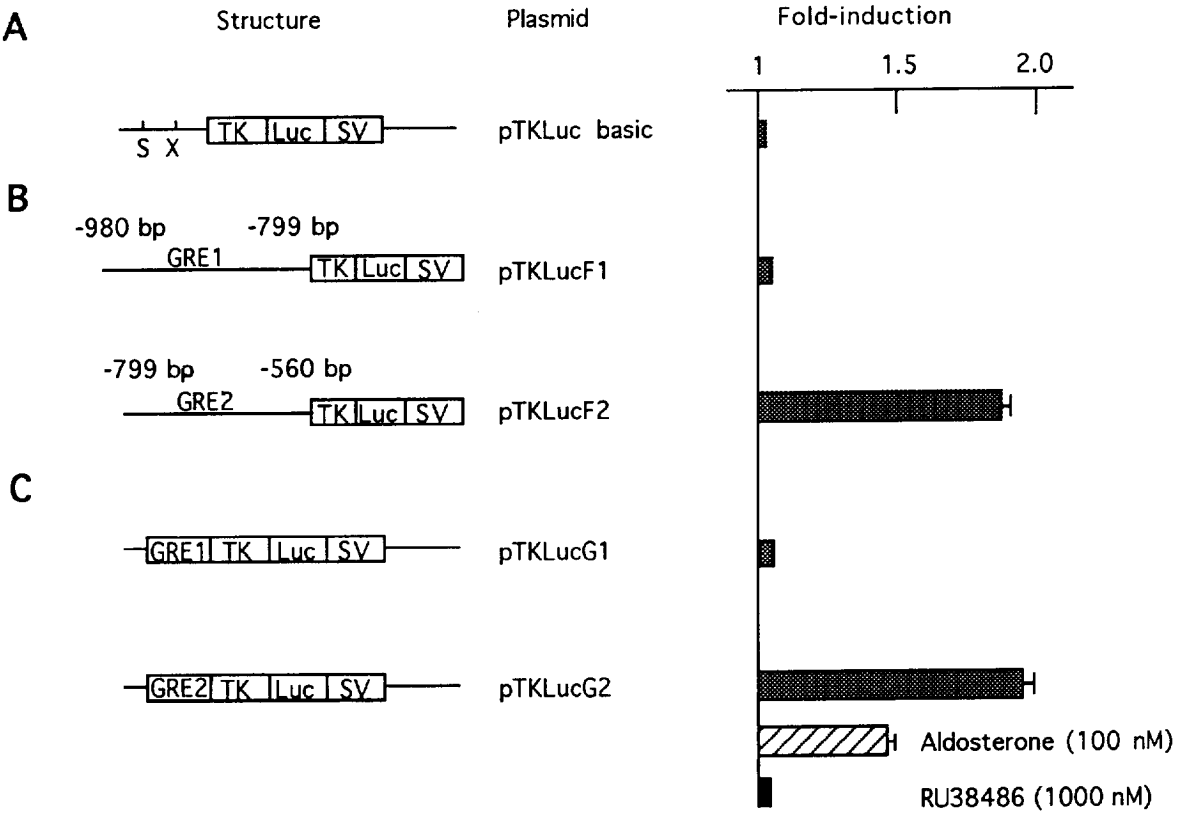

Fig. 2. Function analysis of GREl and GRE2 elements in heterologous context constructs. Tested vectors are illustrated in (A). The pTKLuc basic plasmid consists of, a polylinker that includes SacI (S) and XhoI (X) restriction sites, the herpes simplex virus thymidine kinase promoter, the luciferase reporter gene, and the SV40 splice and polyadenylation signals (SV40). Two fragments which were digested with EcoRI–NsiI and NsiI–XhoI were blunted and inserted into the EcoRV site of pBluescript KS (+), then inserted into the pTKLuc basic vector (B). Two oligomers that correspond to GRE1 and GRE2 were inserted into the EcoRV site of the pBluescript vector, then resubcloned into the pTKLuc vector, individually (C). The data for experiments with dexamethasone, aldosterone and RU38486 and dexamethasone are presented as fold-induction (mean ± SEM) of 4, 3 and 3 independent transfections, respectively.

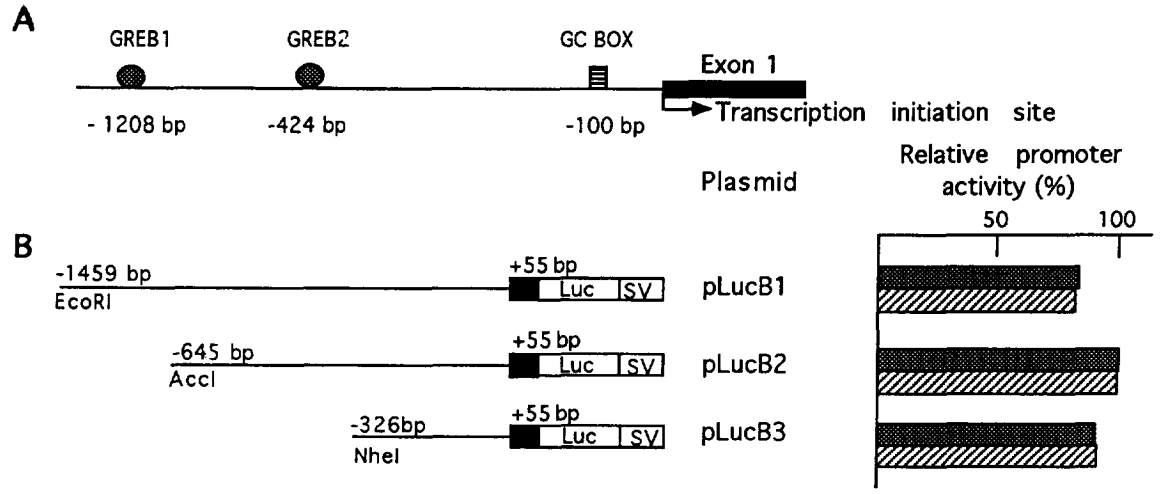

Fig. 3. Analysis of rat AT_{1B} receptor promoter/luciferase reporter gene constructs in transfected VSMC. The glucocorticoid responsive elements (GRE) in the rat AT_{1B} promoter are illustrated in (A). The chimeric plasmid (pLucB 1) consists of the rat AT_{1B} promoter region between −1459 bp to +55 bp relative to the transcription initiation site [18], the luciferase reporter gene, and the SV40 splice and polyadenylation signals (SV40). The structure of the constructs and their relative luciferase activity are shown in (B). The insertion of a 1514 bp fragment into the KpnI–SacI sites of the pLuc basic plasmid created pLucB1. Values are expressed as percentages of activity obtained with the construct pLucB2 (100%), which gave the highest activity, and presented as means of three experiments. ▨, with dexamethasone treatment; ■, without dexamethasone treatment.

for 16 h, and the luciferase activity was measured. The induction by dexamethasone was found only with pTKLucF2, but not with pTKLucFl. These data suggest that GRE2 may be functional in VSMC. To further ascertain this postulate, two 25-mers of double-strand oligomers were inserted into the heterologous pTKLuc basic vector. VSMC transfected with pTKLucGl or pTKLucG2 were incubated with dexamethasone (1 μM) for 16 h, then the luciferase activity was determined as above. Induction by dexamethasone was seen only with pTKLucG2, but not with pTKLucG1 [Fig. 2(C)]. No induction was observed with the pTKLuc basic vector alone. Together, both longer and shorter constructs containing GRE2 responded to dexamethasone, but those containing GRE1 did not. Thus, it is safe to conclude that only the second glucocorticoid responsive element of rat AT_{1A} receptor gene is functional.

Aldosterone can stimulate a gene expression with a GRE sequence. To test if aldosterone affects the AT_{1A} promoter function, VSMC transfected with pTKLucG2 were treated with 100 nM aldosterone for 16 h, and the luciferase activity was measured. Aldosterone was found to increase promoter activity 1.5-fold [Fig. 2(C)]. Evidence that effects of dexamethasone on the AT_{1A} gene expression are mediated by the specific glucocorticoid receptor, was obtained by the inhibition of the effects by RU38486, a specific receptor anatagonist. Cells transfected with pTKLucG2 were incubated with 1 μM of RU38486 and dexamethasone for 16 h, the induction of luciferase activity by dexamethasone was completely inhibited by RU38486 [Fig. 2(C)].

As shown in Fig. 3(A), two putative GREs were found in the rat AT_{1B} promoter by homology search. They were located at positions; −424 to −438 bp and −1208 to −1222 bp upstream from the transcription initiation site of the rat AT_{1B} receptor gene, respectively [18]. Three deletion mutants of the AT_{1B} promoter/luciferase reporter gene construct were generated. VSMC transfected with pLucB2 gave the highest luciferase activity; therefore, its luciferase level was used as a reference (set as 100%) in each series of experiments. The promoter/luciferase constructs and relative luciferase activity of each construct are shown in Fig. 3(B). Compared with the highest luciferase expression in pLucB2, pLucB1 and pLucB3 gave 84 and 90% of pLucB2, respectively. The sequence of the first construct, pLucB1, contains both GREs, and pLucB2 contains only the second GRE, but pLucB3 does not have a GRE. No significant induction by dexamethasone (1 μM) for 16 h was observed in VSMC transfected with each of pLucBl to pLucB3 constructs [Fig. 3(B)].

DISCUSSION

In this study, we identified a functionally active *cis*-acting GRE in the promoter region of the rat AT_{1A} receptor gene, and investigated the effect of glucocorticoid and aldosterone on AT_{1A} receptor gene expression in VSMC. Dexamethasone induced the gene expression of the AT_{1A} receptor through the glucocorticoid-specific receptor binding to GRE in the AT_{1A} promoter in VSMC. Adrenal steroids as well as Ang II

itself are well known stimulators of hepatic angiotensinogen production [19]. Furthermore, it has been reported that Ang II exerts a positive feedback effect on the adrenal AT_1 receptor [20]. These positive interactions between Ang II and adrenal steroids could have potentially deleterious effects on the maintenance of homeostasis of blood pressure and electrolytes. Recent cloning of Ang II receptor and sequence analysis of the promoter sequences of AT_1 receptor subtypes showed the presence of several potential glucocorticoid responsive elements. Measurements of AT_1 mRNA in cultured cells confirmed a positive effect of dexamethasone on AT_1 expression in cardiomyocytes and VSMC [10, 12]. However, it is not clear which of the potential glucocorticoid responsive elements are functionally active. In view of an interesting overlap of a potential GRE and an AP-1 site, it is of great importance for us to delineate the exact mechanism of the positive effect of adrenal steroids on AT_1 regulation. The intriguing upregulation of AT_1 by Ang II [20] in the adrenal is in contrast to down regulation observed in other tissues. The clarification of such a mechanism also requires understanding of the precise mechanism of the regulation of receptor expression by adrenal steroid.

Nucleotide sequence analyses suggested the presence of three putative GRE-like sequences, GRE1, GRE2 and GRE3 in the rat AT_{1A} promoter. The present studies produced evidence that only GRE2 is functionally active, responding positively to dexamethasone. This conclusion is supported by three layers of experimental evidence. Deletion mutants of the promoter/luciferase constructs produced results indicating either GRE1 or GRE2 or both are responsive to dexamethasone whereas GRE3 is inactive. Heterologous context constructs showed that GRE1 was not active, but GRE2 was clearly responsive to dexamethasone. As shown in Fig. 3, no significant induction by dexamethasone was observed in VSMC transfected with each of three rat AT_{1B} promoter/luciferase reporter gene constructs. Taken togther, these are in accord with the finding that dexamethasone did not affect the transcription level of AT_{1B} receptor in cardiomyocytes and cardiac fibroblasts [12].

Glucocorticoids increase the circulating level of Ang II due to an increase in the hapatic production of angiotensinogen in Cushing's syndrome's patients. The mechanisms of glucocorticoid-induced hypertension may be predicted; (1) glucocorticoid itself increases vascular sensitivity to Ang II by enhancing the promoter activity of the vascular AT_{1A} receptor gene; and (2) an elevated concentration of Ang II derived from elevated angiotensinogen enhances steroidogenesis in the adreal glands. Although the exact mechanism of glucocorticoid-induced hypertension await further studies, the present study demonstrated that a functionally active GRE is present in the AT_{1A} promoter. It is involved in the glucocorticoid and aldosterone induced expression of the AT_{1A} receptor gene.

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