EXPERIMENTAL STUDY

Effect of activin on production and secretion of prolactin and growth hormone in cultured rat GH3 cells

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Abstract

Objective: To evaluate the effect of the growth factor activin A on the secretion of prolactin (PRL) and GH in cultured GH3 cells.

Methods: The concentrations of PRL and GH secreted from GH3 cells cultured in media with and without activin A were measured by RIA, and the expression of PRL mRNA and GH mRNA were analyzed using the Northern blot method.

Results: Activin A significantly inhibited PRL release from GH3 cells cultured for 48 h in a dose-dependent manner (activin: 0.3–3 nM). The inhibitory effects of 3 nM activin A were observed in the culture from 12 h to 48 h (53.2% of control). Activin A (3 nM) also significantly inhibited the expression of PRL mRNA at 24 h (33.8% of control). In contrast, activin A significantly stimulated GH release from GH3 cells cultured for 48 h in a dose-dependent manner (activin: 0.3–3 nM). The stimulatory effect of 3 nM activin A was observed in the culture for 48 h (157.6% of control). Activin A (3 nM) also significantly stimulated the expression of GH mRNA at 24 h (183.6% of control). In spite of these significant changes in PRL and GH secretion, pit-1 mRNA levels were not significantly changed by activin A.

Conclusions: These findings indicated that activin A modulates PRL and GH secretion through the regulation of PRL and GH gene transcription in GH3 cells, but that these effects are unrelated to pit-1 gene expression.

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Introduction

Activin is a member of the transforming growth factor β (TGF- β) superfamily. Proteins of this superfamily exert potent growth-regulatory effects on their respective target tissues via a family of specific cell surface receptors. Activin mRNAs are expressed in a variety of tissues, including the ovary, testis, placenta, brain, bone marrow, and pituitary (1), and activin receptor (typeIA, typeII and typeIIB) mRNA expression has been reported in the rat (2, 3). Effects of activin on cellular proliferation and/or differentiation have also been observed in primary cultured cells and established cell lines (4–6).

Activin was initially isolated from gonadal fluids characterized by their ability to stimulate follicle-stimulating hormone (FSH) secretion from cultured rat pituitary cells (7, 8). Moreover, in cultured rat pituitary cells, activin inhibits basal growth hormone (GH) (7, 9, 10), prolactin (PRL) (11) and adrenocorticotropin (7) secretion. Activin also inhibits thyrotropin-releasing hormone (TRH)-stimulated PRL secretion and GH-releasing factor (GRF)-stimulated GH secretion (12). Activin thus plays important roles in hormone secretion from the

pituitary. However, the detailed mechanisms of the regulation of each hormone by activin are not yet clear.

GH3 cells, an established cell line from rat pituitary tumor cells, secrete both PRL and GH (13). It has been shown that GH3 cells retain their ability to respond to TRH (14), epidermal growth factor (EGF) (15, 16) and nerve growth factor (NGF) (17). Recent studies have demonstrated that activin receptors (typeIA, typeII and typeIIB) are present on GH3 cells (18, 19). GH3 cells are thus a useful model with which to study the roles of activin in hormone secretion by the pituitary. In this study, we examined the effects of activin A on the production and secretion of PRL and GH in GH3 cells, in order to clarify the process of secretion of these hormones from the pituitary.

Materials and methods

Cell culture

Activin A was kindly provided by Dr Y Eto (Central Research Laboratory of Ajinomoto Co. Inc., Japan). The

GH3 cells were plated into 24-multiwell plates (Nunc Inter Med., Denmark) (2×10^5 cells/well) and cultured in Ham's F10 medium (Gibco, Grand Island, NY, USA) with 15% horse serum and 2.5% fetal calf serum and penicillin (100 µg/ml) at 37°C under a humidified atmosphere of 5% CO₂ and 95% O₂. After pre-culture for 24 h, each medium was exchanged for medium with various concentrations of activin A. After incubation for various times, the medium was collected and stored at -40°C until assay. In the same fashion, GH3 cells were seeded into 6 cm dishes (Nunc) at a density of 3×10^6 /dish and incubated in culture medium with activin A (3.0 nM). After incubation for various times, cells were collected and used for RNA analysis.

Measurement of PRL and GH concentrations

PRL concentrations in culture medium were determined with a double-antibody RIA kit supplied by the National Pituitary Program, Bethesda, MD, USA. GH levels were measured with a rat GH assay system purchased from Amersham International plc, Aylesbury, Bucks, UK. Coefficients of intra- and interassay variation were less than 10% for both PRL and GH assays.

Cloning of rat PRL, GH and pit-1 cDNA

The partial cDNAs corresponding to rat PRL (434bp), GH (439bp) and the pituitary-specific transcription factor pit-1 (662bp) were subcloned into vectors (pBluescript KS-, Stratagene, La Jolla, CA, USA) by RT-PCR. RT-PCR was carried out with rat pituitary mRNA with the following sense primers and antisense primers: GH sense primer 5'-TCGAGCGTGCCCTACATTCC-3', GH antisense primer 5'-TTGAGCAGAGC GTCATCG-3', PRL sense primer 5'-TTGACCGTGTGGTCATGC-3', PRL antisense primer 5'-GGATTCTTCATCAACTCCTTGC-3', pit-1 sense primer 5'-CTGAGAATGCACCACAGTGC-3' and pit-1 antisense primer 5'-ATGATCTCCTGCGAAGAAGG-3'. Each inserted cDNA was confirmed to correspond to rat PRL, GH or pit-1 by a DNA sequencing method.

RNA extraction and Northern blot analysis

Total RNA was extracted from cultured cells by the acid guanidinium thiocyanate-phenol-chloroform method (20). Each total RNA (4 µg) was electrophoresed in 1% agarose gel, and transferred to nylon membrane (Hybond-N; Amersham International plc). Prehybridization was performed for 2 h at 65°C in hybridization buffer (5 × SSC, 50% formamide, 5 × Denhardt's solution, 0.5% sodium dodecyl sulfate (SDS), and 0.02 mg/ml denatured sonicated salmon sperm DNA (Sigma Chemical Co, St Louis, MO, USA). The membranes were hybridized overnight at 65°C in exchanged hybridization buffer with [³²P]-labeled DNA probe corresponding to rat PRL, GH or pit-1 cDNA. Probes

were obtained with a random primer labeling kit (Rediprime; Amersham International plc) with rat PRL, GH and pit-1 cDNA. After hybridization with each probe, the membranes were washed twice with 2 × SSC plus 0.1% SDS at 65°C for 1 h. After cross-linking by u.v. light, autoradiography was performed at -80°C for 60 h. The membranes were then exposed to imaging plates (Fuji Photo Film Co. Ltd, Tokyo, Japan) for 12–24 h at room temperature. The relative amounts of the respective mRNAs were detected and

Figure 1 Inhibitory effect of activin A on PRL secretion from cultured GH3 cells. (A) The treatment of GH3 cells for 48 h with 0–3 nM activin A significantly decreased the basal PRL release in a dose-dependent manner ($P < 0.01$, ANOVA). Values are means $\pm$ S.E.M. ($n = 4$). * $P < 0.01$ (t -test) compared with control. (B) Time-course study of basal PRL release. Cells were co-cultured with 0 nM (○) or 3 nM activin A (●) for various time-periods. Treatment with 3 nM activin A significantly inhibited basal PRL secretion from 12 to 48 h. Values are means $\pm$ S.E.M. ($n = 4$). * $P < 0.01$ (t -test) compared with control.

quantitated using an imaging analyzer (Fuji BAS 2000; Fuji Photo Film Co. Ltd) and expressed as percentages of the time 0 mRNA level.

Data analysis

Results are presented as means $\pm$ S.E.M. The dose responses for activin addition were determined using ANOVA. For comparison between the two groups, Student's *t*-test was used. Findings of $P < 0.05$ were considered significant.

Results

Effect of activin A on PRL secretion

Activin A inhibited basal PRL secretion from GH3 cells in a dose-dependent manner (Fig. 1A). PRL concentration in culture medium significantly decreased with the addition of 0.3 nM activin A, and reached 53.2% of the control level with 3 nM activin. The significant inhibitory effect of activin A (3 nM) on PRL secretion was observed in culture from 12 to 48 h (Fig. 1B).

Figure 2 shows the effect of activin A on PRL mRNA levels in GH3 cells determined by Northern blot analysis. The addition of 3 nM activin A suppressed the expression of PRL mRNA at 12 h and 24 h, and maximum suppression (33.8% of control) was observed at 24 h. However, this inhibitory effect was no longer

present at 48 h, at which time the expression of PRL mRNA had returned to control level.

Effect of activin A on GH secretion

Activin A stimulated basal GH secretion from GH3 cells in a dose-dependent manner (Fig. 3A). GH concentration in culture medium significantly increased with the addition of 3 nM activin A (157.6% of control). A stimulatory effect of activin A on GH secretion was observed in culture for 48 h (Fig. 3B).

Figure 4 shows the effect of activin A on GH mRNA levels in GH3 cells determined by Northern blot analysis. Major bands corresponding to GH RNA species of 2.3 and 1.0 kb were found. The 1.0 kb species is a mature GH mRNA, and the 2.3 kb species is possibly a GH precursor RNA. The addition of 3 nM activin A enhanced the expression of GH mRNA, and a significant increase in this expression (183.6% of control) was observed at 24 h. However, this stimulatory effect on mRNA tended to decline at 48 h.

Effect of activin A on expression of pit-1 mRNA

Pit-1 is a pituitary-specific transcription factor regulating the expression of PRL and GH genes. To determine the mechanism of the effect of activin A on PRL and GH secretion from GH3 cells, the effect of activin A on the expression of pit-1 mRNA in GH3 cells was examined.

Figure 2 The effect of activin A on the expression of PRL mRNA in cultured GH3 cells. GH3 cells were co-cultured with and without activin A for various time-periods. Total RNA was extracted from cells. Northern blot analysis and quantitation of PRL mRNA was performed. (A) A representative autoradiogram showing the inhibited expression of PRL mRNA in response to activin A: 4 μ g total RNA was used. (B) Values quantitated from autoradiographic signals using an imaging analyzer are given. Changes in the amount of PRL mRNA with (●) or without (○) activin A (3 nM) are shown. Results are plotted as percentages of the time 0 mRNA levels. Values are means $\pm$ S.E.M. ($n = 3$). * $P < 0.01$ (*t*-test) compared with control.

Figure 3 Stimulatory effect of activin A on GH secretion from cultured GH3 cells. (A) The treatment of GH3 cells for 48 h with 0–3 nM activin A significantly stimulated the basal GH release in a dose-dependent manner ($P < 0.05$, ANOVA). Values are means $\pm$ S.E.M. ($n = 3$). * $P < 0.05$ (t -test) compared with control. (B) Time-course study of basal GH release. Cells were co-cultured with 0 nM ($\circ$) or 3 nM activin A ($\bullet$) for various time-periods. GH secretion was significantly stimulated by activin A after 48 h of incubation. Values are means $\pm$ S.E.M. ($n = 3$). * $P < 0.05$ (t -test) compared with control.

As shown in Fig. 5, activin A had no significant effect on the expression of pit-1 mRNA in GH3 cells.

Discussion

In the present study, we demonstrated that activin A stimulated basal GH secretion and inhibited basal PRL secretion in GH3 cells via modulation of the transcription of the GH and PRL genes. Using a rat pituitary monolayer culture system, Billestrup *et al.* (10) reported that no reduction in GH biosynthesis was observed after 6-h treatment with activin, but that by 24 h GH

biosynthesis was clearly inhibited. In our time-course studies, a significant effect of activin on PRL mRNA and GH mRNA levels also appeared at 24 h after the start of incubation. In addition, by 48 h, both mRNA levels tended to return to time 0 mRNA levels. The decline of the effect of activin on PRL and GH transcription suggests the existence of a negative regulatory system in GH3 cells. It has been suggested that, in the pituitary, activin plays an autocrine or paracrine role through interaction with follistatin, which has been demonstrated to function as a negative regulator of activin as a result of its ability to bind activin.

Pit-1, a tissue-specific transcription factor, plays an important role in the transcription of the PRL and GH genes in pituitary cells. MacCormick *et al.* (21) and Supowit *et al.* (22) demonstrated that repression of both GH and PRL syntheses by activin in somatic cell hybrids between GH3 cells and mouse LB82 fibroblasts occurred as a result of the suppression of pit-1 expression. However, TGF- β was found to inhibit transcription of the PRL gene in GH3 cells, but not that of the GH gene, through a mechanism independent of pit-1 gene expression (23). We also found that pit-1 gene transcript levels in GH3 cells were not significantly affected by activin. These data do not address the possibility of activin-induced post-translational modifications of the pit-1 protein and interactions with the promoter. Gaddy-Kurten & Vale (24) demonstrated that activin decreased GH expression in MtTW15 cells through multilevel regulation of pit-1, involving not only a decrease in pit-1 DNA-binding activity but also a decrease in the stability and synthesis of pit-1.

In a pituitary monolayer culture system, activin has been found to be a negative regulator of pituitary somatotrophs and lactotrophs (7, 9–11). In our study, GH3 cells, which were derived from rat pituitary tumor cells, exhibited a paradoxical GH response to activin. Daniels *et al.* (25) reported that activin significantly increased basal GH secretion from cells of two of six human somatotrophinomas *in vitro*. Similar heterogeneous responses to activin were described in studies of FSH release by human gonadotropin-secreting pituitary tumor cells *in vitro* (26). These findings suggest that inconsistent and paradoxical responses to activin may also occur in some tumor cells.

GH3 cells are an established cell line which may be related to the bipotential somatomammotroph from which both somatotroph and mammotroph cells derive. It has been reported that differentiation of GH3 cells into mammotroph cells is induced by NGF (17) and EGF (16). In contrast, it has been reported, based on results of fluorescence-activated cell sorting that GRF promotes the differentiation of GH3 cells into somatotroph-like phenotypes (17). The present findings that synthesis of PRL was inhibited and synthesis of GH was stimulated by activin suggest the possibility that differentiation of the GH3 cells into somatotroph-like cells may occur.

Figure 4 The effect of activin A on expression of GH mRNA in cultured GH3 cells. GH3 cells were co-cultured with and without activin A for various time-periods. Total RNA was extracted from cells. Northern blot analysis and quantitation of GH mRNA was performed. (A) A representative autoradiogram showing the stimulated expression of GH mRNA in response to activin A. Mature GH mRNA (1.0 kb) and a possible GH RNA precursor (2.3 kb) are indicated: 4 μ g total RNA was used. (B) Values quantitated from autoradiographic signals using an imaging analyzer (only mature GH mRNA) are given. Changes in the amount of GH mRNA with (●) or without (○) activin A (3 nM) are shown. Results are plotted as percentages of the time 0 mRNA level. Values are means $\pm$ S.E.M. ($n=3$). * $P<0.05$ (t -test) compared with control.

In summary, our study has demonstrated that activin A modulates basal PRL and GH secretion in GH3 cells through the regulation of PRL and GH gene transcription without affecting pit-1 mRNA level.

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Figure 5 The effect of activin A on expression of pit-1 mRNA in GH3 cells. Hybridization studies of pit-1 mRNA revealed three bands, one major band and two minor bands. The major band was 2.5 kb, and the minor bands were 4.1 and 1.6 kb. The addition of 3 nM activin A had no significant effect on these bands.

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