EXPERIMENTAL STUDY

Human GnRH-secreting cultured neurons express activin β A subunit mRNA and secrete dimeric activin A

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Abstract

Objective: To evaluate the expression of activin β A-subunit mRNA and the secretion of activin A in/from cultured GnRH-secreting neuronal cells cloned from human olfactory epithelium (FNC-B4), which showed biochemical and antigenic properties of GnRH-secreting neurons.

Design: FNC-B4 cells were cultured in basal and conditioned media.

Methods: Reverse transcription-polymerase chain reaction (RTR-PCR) evaluated the expression of activin β A-subunit mRNA. By using a specific ELISA, dimeric activin A concentrations were measured in culture media, in the absence or presence of carvone or forskolin and with different doses of progesterone, GnRH, and estradiol.

Results: RT-PCR experiments performed on total RNA isolated from FNC-B4 cells, using specific primers for the activin β A gene, showed a 787 bp DNA band corresponding to the β A gene. FNC-B4 cells secreted activin A, and the highest accumulation in conditioned medium was achieved after 3 h culture: the addition of forskolin, but not of carvone, was able to stimulate the release of activin A from cultured neuronal cells ($P < 0.01$). When progesterone or GnRH was added, a significant accumulation of activin A was observed ($P < 0.01$), while estradiol administration did not significantly affect activin A secretion.

Conclusion: To date, this is the only study, in an *in vitro* human model reporting, that GnRH-secreting neuronal cells expressed activin β A-subunit mRNA, and released dimeric activin A in culture medium. The expression and secretion of activin suggests that in these cells activin A might exert its action by autocrine/paracrine mechanisms.

European Journal of Clinical Endocrinology 143 133–138

Introduction

Activin A is a homodimer glycoprotein composed of two β A-subunits and is structurally related to the transforming-growth factor β (TGF- β) family (1). It is expressed in a variety of tissues, including granulosa cells of the ovary, Sertoli and interstitial cells of the testis, placenta, bone marrow, adrenal, kidney, spleen, brain and pituitary (2–4). In these organs, activin A affects hormone synthesis and release, and at the same time, may play a role as differentiation and growth factor in the early embryonic development (5, 6).

With regards to activin A action within the hypothalamo–pituitary–gonadal axis, the stimulatory role on FSH release from pituitary cells is well documented (1, 7, 8). In fact, activin induces a rapid and marked increases in FSH β -subunit mRNA and FSH release (1, 7). Furthermore, activin A may regulate the reproductive function modulating not only pituitary FSH release and biosynthesis, but also hypothalamic gonadotropin-releasing hormone (GnRH) release (9–11).

It is well known that the FSH β gene is stimulated by low-frequency pulses of GnRH. At the same time, this GnRH stimulation seems to be mediated by local expression of activin, requiring GnRH stimulation of FSH β mRNA endogenous activin (12).

Since activin subunits are localized predominantly in olfactory regions (13, 14), the expression of activin β A subunit mRNA and the mechanisms regulating activin A secretion *in vitro* were investigated in cloned long-term cultures of primary human olfactory neuroblast cells (FNC-B4 cells), which express both biochemical and antigenic properties of mature neurons (15, 16).

Materials and methods

Preparation of cell culture from human fetal olfactory epithelium

FNC-B4 cell long-term cultures were isolated, cloned, and propagated *in vitro* from the human fetal olfactory epithelium as previously described (15, 16). Although

these cells have the same properties as immature neurons, they can differentiate and express both neuronal proteins as well as olfactory specific markers, are electrically excitable and are GnRH-secreting neurons (16, 17). These findings suggest that these cells originate from the 'stem cell' compartment which gives rise to mature olfactory neurons throughout life. They grow as a monolayer, are non tumorigenic, have a normal karyotype and have properties of mature neurons (13, 14); the biochemical characteristics of these long-term primary cultures are highly stable.

Dissociated FNC-B4 cells were resuspended (5×10^5 cells/cm² in 60 mm plastic tissue-culture dish) and cultured in Coon's modified Ham's F12 medium supplemented with 10% heat-inactivated fetal calf serum (FCS). The cultures were maintained at 37 °C in a 5% CO₂ atmosphere.

All experiments were performed on early passages from isolated cells and analysis of various markers was performed before and after the addition of stimuli, with similar results.

Basal activin A secretion After plating, basal activin A secretion was evaluated on conditioned medium after 15, 30, 45 min, and thus 1, 3, 6, and 24 h of culture (four experiments).

Effect of carvone and forskolin on activin A release

After 6 h culture, cells were incubated for 3 h with increasing doses (from 10^{-13} to 10^{-8} M) of carvone (an odorant compound able to increase odorant-dependent cAMP accumulation) (Aldrich Chimica, Milano, Italy) or forskolin (from 10^{-13} to 10^{-8} M) (Sigma, St Louis, MO, USA), or vehicle (Coon's modified Ham's F12 medium supplemented with 10% heat-inactivated FCS) (four experiments). Cells were then harvested and viability evaluated by trypan blue exclusion test, as previously described (15).

Effect of GnRH, progesterone, and estradiol on activin A release FNC-B4 cells were incubated for 3 h in presence of various doses (from 10^{-12} to 10^{-6} M) of GnRH, progesterone, estradiol, or vehicle (ethanol; SIGMA) in culture medium (four experiments). Cells were then harvested and viability evaluated by trypan blue exclusion test, as previously described (15).

In all the experiments, the conditioned media were collected and frozen at -20 °C, in order to assay activin A levels in all samples simultaneously.

Reverse transcriptase polymerase chain reaction (RT-PCR) Total RNA was extracted from untreated cultured cells (18) and quantified by UV absorption at 260 nm.

The presence of activin β A mRNA was demon-

strated by amplifying the target sequence with PCR according to the instructions provided with the GeneAmp amplification reaction kit (Perkin Elmer, Milan, Italy). One microgram of total RNA was reverse transcribed to prepare complementary DNA (cDNA). The PCR was performed on the entire cDNA product with Taq (*Thermus aquaticus*) DNA polymerase with the manufacturer's recommended buffers. Reaction conditions for reverse transcription were as follow: 1 mmol/l each deoxynitrophenyl triphosphate, 1 unit RNasin, 100 pmol random hexamers and 200 units RT. The reaction was run at 42 °C for 1 h. The mixture was then heated at 99 °C for 5 min and quick chilled on ice.

Specific oligonucleotide primers were designed to amplify sequences of activin β A subunits (19). The sequence of the sense primer was 5'-GTTTGCCGAGTCA GGAACAG-3' (Gene Bank accession number M13436; 611–630 bp); the sequence of the antisense primer was 5'-GAGGTTGGCAAAGGGGCTATGGCCCCGCAT-3' (Gene Bank accession number M13436; 1397–1368 bp).

Computer analysis performed to study the possible secondary structure of the different cDNAs and to compare the synthesized oligomers to the human sequences in the MicroGenie (Beckman, Palo Alto, CA, USA) gene database bank revealed no more than 74% homology in the former and 72% in the latter among all the other genes. For the activin β A subunit, cDNA amplification was performed with 35 thermal step cycles (94 °C, 1 min; 60 °C, 1 min; 72 °C, 3 min), followed by a 10 min extension at 72 °C. The 787 bp amplified fragment corresponding to bases 611–1397 of the activin β A cDNA sequence was digested with the restriction enzyme PstI, which has a unique restriction site in the entire fragment sequence, generating two digested fragments of 508 and 279 bp respectively. Amplification and digestion products were visualized on a 4% agarose gel stained with ethidium bromide and viewed on an UV light box. In all the amplification procedures, the negative control was a blank prepared using all reagents and substituting 2 l water for RNA. Quantity, integrity and possible genomic contamination of all RNA samples were controlled by RT-PCR of the constitutively expressed human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene. All GAPDH amplifications revealed the correct 240 bp fragment but no 354 bp product that would indicate genomic contamination.

Activin A ELISA

Activin A concentration was measured in culture medium using a specific and sensitive ELISA for activin A, as previously described (20). Briefly, standard (recombinant human activin A) and samples were diluted as appropriate in phosphate-buffered saline containing 10% (w/v) BSA and 0.1% (w/v) sodium azide; 125 l of diluted samples and standards were

Figure 1 A, detection of activin β A-subunit (787 bp) mRNA by RT-PCR in cultured fetal olfactory neuronal cells (FNC-B4). Ethidium bromide-stained agarose gels were used to separate the products of the RT-PCR. Signals are indicated by the arrows on the right. M, DNA size marker; lane 1, negative control without template; lane 2, FNC-B4 cells; lane 3, placental trophoblast (positive control). B, digestion of the 787 bp amplified activin β A fragment by using the restriction enzyme PstI. M, DNA size marker; lane 1, 787 bp undigested fragment; lane 2 508 bp digested fragment and lane 3, 279 bp digested fragment (3). The size marker is BioMarker LOW (1000, 700, 525/500, 400, 300, 200 bp) (Bio Ventures, Inc.).

transferred to 1.5 ml microfuge tubes and mixed with an equal volume of distilled water containing 10% (w/v) SDS. After 3 min incubation at 100 °C in a water bath, the tubes were cooled before adding 20 l hydrogen peroxide solution (30% v/v). After 30 min incubation at room temperature, denatured and oxidized samples/standards were transferred to the activin assay plate (Serotec Limited, Kidlington, Oxford, UK) containing 25 l ELISA plate buffer/plate (0.1 mol/l Tris-HCl, 0.15 mol/l NaCl, 10% (w/v) BSA, 5% (v/v) Triton X-100, and 0.1% (w/v) sodium azide, pH 7.5). After the addition of 25 l biotinylated monoclonal antibody (mouse anti-inhibin β A subunit) to each well, plates were incubated with agitation overnight in a humidified box at ambient temperature. After washing with ELISA wash buffer (0.05 mol/l Tris-HCl, 0.15 mol/l NaCl, 0.05% (v/v) tween-20, and 0.05% (v/v) sodium azide, pH 7.5), 50 l diluted alkaline phosphate-conjugated extravidin (1:10000, v/v) was added, and plates were incubated for 1 h with agitation at room temperature in a humidified box. Plates were washed and bound

alkaline phosphatase was quantitated using a commercially available ELISA amplification kit (Immuno Select ELISA Amplification System, Dako, Milano, Italy) which was used according to the supplier's instructions. The plate was read at 490 nm by using an automated reader (BRIO: Basic Radim Immunoassay Operator; Radim spa, Pomezia, Italy). The assay limit of detection was < 100 pg/ml. The inter- and intra-assay coefficients of variation were < 7%. The antiserum did not cross-react with recombinant human activin B, inhibin A, inhibin B, follistatin, and 2-macroglobulin. Activin A concentrations in culture media were expressed as ng/ml secreted per dish (5×10^5 cells), and each sample was assayed in duplicate.

Statistical analysis

Each data point represents the mean $\pm$ S.E.M. Statistical analysis of the results was performed by means of analysis of variance, followed by the Duncan test.

Results

RT-PCR experiments performed on total RNA isolated from FNC-B4 cells, using specific primers for the activin β A gene, showed a 787 bp DNA band corresponding to the expected length (Fig. 1A). In addition, the amplified fragment was digested with the restriction enzyme Pst I, generating two digested fragments of 508 and 279 bp respectively (Fig. 1B).

In addition to expressing activin mRNA, these cells also secreted the protein into the culture medium, with the maximal accumulation of activin A being achieved in the conditioned medium (as results of the secretion/degradation rate) after 3–6 h of culture (Fig. 2).

The addition of carvone, an odorant compound, failed to increase activin A levels but, conversely, forskolin stimulated the release of the protein from cultured FNC-B4 cells in a dose-dependent manner: this effect was evident starting from the lowest dose and reached the highest effect at 10^{-9} M forskolin ($P < 0.01$) (Fig. 3).

Activin A accumulation was also augmented in presence of increasing doses of GnRH: this addition was effective starting from 10^{-10} M and was highly significant at higher concentrations of GnRH (10^{-9} – 10^{-6} M; $P < 0.01$) (Fig. 4).

The addition of progesterone induced a significant increase of activin A accumulation, starting from 10^{-9} M: the highest levels of activin achieved in the conditioned medium were reached in presence of the highest progesterone dose used ($P < 0.01$) (Fig. 4). By contrast, estradiol administration did not significantly affect activin A secretion, at all tested doses (Fig. 4).

Discussion

In mammals, the olfactory neurons reside within the posterior recesses of the nose, whereas the GnRH

Figure 2 Mean $\pm$ S.E.M. activin A accumulation in culture media of FNC-B4 cells. The maximal levels of activin A were achieved in the conditioned medium after 3 h of culture ($P < 0.01$).

neurons reside within the hypothalamus. During organogenesis, both olfactory and GnRH neurons originate in the olfactory placode that will eventually give rise to the olfactory epithelium. Although the olfactory cells project their axons to the olfactory bulb, the GnRH neurons migrate along the pathway of the olfactory nerve, cross the nasal septum and arrive at the septal-preoptic area and hypothalamus (21, 22). Therefore, the targeting of the olfactory axons and the migration of the GnRH neurons appear to be time-correlated (23, 24); thus, olfactory neurons and GnRH neurons have a common origin during organogenesis (21, 22).

FNC-B4 cells derive from fetal olfactory neurons (8- to 12-week-old human fetuses) (15, 16), and morphological, immunocytochemical, biochemical, and functional properties support their identification as human olfactory neurons (15, 16). In addition, these cells synthesize and release carnosin (15), a potential olfactory neurotransmitter (24) and secrete authentic

endothelin-1 (25) and GnRH (16, 25), which leads us to support a neuroendocrine identity for these olfactory neurons.

Thus, the present study showed that, in an *in vitro* human model, GnRH-secreting neuronal cells derived from olfactory neurons i.e. FNC-B4 cells, express activin β A-subunit mRNA, and release activin A in culture medium under different stimuli. In particular, we report that progesterone and GnRH increased significantly the release of activin A from FNC-B4 cells. The present data and the fact that these cells secrete both GnRH (16, 25) and activin A in culture medium, suggest a reciprocal autocrine regulation. Although no data are to date available on activin action on FNC-B4 cells, reports that activin increases GnRH secretion from a GnRH-secreting cultured neuronal cell lines (11) and that activin A can stimulate the expression of GnRH receptor gene at transcriptional level (10), suggest that activin could also act in this way.

Figure 3 Mean $\pm$ S.E.M. activin A levels in culture media of FNC-B4 cells after addition of carvone or forskolin. Forskolin significantly increased the release of activin A from cultured FNC-B4 cells ($P < 0.01$).

Figure 4 Mean $\pm$ S.E.M. activin A levels in culture media of FNC-B4 cells incubated in the presence of progesterone, GnRH, estradiol. Estradiol administration did not significantly affect the secretory pattern of FNC-B4 cells.

On the basis of these findings an interaction between activin and GnRH neuronal system in the human hypothalamus could be hypothesized. In fact, in the rat, activin β A-subunit-immunostained fibers were distributed throughout the hypothalamus and GnRH-positive perikarya, and fibers were in close association with β A subunit-immunoreactive fibers (26). In addition, it was reported that FSH secretion is stimulated over 6-fold by concomitant GnRH and activin A administration (8). So, the presence of activin A in those cells containing and secreting GnRH (FNC-B4) suggests that these peptides may regulate the reproductive function modulating pituitary FSH release and biosynthesis; GnRH stimulation of activin peptide production provides regulatory control over the production of FSH.

We reported that FNC-B4 cells respond to odorants secreting GnRH (16, 25), but not activin A. The fact that the addition of forskolin, but not of carvone, increased the secretion of activin A suggests that cAMP has a major role as second messenger in mediating the release of activin A from FNC-B4 cells, these cells being responsive to cAMP accumulation in presence of forskolin, but not in presence of carvone (15). In addition, recent studies have provided some clues concerning the neuroendocrine integration of olfaction and reproduction. In fact, an interaction between olfactory and reproductive function has been largely confirmed by the reports that gonadal hormones are involved in olfaction (27), that olfactory cues affect copulatory behavior and gonadotropin secretion (28) and that pheromones may regulate ovulation in humans (29). The involvement of activin A in the interaction between olfaction and reproductive behaviour could be mediated by the increase in carvone-induced GnRH secretion (16).

It is well documented that activin A acts as signal in the formation of neuronal tissue during development of lower vertebrates (3, 14, 30) and as neuronal cell survival factor (31, 32). Therefore, the presence of

activin A in GnRH-secreting neurons may point towards a role of activin A in the maintenance of neuronal organization and/or functioning throughout autocrine/paracrine mechanisms.

In conclusion, the recent development of GnRH-secreting neuronal cells provides a model for the study of neuroendocrine regulation of activin A in an *in vitro* human model. The evidence that activin is expressed in both the central nervous system and peripheral tissues would suggest further multiple roles for activin A as a secreted neurohormone, as a candidate transmitter/modulator in the brain and a polypeptide growth and/or survival factor. Activin may serve a role in GnRH-secreting olfactory neurons mediating some aspects of reproductive function, affecting local neuronal circuits.

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Received 27 July 1999

Accepted 29 December 1999