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RAT FOLLISTATIN: ONTOGENY OF STEADY-STATE mRNA LEVELS IN DIFFERENT TISSUES PREDICTS ORGAN-SPECIFIC FUNCTIONS

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Follistatin (FS), a monomeric glycoprotein which specifically binds activin, is expressed in many tissues. This study investigated 1) the ontogeny of the steady-state FS mRNA levels in different extragonadal tissues and 2) whether the ratio of the differential splicing products, FS 344 or its carboxy-truncated form FS 317, is changed during postnatal development. Whereas the levels of FS mRNA 344 in the kidney showed a profound increase from the day of birth to adulthood, the levels in the muscle peaked during the infantile period and then declined. Brain cortex, heart and thymus also showed tissue specific expression in the steady-state mRNA level of FS during postnatal development. None of the tissues showed a measurable change in the ratio of the mRNA for FS 344 and FS 317. The FS mRNA 344 levels in male and female kidney were not different. It is concluded that the ontogeny of steady state FS mRNA varies in a tissue specific manner during postnatal development of the rat and may be involved in modulating the outcome of activin.

In 1987 peptides were isolated from ovarian follicular fluid, which were structurally different to inhibin (1,2) but which also inhibited pituitary FSH-release in vitro (3,4) and in vivo (5). These glycoproteins were named FSH-suppressing protein (FSP) or follistatin (FS) respectively. In contrast to inhibin which consists of an α subunit covalently bound to one of two different β subunits, follistatins are single chain glycoproteins. They are present as different molecular weight forms (6) which differ in their potency to inhibit FSH-release. The gene structure of porcine (7) and human (8) FS revealed two precursors of single chain proteins (FS 344 and a carboxytruncated form FS 317) which are generated by alternative splicing.

Activin, in contrast to FS and inhibin, is an enhancer of pituitary FSH-release (9,10) and also shows a large variety of other biological functions, e.g.: regulation of erythropoesis (11), as a nerve cell survival molecule (12), regulation of growth hormone biosynthesis and secretion in somatotrophs (13,14,15), stimulation of pancreatic insulin secretion (16), induction of mesoderm (17) and inhibition of neural differentiation (18). Recently Nakamura et al. demonstrated, that FS is a binding protein of activin (19) and Shimonaka et al. (20) provided evidence for binding of inhibin to FS through its β subunit. Binding of activin to FS results in a tetrameric complex

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(21,20) which then lacks the stimulatory activity of activin on FSH-release in vitro (21). Although FS is found in many tissues where as yet, no apparent effects of activin are known, it is conceivable that FS regulates the actions of activin or some related peptide(s) in different organs through its binding properties.

Tissue distribution studies of FS mRNA in the rat revealed that the FS gene is expressed at different levels in many adult tissues (22). Because of the differentiating properties of activin, and particularly as a mesodermal organizer, it is possible that FS may play a regulatory role by virtue of its activin-binding properties, during pre- and postnatal development. FS has recently been shown to antogonize the mesodermal inducing properties of activin in Xenopus (23). We therefore studied the ontogeny of the steady-state levels of FS mRNA during postnatal development in different extragonadal tissues to examine whether the FS mRNA levels in these organs follow the same pattern or whether they are expressed in a tissue-specific manner. We also examined whether or not the ratio of the two different forms of FS (FS 344 to FS 317) is changed in these organs during postnatal development.

Materials and Methods

Animals: For tissue preparation rats were decapitated and tissue was collected into liquid nitrogen and then stored at -80 °C till use. In the first study for every tissue examined individual RNA samples from 4 rats (aged 1d, 6d, 18d and 100d) were prepared. In the second study, RNA was prepared from pooled tissues of 2 (day 1 to day 12) or 1 rat (older than day 15). The concentration of the RNA was determined by measuring the O.D. at 260 nm and the integrity of the RNA of each sample was verified by analysing an aliquot on a nondenaturing gel. The animal experiments were approved by the local institutional ethics committee and conformed to the code of practice on animal experimentation endorsed by the National Health and Medical Research Council of Australia.

RNA preparation: RNA was prepared by the guanidine isothiocyanate method (24) or for small amounts of tissue by a small-scale preparation method used in our laboratory: 25 - 50 mg of tissue were homogenized in 200 ul cold 0.3 m AT-buffer (10 mM TRIS pH 8, 3 mM $CaCl_2$ 2 mM $MgCl_2$, 0.5 mM DTT, 0.3 mM sucrose and 0.15 % Triton-X) with RNAsin, layered over 400 ul 0.4 M AT-buffer (see above except 0.4 M sucrose) and spun for 10 minutes at 6000 rpm in a 1.5 ml tube at 4 °C in a bench top centrifuge. The supernatant was transferred to a 1.5 ml tube and a tenth of the volume of set-buffer (10 % SDS, 50 mM EDTA, 100 mM TRIS pH 8) and one hundredth of the volume of proteinase K (10 mg/ml) was added. The solution was incubated for 1 h at 45 °C, phenol/chloroform extracted and the upper phase was transferred to a tube containing 0.5 M EDTA (100 ul 0.5 M EDTA/500 ul aqueous phase). After 15 minutes one tenth volume of 5 M ammonium acetate and 1 volume of isopropanol was added and the RNA was precipitated overnight.

S1-Nuclease Analysis: Our FS probe corresponds to the last 157 nucleotides of the 3' end of exon 5 and to the first 71 nucleotides of exon 6 of the rat FS gene. S1-Nuclease digestion of probe hybridized to total RNA results in 3 different sized fragments: One signal with 228 nucleotides which corresponds to the mRNA of FS 344 and two smaller fragments (157 and 71

nucleotides) which correspond to the mRNA of FS 317. S1-Nuclease analysis was performed as previously described (22). In brief, total RNA was hybridized to an excess of radiolabeled probe at 50 °C for 12-14 h and the samples were than subjected to S1-Nuclease digestion for 50 minutes using 750 U of enzyme. The digestion was stopped with 4 M ammonium acetate 0.1 M EDTA, phenol/chloroform extracted and precipitated with 1 volume of isopropanol in the presence of 20 ug yeast tRNA. The samples were washed in 70 % ethanol, dried and resuspended in 5 ul TE (pH8) and 4 ul loading buffer. An aliquote of each samples was analyzed on a denaturing polyacrylamide gel and autoradiography was performed on X-ray film with an intensifying screen at -80 °C.

Data Analysis and presentation: As described above S1-analysis of total RNA with our probe leads to 3 different sized protected fragments: One signal with 228 nucleotides which corresponds to FS 344 and two smaller fragments which correspond to the RNA of FS 317. The relative intensity of hybridization signals were determined by transmittance scanning densitometry of the band for FS 344 and relative levels are expressed as percent of oldest age examined (= 100%). In experiments where triplicate or quadruplicate samples of each tissue were analyzed, the values are expressed as means ± SD. Different ages within one experiment were compared by one-way analysis of variance and significant differences ($p<0.05$ or less) are shown by different letters above the bars in the figures.

Results and Discussion

In most cases the signal for the carboxy-truncated form of FS (FS 317) was below the limit of detection and during the whole study we were not able to detect any obvious changes in the ratio of message for FS 344 : FS 317 (data not shown).

In Fig. 1 the age-dependent profiles of the steady-state levels of FS mRNA from female rat skeletal muscle (a) and kidney (b), and in figure 2 the ontogeny of steady-state levels of rat brain cortex (a), heart (b) and thymus (c) are shown. The results indicate that within each organ the level of FS-mRNA during postnatal development of the female rat did not follow an overall, age-dependent pattern, but rather exhibited a tissue-specific profile. The ontogeny of FS mRNA in the muscle showed a distinct peak during the infantile period with a rapid increase between day 1 and day 6 and a fall to the adult level between day 6 and day 60. In the kidney the level of FS mRNA increased ten- to twelve-fold from day 6 to day 60 with no difference between day 60 and day 100. Whereas brain cortex had a nadir on day 6, the profile of the steady-state level of FS mRNA during development of the heart did not change significantly between the age groups examined except on day 60 when the levels were twice those on days 18. The steady-state mRNA level of FS in the female thymus revealed a two-fold increase between day 6 and day 18 when the level plateaued at the adult level.

Fig. 3 depicts the patterns of steady-state levels for FS from female muscle (a) and female and male kidney (b and c) during postnatal development of the rat. This study was performed at more frequent time points in individual animals to define more precisely the timepoints of changes in FS mRNA level in these tissues. The peak of FS mRNA was between day 3 and 15 in the female muscle and the onset of the rise of the FS mRNA level in the female kidney was at day 21. Although the onset of the rise in FS mRNA level in the male kidney seems to be delayed to the timespan between day 21 and day 25, the ontogeny of FS mRNA level of the male resembled the corresponding profile in the female tissue.

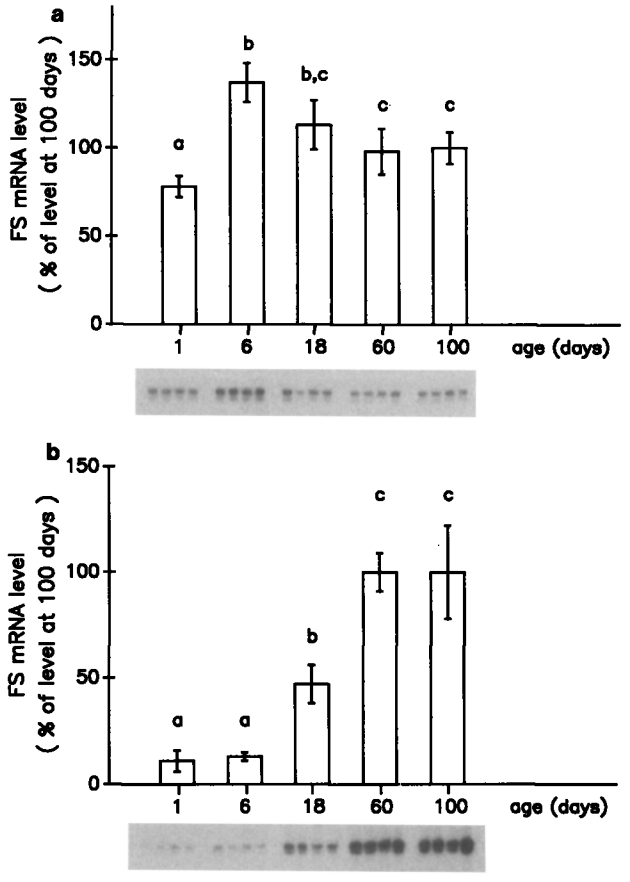

Fig. 1. Steady-state levels of FS mRNA in muscle (a) and kidney (b) during postnatal development of the female rat (n = 3 or 4 per time point). The band for FS 344 on the autoradiograph (underneath the bar graph) was scanned by densitometer, and the integration values (mean ± SD) are expressed as percent of mRNA level at 100 days. Different letters indicate significant differences between age groups ($p<0.05$ or less).

It was recently shown that bovine granulosa cells in vitro secrete immunoreactive FS (25). Due to the lack of a specific RIA for rat FS we do not know how much of the transcribed message for FS is translated into bioactive hormone, nor do we know what amount of hormone is secreted by the tissues exhibiting FS gene expression. This means that the major source of circulating FS still remains to be determined. Although Sugawara et al. (26) presume that the major source of FS in the human is the ovary, our studies suggest that this is not necessarily the case during the developmental phase. Our data show that the muscle already has a high level of FS mRNA expression before onset of puberty. If FS mRNA expressed in the muscle is also translated into bioactive hormone and if muscle contributes a high percentage to the whole body mass, it is likely, that before the end of puberty the muscle could be a major source of FS. At the onset of adulthood all tissues examined appeared to reach their individual plateaus which then might leave the ovary as the only variable source of FS-secretion depending on external and/or internal regulators.

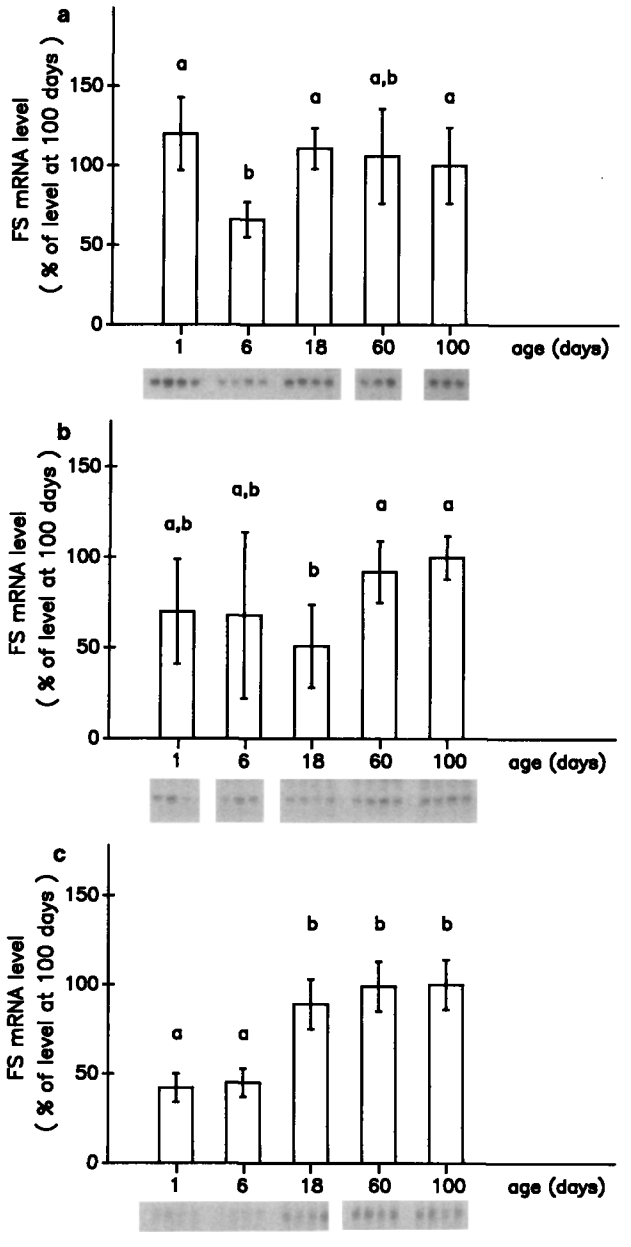

Fig. 2. Steady-state levels of FS mRNA in brain cortex (a), heart (b) and thymus (c) during postnatal development of the female rat (n = 3 or 4 per time point). The band for FS 344 on the autoradiograph (underneath the bar graph) was scanned by densitometer, and the integration values (mean ± SD) are expressed as percent of mRNA level at 100 days. Different letters indicate significant differences between age groups ($p<0.05$ or less).

Besides its stimulatory effect on FSH-secretion (9,10) activin also inhibits GH-secretion and synthesis as well as proliferation of the somatotrophs (13,14,15); thus activin controls two major pituitary hormones which are involved in the regulation of reproduction and growth. Activin function is thought to be regulated by its binding to FS which leads to an apparently inactive

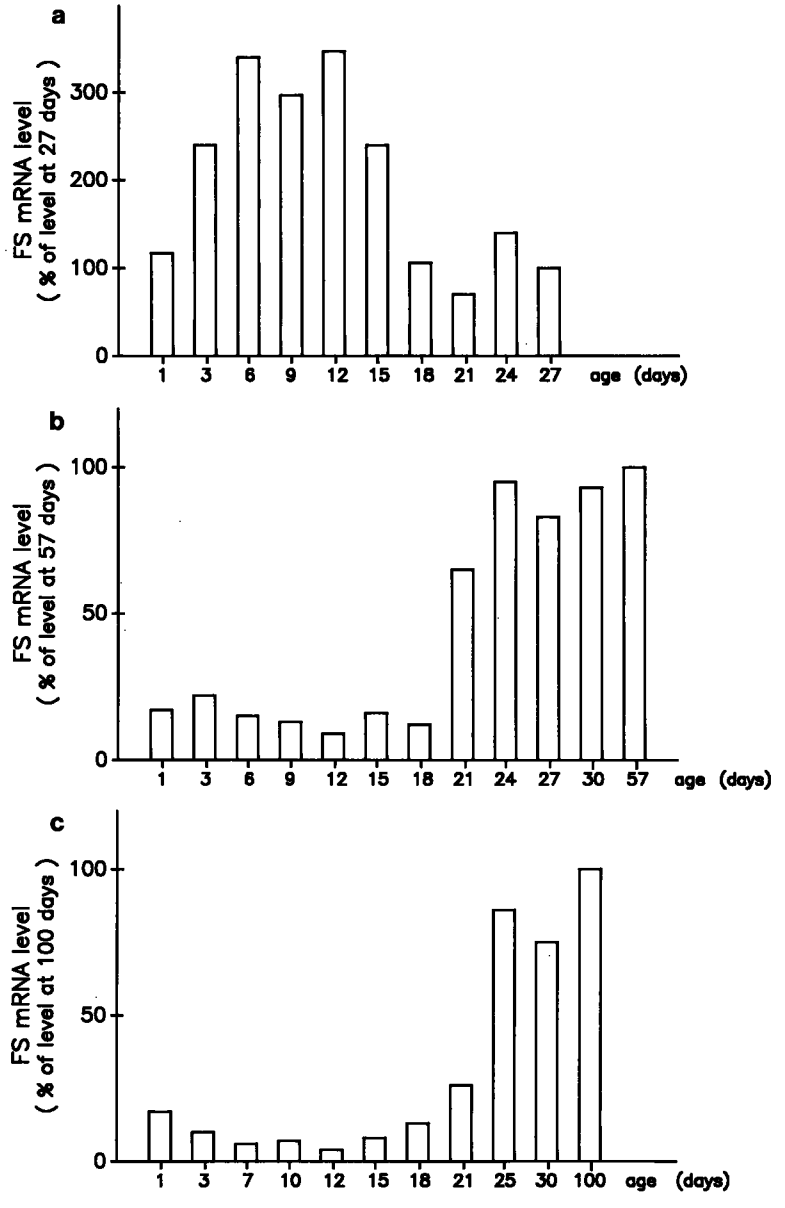

Fig. 3. Steady-state level of FS mRNA in female muscle (a) and female (b) and male kidney (c) during postnatal development (n=1 per day) of the rat. The band for FS 344 on the autoradiograph was scanned by densitometer and the integration values are expressed as percent of mRNA level at day 27 (fig. a), day 57 (fig. b) or day 100 (fig. c).

tetrameric complex (21). Comparison of serum FSH- and GH-levels in the female rat depict inverse patterns during a phase of prepubertal development (27,28) and studies in the human show that the initiation of the adolescent growth spurt is likely to be triggered by the attainment of a critical body weight (29). It is conceivable that FS derived from muscle is an important factor involved in regulating these events. It is tempting to speculate that at a certain body mass, and/or time when organ specific production of FS is increased during a distinct phase of

prepubertal development (correlated with the observed peak of FS mRNA levels in our study), there is an FS related suppression of activin dependent FSH-secretion and an increased output of GH, thus promoting the growth phase. A decrease of circulating FS hormone level or an altered responsiveness of gonadotrophs and somatotrophs to activin could reverse FS function thus providing a switch from growth to reproductive status.

The fact that the ontogeny of FS mRNA expression varies between different tissues during the postnatal development points towards an organ related function of FS. Although FS mRNA was found in many tissues where no effects of activin (or inhibin) are known, it is likely that FS is involved in controlling actions of activin (or inhibin) related peptides in many tissues during pre- and postnatal development.

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