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GENE TRANSCRIPTION OF ANGIOTENSIN II TYPE 2 RECEPTOR IS REPRESSED BY GROWTH FACTORS AND GLUCOCORTICOIDS IN PC12 CELLS

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Summary: The rat angiotensin II type 2 receptor (AT2-R) expression was markedly downregulated by the mitogenic action of serum, growth factors and dexamethasone. The regulation by serum or growth factors did not affect the AT2-R mRNA half-life (18 h), whereas the AT2-R half-lives of dexamethasone-treated cells and proliferating cells decreased to 10 h and 15 h, respectively. Nuclear run-off assays indicated the mechanism of repression of AT2-R expression by serum, growth factors and dexamethasone or in proliferating cells to be, in large part, transcriptional. These findings indicate that transcription of the AT2-R gene is regulated in a growth state-dependent manner and suggest that this regulation provides a means by which cells can modulate their responsiveness to the actions of angiotensin II mediated through AT2-R.

Angiotensin II (Ang II) plays an important role in cardiovascular regulation, fluid volume homeostasis, electrolyte balance, neuroendocrine regulation, and cellular growth (1). Two types of Ang II receptors were identified based on their pharmacological and biochemical properties, and designated as type 1 receptor (AT1-R) and type 2 receptor (AT2-R) (2,3). To date, most if not all of the known effects of Ang II in adult tissues are attributable to AT1-R (4). Very recently, several laboratories have cloned rat (5,6), mouse (7-10) and human (11-14) cDNAs and genes encoding the AT2-R. Although the AT2-R has been suggested to activate potassium currents (15) and inhibit T-type calcium current (16), the exact signaling pathways and physiological roles of AT2-R remain to be determined.

The AT2-R is abundantly and widely expressed in fetal tissues (17,18), and its expression is activated in skin wound (19), the neointima following vascular injury (20), and cardiac remodeling (21,22). These patterns of expression of the AT2-R suggest an important role in growth and development. In vitro binding studies including ours (23) have shown that the PC12 (23) and PC12W (24,25) cells or R3T3 (26,27) fibroblasts abundantly express the AT2-R, and that growth factors and serum downregulate the AT2-R number. Tanaka et al. have reported that the AT2-R is

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involved in apoptosis in ovarian granulosa cells (28). However, these findings are examined on the protein level using the binding assay and the underlying molecular mechanisms are not studied yet. Although glucocorticoids also have a mitogenic action on fibroblasts (29) and ovarian granulosa cells (30) and the glucocorticoids-responsive element (GRE) is present in the human AT2-R gene (14), the effects of glucocorticoids on the AT2-R gene expression remain to be determined. Here, we report using the PC12 cells that the AT2-R gene expression is regulated in a cell-density dependent manner and repressed by growth factors, serum and glucocorticoids, and that these regulations are mediated by in large part inhibiting the gene transcription rather than the mRNA stability.

METHODS AND MATERIALS

Cell culture PC12 cells were generously provided by Dr. Eva J Neer (Harvard Medical School, Boston, MA). PC12 cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco-BRL, Gaithersburg, MD) containing 10 % (vol/vol) fetal calf serum (Gibco-BRL), penicillin (100 units/ml) and streptomycin (100 μ g/ml) using the plates precoated with poly-L-lysine (1 %, Sigma, St. Louis, MO) as previously described (23). Cells were fed every 3 days, passed weekly, and maintained at 37°C in a humidified atmosphere of 5 % CO^2/95 % air.

[^{125}I] AngII binding assay and Northern blotting The receptor binding assays were performed using the membrane fractions as previously described (23). The Bmax and Kd values were determined by Scatchard analyses (23,31). The cDNA fragment encoding the whole part of the coding region was obtained by reverse transcriptase (RT) and polymerase chain reaction (PCR) using the total RNA from PC12 cells as previously reported (22), and was used as a probe. The used filters were boiled and rehybridized to a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe (23,31). The obtained mRNA signals were counted by a densitometer.

Transcript stability analysis and nuclear run-off assay The confluent PC12 cells were exposed to the serum-depleted medium and subsequently incubated with NGF (50 ng/ml), EGF (100 ng/ml) or dexamethasone (1 μ mol/L) in the presence of actinomycin D (5 μ g/ml). After various times of incubation, total RNA was isolated from individual dishes and the disappearance of mRNA abundance was determined by the Northern blotting (23,31). The preparation of nuclei and run-off assays were performed as described previously (23,31). The confluent PC12 cells were treated with NGF (50 ng/ml), EGF (100 ng/ml) or dexamethasone (1 μ mol/L) for 48 h in the presence of serum-depleted medium. Nuclei were incubated for 20 min at 30° C in the presence of [α -^{32}P]UTP followed by digestion with RNase-free DNase I and proteinase K. The radiolabeled RNA (4 $\times 10^6$ cpm) was hybridized at 42° C for 48 h with linealized pGEM vector containing rat AT2-R cDNA (15 μ g) or GAPDH cDNA (5 μ g) fragments. After washing the membrane the bound radioactivity was determined by scintillation counting.

Reagents and Statistical Methods All reagents were purchased from Sigma Chemical Co., St Louis, MO, unless otherwise indicated below. The results are expressed as mean ± SE. Analysis of variance and the Fisher's PLSD test were used for multigroup comparisons. Differences with $p< 0.05$ were considered significant.

RESULTS AND DISCUSSION

AT2-R mRNA level is upregulated in confluent PC12 cells and by serum-deprivation

We initially examined the AT2-R expression in the proliferating (~50 % confluent) or 100 % confluent cells cultured with serum-supplemented medium. Northern blot analyses indicated that

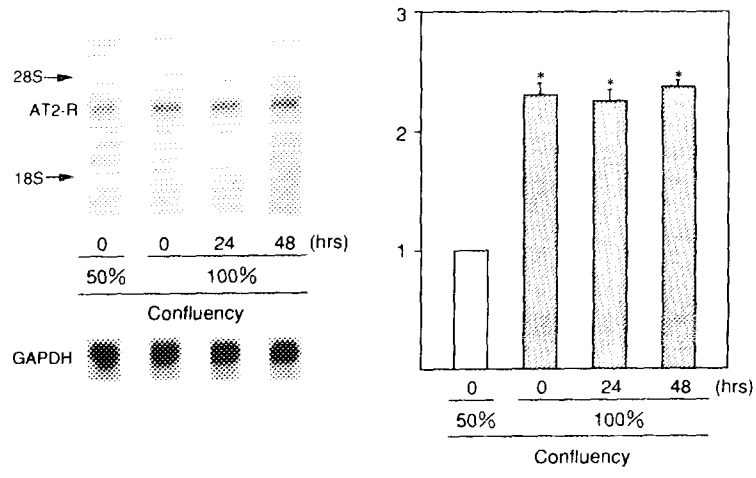

Figure 1. Northern blot analyses of AT2-R mRNA levels in proliferating (50 % confluent) and 100 % confluent PC12 cells cultured with serum-supplemented medium. The 100 % confluent cells were maintained in serum-supplemented medium for subsequent 24 and 48 hrs and the changes of AT2-R mRNA levels were examined. AT2-R mRNA levels are expressed relative to GAPDH mRNA levels, and the relative values in proliferating cells were normalized to "1" for easy comparison. The results are shown as the means ± SE of five separate experiments. *p<0.01 vs proliferating cells.

PC12 cells express a single size (~3.5 Kb) of AT2-R mRNA as previously reported in PC12W cells (11), and that the accumulation of AT2-R mRNA in the confluent cells increased 2.3-fold as compared with that in the proliferating cells (Fig. 1). Similar mRNA levels of the AT2-R were sustained even after the confluent cells were cultured for subsequent 48 h in the same medium, suggesting that the AT2-R mRNA level is maintained in the static state level once the cells proliferate to the confluent phase.

We next examined the effect of serum-deprivation on the AT2-R expression in the confluent PC12 cells. Serum-deprivation for 24 h caused a marked increase (2.6-fold) in the AT2-R mRNA level as compared with that in serum-supplemented cells, and a similar increase continued for 48 h incubation (Fig. 2).

Growth factors and glucocorticoid downregulate AT2-R mRNA level

We examined the effects of growth factors and dexamethasone on the AT2-R expression in the confluent PC12 cells in a serum-depleted condition. As shown in Fig. 2, the exposure to NGF, EGF or dexamethasone for 48 h markedly reduced the increase in the AT2-R mRNA level induced by serum deprivation; the decreased levels were similar to that in the serum-supplemented confluent cells. These effects were significant 24 h after exposure to the compounds and maximal in 48 h incubation (NGF 70 %, EGF 71 %, dexamethasone 74 % inhibition vs serum-depleted control). Similar inhibitions were observed when the serum-supplemented confluent cells were treated with growth factors and dexamethasone (data not shown). To examine whether these inductions require new protein synthesis, the confluent cells were pretreated with cycloheximide for 4 h in serum-depleted medium and then NGF, EGF or dexamethasone were added. The coincubation of

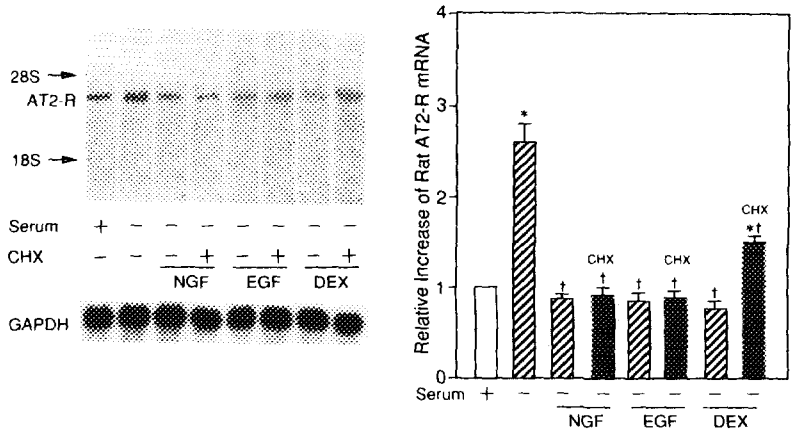

Figure 2. Northern blot analyses of growth factors and dexamethasone-induced changes of rat AT2-R mRNA levels in PC12 cells. The confluent PC12 cells were incubated with NGF (50 ng/ml), EGF (100 ng/ml) or dexamethasone (1 μ mol / L) for 48 h in serum-depleted medium. The confluent cells were pretreated with cycloheximide (5 μ g/ml) for 4 h in serum-depleted medium and then exposed to NGF, EGF or dexamethasone. AT2-R mRNA levels are expressed relative to GAPDH mRNA levels, and the relative values in serum-supplemented cells were normalized to "1" for easy comparison. The results are shown as the means ± SE of five separate experiments. *p<0.01 vs serum-supplemented cells. †p<0.01 vs values in serum-depleted cells. DEX: dexamethasone, NGF: nerve growth factor, EGF: epidermal growth factor, CHX: cycloheximide.

cycloheximide with NGF or EGF did not influence the effects of the growth factors, whereas the effect of dexamethasone was partially blocked (Fig. 2).

Effects of growth factors and glucocorticoid on AT2-R mRNA stability, gene transcription and protein levels

The stability of the AT2-R mRNA was examined by inhibiting new mRNA transcription with actinomycin D. A half-life of 18.4 ± 0.2 h for the AT2-R mRNA was obtained from serum-depleted confluent cells. The treatments with NGF and EGF did not affect the half-lives (NGF: 17.9 ±0.2 h, EGF: 18.1 ±0.2 h), whereas the half-life in dexamethasone-treated cells decreased to 9.7 ± 0.1 h (~47 %, $p<0.01$). We also examined the change for the half-life between proliferating (50 % confluent) and confluent cells in the presence of serum-supplemented medium (Fig. 3). A half-life of 18.8 ± 0.2 h for AT2-R mRNA was obtained from serum-supplemented confluent cells, which did not significantly differ from that in serum-depleted confluent cells, whereas a half-life of AT2-R mRNA in proliferating cells decreased to 15.2 ± 0.1 h (~19 %, $p<0.05$).

We next examined the effects of growth factors and dexamethasone on the transcriptional level using a nuclear run-off assay as previously established (22,31). As shown in Fig. 4, the relative rate of the AT2-R gene transcription in serum-supplemented proliferating cells was assigned to "1" for easy comparison. Comparison between confluent and proliferating cells indicated that the transcriptional rate of the AT2-R gene in the confluent cells is significantly higher (1.6-fold) than that in the proliferating cells. The rate of AT2-R gene transcription in serum-depleted confluent cells was increased 2.1-fold compared with that in serum-supplemented confluent cells, whereas the treatments

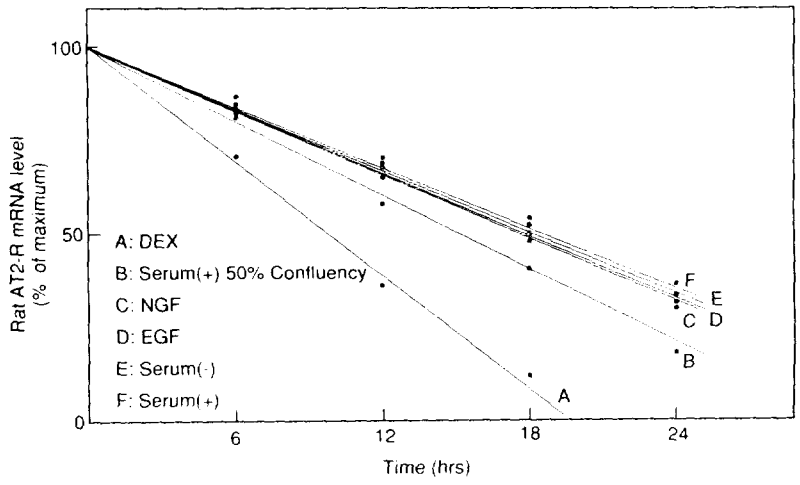

Figure 3. Effects of growth factors and dexamethasone on stability of AT2-R mRNA. Stability of AT2-R mRNA was estimated by inhibiting gene transcription with actinomycin D (5 μ g/ml). The decay of AT2-R mRNA abundance at 6, 12, 18, 24 h after exposure to actinomycin D was detected by the Northern blotting. The confluent PC12 cells were exposed to serum-depleted medium and subsequently incubated with NGF (50 ng/ml), EGF (100 ng/ml) or dexamethasone (1 μ mol / L) in the presence of actinomycin D. The data shown are the means of five separate experiments. Abbreviations are same as in Figure 2.

with NGF, EGF and dexamethasone inhibited this increase significantly; NGF and EGF reduced the transcriptional rates to the control levels of serum-supplemented proliferating cells but those in dexamethasone-treated cells were relatively higher than the control levels.

We have previously shown that the serum-supplemented confluent PC12 cells exclusively express the AT2-R with a single binding site and a high binding affinity (Bmax = 168±3 fmol/mg protein, Kd = 0.52±0.02 nM) (23). The receptor densities (81±2 fmol/mg) in the proliferating cells were lower (52 %) than those in the confluent cells and serum-deprivation of the confluent cells upregulated the densities by 2.6 fold (437 ±11 fmol/mg) , whereas the exposure to growth factors or dexamethasone markedly reduced the AT2-R densities to the levels in the proliferating cells (NGF: 108±4, EGF: 97±5, dexamethasone: 94±3 fmol/mg). The receptor affinities were not significantly changed by growth factors and dexamethasone, and were similar between the proliferating and confluent cells (data not shown)

The present findings showed that both NGF and EGF induce a decrease in the AT2-R gene transcriptional rate without affecting its mRNA stability. The state of growth arrest, induced by serum deprivation, was also associated with increased expression of the AT2-R. This regulation was induced at the gene transcriptional level. Similar regulations by serum deprivation were observed in the receptors for platelet derived growth factor (PDGF)-β (35) or insulin (36) in BALB/c-3T3 cells. PDGF stimulation of BALB/c-3T3 cells causes a decrease in EGF receptor expression (37). NGF treatment downregulates EGF receptors in PC12 cells (38). Transforming growth factor-β downregulates expression of the PDGF-α receptor in Swiss 3T3 cells (39). Glucocorticoid also causes the mitogenic action on the fibroblasts (29) and ovarian granulosa cells (30). Tanaka et al.

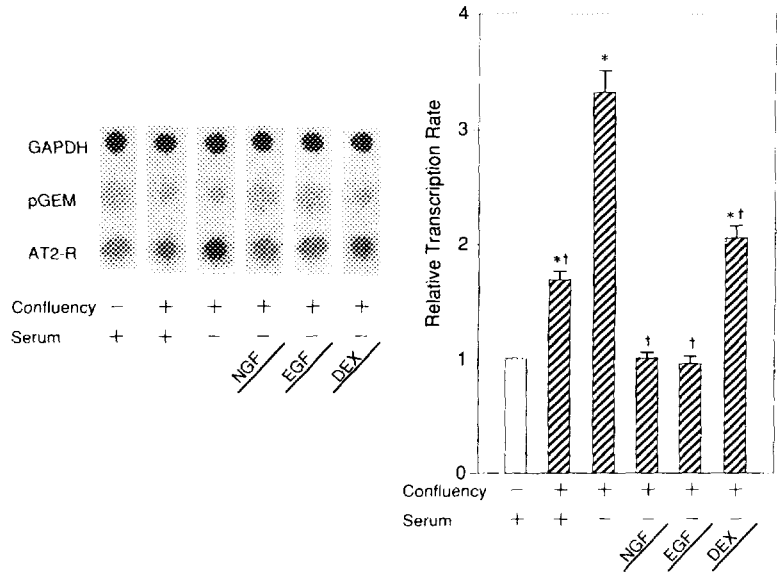

Figure 4. Nuclear run-off assays of growth factors and dexamethasone-induced changes in transcriptional rate of the rat AT2-R gene. The confluent PC12 cells were treated with NGF (50 ng/ml), EGF (100 ng/ml) or dexamethasone (1 μ mol / L) for 48 h in the presence of serum-depleted medium, and the nuclei were isolated. Confluency (-) indicates the cells in proliferating (50 % confluent) phase. The radiolabeled RNA was hybridized with linealized pGEM vector alone (15 μ g, negative control), pGEM containing rat AT2-R cDNA (15 μ g) or GAPDH (5 μ g), and the bound radioactivities were determined by scintillation counting. AT2-R transcriptional rates are expressed relative to those of GAPDH gene after their background levels (pGEM vector alone) were reduced, and the relative values in serum-supplemented proliferating cells are normalized to "1". The results shown are the means ± SE of three separate experiments. *$p<0.01$ vs values in serum- supplemented proliferating cells. †$p<0.01$ vs values in serum-depleted confluent cells. Abbreviations are same as in Figure 2.

have reported that follicle stimulating hormone downregulates the AT2-R density in ovarian granulosa cells (28). This study showed that glucocorticoids downregulate the AT2-R expression through the gene transcriptional mechanism as well as the mRNA instability, suggesting that the gene transcription of the AT2-R is repressed in a mitogenic or differentiated state as observed in other growth factor receptors (34-39). Several cis-regulatory elements involved in cellular growth and development or regulations by growth factors as well as the GRE are present in the rat (Murasawa et al., unpublished observation), mouse (8) and human (14) AT2-R genes. Deletion analysis of the rat AT2-R promoter using the PC12 cells will allow us to begin to characterize the cis-elements responsible for the regulation of rat AT2-R transcription.

In conclusion, the present findings demonstrate that the AT2-R expression in PC12 cells is upregulated when cell growth is arrested by serum deprivation or contact-inhibition. When the growth-arrested cells are stimulated to reenter the cell cycle by the mitogenic action of serum, EGF and glucocorticoid or differentiate to neurons by NGF, AT2-R expression is markedly repressed. Thus, these findings indicate that the expression of AT2-R in PC12 cells is positively and negatively regulated in a growth state dependent manner, and suggest that this regulation provides a means by which cells can modulate their responsiveness to the actions of Ang II mediated through AT2-R.

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