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OVEREXPRESSION OF ACTIVIN-βA SUBUNIT mRNA IS ASSOCIATED WITH DECREASED ACTIVIN TYPE II RECEPTOR mRNA LEVELS IN THE TESTES OF α-INHIBIN DEFICIENT MICE

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Summary- Activins and inhibins are polypeptides of the transforming growth factor-β family that participate in differentiation and growth of diverse cell types, and are involved in endocrine/paracrine regulation of the hypothalamo-pituitary-gonadal axis. Mice with α-inhibin subunit gene deletion develop large testicular tumors. In these animals, a 200-fold increase in testicular expression of activin βA subunit mRNA was detected using S1-nuclease protection analysis. Northern blot analysis demonstrated that a predominant mRNA form of approximately 6.5 kb and a second minor form of 4.5 kb were overexpressed in the testes of the α-inhibin deficient animals. Testicular expression of the type II activin receptor was decreased 3-fold in these mice. In contrast, hypothalamic βA and type II activin receptor mRNA levels remained unaltered. α-Inhibin may play a role to suppress the expression of βA mRNA in the mammalian testes. These results demonstrate that increased expression of activin is accompanied by a tissue specific reduction in the expression of its own receptor mRNA in vivo.

The activins and inhibins are proteins that have structural homology to a large number of proteins in the transforming growth factor-β (TGF-β) superfamily that include Mullerian inhibiting substance, bone morphogenic proteins, Xenopus Vg1 proteins and the product of the Drosophila decapentaplegic gene complex (1-4). The activins and inhibins were originally characterized from gonadal sources (1, 5) according to their ability to stimulate and inhibit follicle stimulating hormone (FSH) release from the pituitary, respectively. The activins are

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βA/βA (activin-A), βA/βB (activin-AB) and/or βB /βB (activin-B) subunit homodimers, and the inhibins are α/βA (inhibin-A) and α/βB (inhibin-B) subunit heterodimers. All three subunits are highly conserved in evolution (3,4,6). In addition to endocrine regulation of pituitary FSH release (1,3,4), the activins and inhibins are involved in local paracrine regulation of gonadal function (for extensive reviews see 1-4). In the ovary, these factors are involved in folliculogenesis, oocyte maturation and regulation of granulosa cell function (3,4). For example, activin has been shown to stimulate inhibin secretion, FSH receptor production and aromatase activity. In the testes, the roles of activin and inhibin are less well understood. In mixed testicular cell type cultures, activin decreased, and inhibin increased luteinizing hormone-stimulated testosterone production (3,7). However, in purified Leydig cell cultures, activin stimulates testosterone production (3). Activins regulate Sertoli cell function in vitro by inhibition of FSH-stimulated aromatase activity (8). Activin can also stimulate spermatogenesis, and in some in vitro systems, inhibin may inhibit spermatogenesis (3). Such opposing activites of inhibin and activin are a common feature for many processes (1-4).

Activin has now been shown to have important extra-gonadal endocrine functions including inhibition of pituitary growth hormone release (9), stimulation of placental progesterone production (10) and stimulation of hypothalamic corticotropin-releasing factor release (11). Activins have been suggested to be important for early embryonic development in Xenopus laevis (12,13), and for survival and differentiation of several cell lines (14,15). The majority of the studies on the effects of activin and inhibin are performed in vitro and the physiological significance of these results remains unclear.

Furthermore, numerous reports on the distribution of the inhibin/activin molecules in vivo, in particular in gonadal tissues (3,4,5,16), have not yet established a clear relationship between the levels of expression of the α, βA and βB subunits. Recently, Matzuk et al. (17) have shown using α-inhibin gene deleted mice, that inhibin has testicular tumor suppressor activity in vivo. In the present study we use this novel model to demonstrate that the normal presence of α-inhibin suppresses the expression of testicular activin βA mRNA in vivo. Furthermore, overexpression of activin βA in the α-inhibin deficient mouse is associated with a dramatic reduction of testicular activin type II receptor mRNA.

MATERIALS AND METHODS

Animals. Three month old male wild-type or homozygous α-inhibin deficient mice, generated as described previously (17) were used in the present study. α-Inhibin subunit gene deleted mice had bilateral hemorragic testicular tumors twice as large as testes from wild-type control mice.

Tissue collection and RNA extraction. For collection of hypothalamic tissues (approximate weight 100 mg), mice were decapitated by guillotine and tissues rapidly dissected as described (18), then frozen on dry ice. Whole testes were also rapidly dissected and frozen. Total tissue RNA was isolated by the acid-phenol method of Chomczynski and Sacchi (19). Integrity of isolated RNA was routinely assessed by visualization under UV exposure after electrophoresis on 1.2% agarose gels and staining with ethidium bromide (20).

cRNA probes. Rat βA clone 24 (5) subcloned in pGEM4 was linearized with XmnI. SP6 polymerase directed transcription of this template generates a 370 base ^{32}P-CTP labelled probe with 340 nucleotides being complimentary to β_A sequences. cDNA encoding the extracellular domain of rat type-II activin receptor (ActRII), subcloned from pTZ19 to pBluescript (21) was linearized with AccIII. T3 polymerase directed transcription of this template generates a 245 base ^{32}P-CTP labelled probe with 170 nucleotides being complimentary to ActRII sequences. Tissue content of cyclophilin (CYC) mRNA was also measured as an internal standard and control for RNA loading as previously reported (18,22). All three probes had a specific activity of 0.5-1 X 10^8 CPM/μg.

S1-Nuclease protection analysis. The ^{32}P-labelled β_A (~20x10^3 CPM), ActRII (~20x10^3 CPM) and CYC (~2x10^3 CPM) cRNA fragments were mixed and hybridized to 2-20 μg total mRNA, and samples processed for electrophoresis and densitometry quantitation of specifically hybridized fragments as previously validated (18,20).

Northern blot analysis. Twenty μg total RNA was electrophoresed on a 1.2 % agarose formaldehyde denaturing gel and transferred to Nytran membranes (0.45 μ pore size; Schleicher and Schüell, Keen, NH) by standard procedures. Hybridizations (18 hr) to the 370 base βA probe were performed at 55°C in buffer containing 5XSSPE, 50 % formamide, 2X Denhardt's solution and 0.1 % SDS. The membrane was washed initially in 5XSSPE for 15 min at 45°C, followed by 60 min washes in 2XSSPE, 1XSSPE and 0.2XSSPE at 45°C. The final high stringency wash was in 0.1XSSPE at 65°C for 30 min. Hybridized signals for nuclease protection and Northern analysis were visualized on preflashed KODAK X-OMAT-AR film at -80°C.

RESULTS

Messenger RNA for activin βA subunit could be detected in the hypothalamus and testes of 3 month old wild-type mice (Fig.1), confirming our observations in the male rat (18). In wild-type mice, testicular βA mRNA was often near the limits of detection in the nuclease protection analysis (Fig. 2, for example). In marked contrast, in the α-inhibin-deficient mouse, a prominant 340 base βA subunit mRNA signal in testicular tumors was approximately 200-fold higher compared to wild-type males. Several other smaller bands (approximate size range; 200-300 bases) were also evident (Fig. 1). These observations were confirmed in a second experiment where varying amounts of total RNA were hybridized to the βA subunit probe and run on a nuclease protection gel (Fig. 2). Levels of βA subunit mRNA in hypothalamus or levels of cyclophilin mRNA in both hypothalamus and testes were not affected by α-inhibin gene deletion (Fig.1).

Northern blot analysis demonstrated that a predominant βA subunit mRNA form of approximately 6.5 kb and a second minor form of 4.5 kb were overexpressed in the testicular tumors of the α-inhibin deficient mice (Fig. 3). In contrast to the nuclease protection assay (Fig.1), βA subunit mRNA could not be detected by Northern blot in rat and mouse hypothalamus or in wild-type mouse testes (Fig.3).

Messenger RNA for ActRII was detected in hypothalamus and testes (Fig. 4); yeast tRNA served as a negative control. In α–inhibin deficient mice, testicular tumor levels of ActRII were decreased by approximately 3-fold relative to wild-type males (Fig. 4A and Fig. 4C). In contrast, hypothalamic ActRII mRNA levels remained unchanged (Fig. 4A and Fig. 4B).

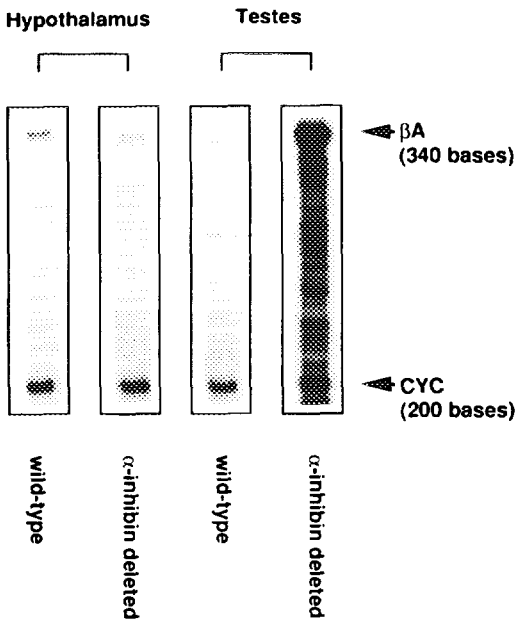

Fig. 1. The expression of activin βA subunit and cyclophilin (CYC) mRNA in hypothalamus and testes of wild-type and α-inhibin deficient mice. Protection of 340 and 200 nucleotide sequences were observed for βA and CYC mRNAs respectively (arrowheads), following S1-nuclease digestion of 20 μg total RNA hybridized to βA and CYC cRNA. Note the low levels of βA mRNA in wild-type testes but overexpression in α-inhibin deficient mice.

DISCUSSION

The present study demonstrates that in vivo deletion of the α-inhibin subunit gene leads to an overexpression of activin βA subunit mRNA in testicular tumors. There appears to be predominantly two mRNA molecular species (6.5 kb and 4.5 kb) overexpressed in the testes of the α-inhibin deficient mouse. Our size estimates in Northern analysis of mouse testicular RNA compare favorably with those in rat ovary where a predominant 7 kb and smaller molecular forms of βA mRNA have been observed (23,24). LaPolt and Hsueh (3) suggest that the presence of multiple species of mRNA for the βA subunit may result from alternative splicing events or the use of multiple polyadenylation signals. Our observations suggest that in vivo, absence of the α-inhibin subunit can inhibit activin production in the gonadal tumors in these mice. The increase in activin may have important consequences for pituitary and testicular function. Overexpression of activin may act as an autocrine growth factor in the development of testicular stromal tumors in the α-inhibin deficient mouse (17,25), especially given activin's morphogenic effects on Sertoli-germ cell cultures in vitro (4). Furthermore, the increase in the steady state levels of mRNA leads to an increase in activin-A protein production since these mice have elevated levels of immunoreactive βA subunit in the blood (26).

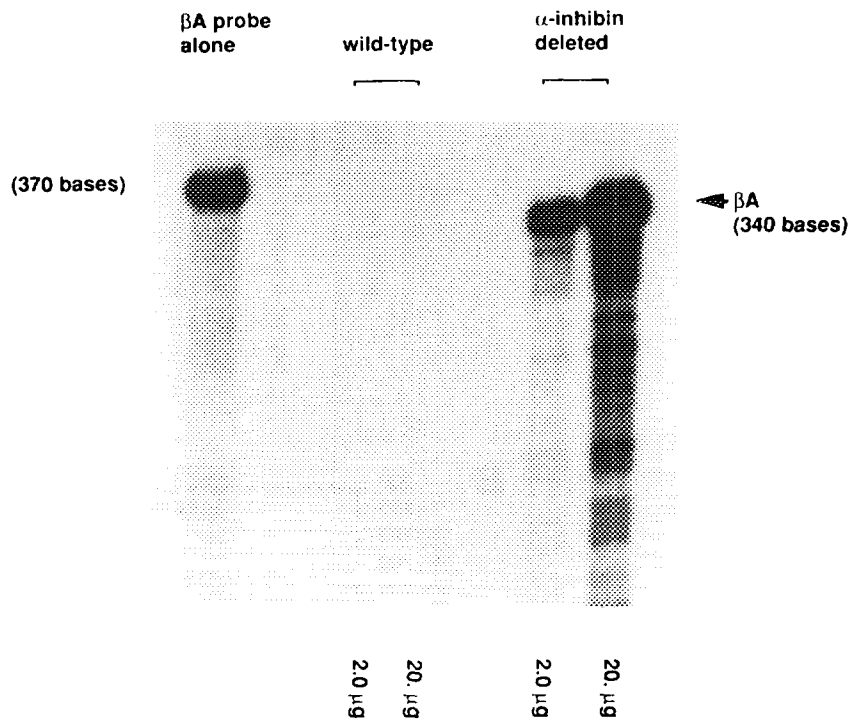

Fig. 2. The expression of activin βA subunit in testes of wild-type and α-inhibin deficient mice. Protection of 340 nucleotide sequences was observed for βA mRNA following S1-nuclease digestion of 2 and 20 µg total RNA from testes of α-inhibin deficient mice hybridized to βA cRNA. Note that in wild-type mice βA mRNA was at the limits of detection.

Activins interact with serine/threonine kinase cell surface receptors to affect cellular function (1,3,27,28). The first reported activin receptor sequence from the mouse AtT20 corticotrope cell line (29) was termed type II activin receptor (ActRII), corresponding to the classification of the three TGF-β receptor types (27,28). In an attempt to measure the first potential target of elevated testicular βA subunit production, we measured mRNA levels for ActRII. In α-inhibin deficient mice, a significant reduction in testicular ActRII mRNA was detected. These observations suggest that a local increase in activin levels *in vivo* leads to a significant down-regulation in activin receptor production. To our knowledge this is the first demonstration of such a control mechanism for the activin/inhibin family of growth factors. Alternatively, the tumorous tissues which are taking over the testes of the inhibin-deficient mice, may produce less activin type II receptor than normal testes.

Previous studies have shown that the Sertoli cell is the major site of activin and inhibin production (3,4,8) although the Leydig cell may produce low levels of α-inhibin. Activin type II receptor mRNA is mainly localized in spermatogenic cell types of the rodent testes (16,21). Therefore, a decrease in ActRII levels may have important inhibitory consequences for

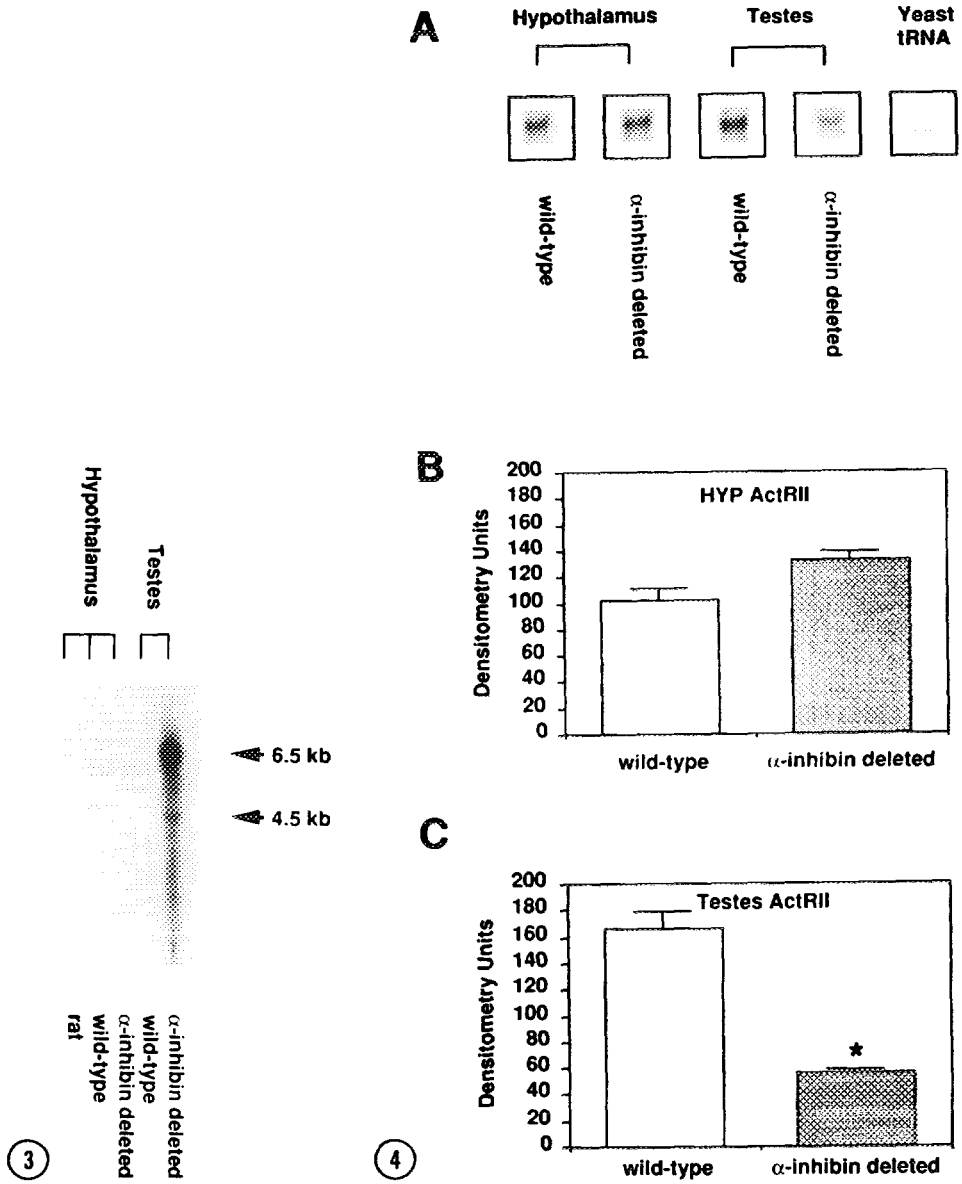

Fig. 3. The expression of activin βA subunit in hypothalamus and testes of wild-type and α-inhibin deficient mice. Twenty μg total RNA was analysed by Northern blot hybridization to βA cRNA probe. Specific bands of approximately 6.5 kb and 4.5 kb are indicated (arrowheads). Note the lack of detectable signals in all lanes except that of testes of the α-inhibin deficient mouse. Adult male rat hypothalamus is included for comparison.

Fig. 4. Levels of activin type II receptor (ActRII) mRNA in the hypothalamus and testes of hypothalamic tissues of wild-type and α-inhibin gene deleted mice. (A) Protection of a 170 nucleotide sequence was observed for ActRII mRNA following S1-nuclease digestion of 20 μg total RNA hybridized to ActRII cRNA. Note the lack of specific signal when 20 μg yeast tRNA was included in the reaction. (B) Hypothalamic (HYP) ActRII mRNA levels (n=3). (C) Testicular ActRII mRNA levels (n=3). For ActRII mRNA levels, densitometry measurements for the 170 nucleotide protected bands are expressed as percentage of the mean within a given experiment. Data are given as mean ± SEM. Group differences were determined using Student's t-test (*; $p<0.05$).

testicular steroidogenesis and spermatogenesis, even though activin levels remain elevated. Indeed, Leydig cell numbers decrease and spermatogenic regression occurs in parallel with tumor growth in α-inhibin deficient males (17). Furthermore, our observations give general support to the tight paracrine relationship between Sertoli and spermatogenic cell regulation by activin and/or inhibin (4,8,16,21,30).

Whereas α-inhibin deficiency increased βA and decreased ActRII mRNA levels in testes, there were no similar changes detected in the hypothalamus. The basis for this significant difference in the regulation of βA and ActRII mRNA expression in hypothalamus versus testes remains to be elucidated. In whole rat brain extracts, levels of α-inhibin mRNA is ~60% of that in testes, and the ratio of α-inhibin to βA mRNAs is ~3 for brain and ~24 for testes (31) further suggesting the existence of different control mechanisms for activin/inhibin subunit production in the CNS. In the adult male rat, long term castration and subsequent estradiol injection had no pronounced effects on hypothalamic βA mRNA expression, but respectively increased and decreased hypothalamic ActRII mRNA levels (18). Clearly, future studies should address the basis for differential regulation of peripheral versus central expression of the activin/inhibin family and their receptors.

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