EXPERIMENTAL STUDY

Comparative analysis of follistatin-, activin beta A- and activin beta B-mRNA steady-state levels in diverse porcine tissues by multiplex S1 nuclease analysis

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Abstract

Objective: The relation of activins (dimers of the beta-subunits of inhibin) and follistatin (FS) (their binding protein) affect the growth and differentiation of many cell types. Activin- and FS-mRNAs show a widespread co-expression throughout the organism, indicating an essential role for the FS/activin system in diverse physiological processes. The present study was performed to investigate FS-, activin beta A-, and activin beta B-mRNA expression in porcine tissues and to compare the relative mRNA tissue distribution by a newly developed multiplex S1 nuclease protection assay.

Methods: Twenty micrograms total RNA from different porcine tissues were subjected to multiplex S1 analysis. Specific mRNA expression was determined by measurements of optical densities on autoradiographs.

Results: Activin beta A-mRNA expression was abundant in the ovary, adrenal gland, fat, vein, artery and uterus, activin beta B-mRNA was highly expressed in the ovary, pituitary, uterus, placenta, aorta and cerebellum. FS-mRNA showed a widespread expression with high levels in ovary, uterus, cerebellum, placenta and fat. The comparison of relative activin beta A-, activin beta B- and FS-mRNA expression within a certain tissue showed a predominance of activin beta A-mRNA in the adrenal gland, fat, artery, spinal cord, cerebrum and colon and of activin beta B-mRNA in pituitary, testis and placenta, while FS-mRNA levels exceeded those of activin subunits in epididymis, liver, lymphoid tissue, muscle, intestine, cerebellum, ovary and uterus.

Conclusions: The presented data provide an overview of FS-, activin beta A-, and activin beta B-mRNA steady state levels in porcine tissues.

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Introduction

Activins are homo- or heterodimers consisting of two homologous beta-inhibin peptide chains structurally related to the transforming growth factor beta family. The most common forms are activin A (beta A-beta A), activin AB (beta A-beta B) and activin B (beta B-beta B) (1, 2). As growth and differentiating factors, activins are involved in the regulation of multiple biological systems including pituitary hormone secretion (2–5), spermatogenesis (6), erythropoiesis (7, 8), embryonic development (9, 10) and neural differentiation (11, 12). Recently, three additional beta-inhibin subunits have been discovered (beta C, beta D and beta E), but so far little is known about their physiological significance (13–15). The biological actions of activins are mediated by a heterotrimeric complex of activin and membrane-bound activin receptors type I and II with serine/threonine kinase activity (16, 17).

Activin is bound by follistatin (FS), a 32–35 kDa single-stranded glycoprotein (18, 19) in a high-affinity complex that itself seems biologically inactive (20). FS inhibits the interaction of activin with its type II receptor (21), and heparan sulfate-bound FS at the cell surface accelerates intracellular activin A uptake and lysosomal degradation in rat pituitary cells *in vitro* (22). As outlined above, the FS/activin system is involved in the paracrine regulation of multiple tissues. In contrast to inhibin, where endocrine effects are well established (23), possible systemic endocrine FS/activin effects are still under debate (24).

Earlier studies focused on the measurement of activin beta A-, activin beta B- or FS-mRNA expression in diverse tissues to determine those organs in which FS/activin interactions might be important for proliferation and differentiation. A broad tissue distribution of activin- and FS-mRNA in rat tissues with the highest levels in ovary and testis was shown by S1 nuclease

analysis (25, 26). Tuuri *et al.* (27) demonstrated a widespread expression of activin- and FS-mRNA in human fetal tissues, suggesting an important role of FS and activin in human development. Since then the expression of FS- and activin-mRNA has been detected in many non-gonadal tissues throughout the organism, and this has increased the interest in non-gonadal effects of the FS/activin system. Recent results showed the regulation of FS and activin in inflammation, acute phase response and tissue repair (28, 29), arteriosclerosis (30), pulmonary fibrosis (31), liver cirrhosis (32) and ischemic brain injury (33). Although a wealth of data has been accumulated about FS/activin expression in different organs, the source of circulating FS and activin protein in serum has not yet been determined and the naturally occurring ratios of FS- and activin-subunit-mRNAs under physiological conditions are still unknown.

In this study we focused on the tissue distribution of FS-, activin A- and activin-B-mRNAs. Our data should facilitate the analysis of paracrine effects of the FS/activin system and add to earlier work on inhibin subunits (25). For this purpose, we developed a single-tube multiplex S1 nuclease assay which permits the direct comparison of FS-, activin beta A- and activin beta B-mRNA expressions. We used this assay to analyze the distribution of activin beta A-, activin beta B- and FS-mRNA expressions in porcine tissues, and to compare the relative ratios of the specific mRNAs within each tissue.

Materials and methods

Yeast tRNA was from Boehringer (Mannheim, Germany), herring sperm DNA type XIV and BSA fraction V from Sigma (Deisenhofen, Germany), S1 nuclease (*Aspergillus oryzae*) from Pharmacia (Freiburg, Germany), SP6 and T7 RNA polymerases and restriction enzymes from Promega (Heidelberg, Germany), and [32 P]UTP (20 μ Ci/ μ l) from Amersham Buchler (Braunschweig, Germany). All further chemicals and reagents were purchased in the purest grade available.

Preparation of RNA from tissues and cell cultures

Porcine tissues were prepared from 9-month-old freshly slaughtered female pigs (*Sus scrofa ferus*) from the local slaughterhouse (except for testis and epididymidis, which were taken from postpubertal 7-month-old male pigs), rapidly frozen in liquid nitrogen and stored at -80°C until use. Arterial tissue was removed from the thoracic aorta and venous tissue from the superior vena cava. Lymphatic tissue was taken from para-aortal lymphatic nodes. RNA preparation using guanidine isothiocyanate and CsCl was performed according to standard methods (34). RNA was dissolved in H_2O

to a concentration of 1.0–2.5 $\mu\text{g}/\mu\text{l}$. The integrity of each sample was checked by analyzing an aliquot on an ethidium bromide-stained, nondenaturing agarose gel and the amount of RNA was determined by measuring the optical density at 260 nm. Cell cultures from endothelial cells of porcine aorta and porcine brain microvessels as well as RNA from the cell cultures were prepared exactly as described recently (34).

PCR products and complementary RNA probes

RNA was extracted from porcine ovaries, and first strand cDNA was produced with random primers according to standard methods (35). The design of the specific PCR products and complementary cRNA probes for porcine follistatin, activin beta A and beta B was described elsewhere (34). All clones were sequenced to confirm the identity of the cloned fragments.

In a first set of experiments the plasmid containing the beta B subunit was cut with EcoRI to produce a 474 bp long cRNA probe. The 273 bp FS insert corresponding to the last 153 bp of exon 5 and the first 71 bp of exon 6 was cut with EcoRI and the plasmid containing the 194 bp beta A subunit insert was cut with HincII. After multiplex S1 nuclease analysis, the appropriate sizes of the digested fragments were 432 bp for beta B, 222 bp for FS and 180 bp for beta A. *In vitro* transcription of the beta A plasmid produced neotranscripts, which caused high background levels. Therefore, in further experiments the plasmids were cut in a different way: the beta B plasmid was cut with NARI, the FS plasmid was cut with EcoRI and the beta A plasmid was cut with KPN2I. The respective sizes of the digested fragments were 410 bp for beta A, 333 bp for beta B and 222 bp for FS.

In vitro transcription

For *in vitro* transcription the Gemini-kit (Promega) was used following the manufacturer's instructions. Antisense cRNAs for all plasmids were transcribed with T7 polymerase. Each radioactively labeled cRNA for FS, activin beta A and beta B was resuspended in a phosphate buffer (0.05 mol/l $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$, (pH 7.2) 0.7% sodiumdodecylsulfate (SDS), 0.1 mmol/l EDTA and 0.1% BSA fraction V) to a final concentration of 300 000–350 000 c.p.m./ μl . Aliquots of each labeled cRNA (10 000 000 c.p.m. (i.e. 28–33 μl)) were transferred to 900 μl hybridization buffer (150 μl 0.5 mol/l PIPES pH 6.8, 120 μl 5 mol/l NaCl, 6 μl 0.5 mol/l EDTA and 624 μl H_2O) and phosphate buffer was added to make up to a final volume of 1000 μl . The concentration of the phosphate buffer in the hybridization buffer was 10% and the final concentration of each labeled cRNA was approximately 10 000 c.p.m./ μl .

Multiplex S1 nuclease analysis

Twenty micrograms total RNA or yeast tRNA (negative control) were dissolved in 20 μ l H₂O, and then 10 μ l hybridization buffer including the labeled cRNAs (10 000 c.p.m./ μ l of each probe) were added to each tube. The hybridization mix was gently vortexed, centrifuged and two drops of mineral oil (Sigma) were added to each sample to avoid evaporation during the hybridization. The tubes were heated to 100 °C for 3 min and then immediately transferred to the incubator; hybridization was performed at 80 °C for 3 h.

After incubation, 470 μ l digestion buffer (per sample: 5 μ l 5 mol/l NaCl, 5 μ l 3 mol/l Na-acetate pH 4.6, 5 μ l herring sperm DNA (10 μ g/ μ l), 20 μ l 0.1 mol/l ZnSO₄, 480 μ l H₂O and 350 U S1 nuclease) were added to each sample. The tubes were gently vortexed and centrifuged. Digestion was performed in a water bath for 60 min at 37 °C. The reaction was stopped by adding 100 μ l 4 mol/l ammonium acetate/0.1 mol/l EDTA. Each sample was precipitated with 700 μ l isopropanol containing 1 μ l dextran blue (20 μ g/ μ l) per sample and 20 μ g yeast tRNA and washed with 500 μ l 70% ethanol. The addition of dextran blue to the isopropanol resulted in a solid blue-coloured pellet, which minimized the loss of pellets during the precipitation step. The samples were resuspended in 5 μ l TE (10 mM TRIS, 1 mM EDTA) pH 8 plus 4 μ l loading buffer (80% formamide; 10 mmol/l EDTA pH 8; 1 mg/ml xylene cyanol FF and 1 mg/ml bromophenol blue). Samples were heated for 3 min to 90 °C and kept on ice for another 3 min. Thereafter, 2.5 μ l aliquots were loaded on denaturing acrylamide gels (7% acrylamide/8 mol/l urea). Autoradiography was performed on X-ray films with intensifying screens at -80 °C for varying time spans.

Data analysis and presentation

Hybridization signals on X-ray films were scanned with an Epson 9000 scanner and the Epscan-ScanPack 2 software analysis program. Quantification of the signals was achieved with the NIH Image 1.54 program. If the FS signals consisted of double bands as sometimes observed in earlier studies (36), both bands were scanned for quantification. To ensure the use of equal amounts of total RNA for each S1 nuclease analysis, total RNA was quantified by optical density measurements, and integrity of the RNA was examined by non-denaturing agarose gels (as described in Materials and methods). Data presented in the figures represent results confirmed in at least two other similar experiments.

Results

Evaluation of the multiplex S1 nuclease protection assay

To determine the accuracy and the linearity of the mRNA measurements, standard curves using 2.5, 5, 10

and 20 μ g total RNA from porcine ovary, cultured endothelial cells from porcine aorta (AEC) and from porcine brain microvessels (BMVEC) were produced. Figure 1 summarizes the results of activin beta A-, beta B- and FS-mRNA expression in these tissues and demonstrates the reproducibility of the measurements in the range from 2.5 to 20 μ g total RNA. There was no obvious interference between the three labeled plasmids (Fig. 1A). Activin beta B-mRNA was not detectable in AEC (Fig. 1B), and FS-mRNA was not detectable in 2.5 and 5 μ g but was detectable in 10 and 20 μ g total RNA from BMVEC (Fig. 1C).

Distribution of activin beta A-, beta B- and FS-mRNA in different porcine tissues

The distribution of activin beta A-, beta B- and FS-mRNA in porcine tissues is demonstrated in Fig. 2. Highest steady state levels of all three mRNAs were found in the ovary. FS showed a widespread expression with high levels in ovary, uterus, cerebellum, placenta and fat, while it was below the limit of detection in pituitary, spinal cord, testis, blood cells, and stomach (latter two not shown) (Fig. 2A). An unexpected result was the FS-mRNA levels in the kidney which revealed a high individual variation between different analyses. Activin beta A-mRNA expression was abundant in the ovary, adrenal gland, fat, vein, artery and uterus. We could not find specific signals for activin beta A-mRNA in lymphoid tissue, pituitary, epididymis, muscle, lung, intestine, liver, blood cells and stomach (latter two not shown) (Fig. 2B). Activin beta B-mRNA was highly expressed in the ovary, pituitary, uterus, placenta, thoracic aorta and cerebellum. No specific signal could be detected in lymphoid tissue, adrenal gland, epididymis, fat, muscle, heart, kidney, gut, liver, blood cells and stomach (latter two not shown) (Fig. 2C).

Comparison of relative activin beta A-, activin beta B- and FS-mRNA levels within single porcine tissues

In order to compare the proportion of activin beta A-, activin beta B- and FS-mRNA expressions in a certain porcine tissue, the relative ratios of the specific signals (as a percentage of the total radioactivity detected) are listed in Table 1. Activin beta A-mRNA predominated in the adrenal gland, fat, artery, vein and spinal cord. For activin beta B the relative amount was highest in pituitary, testis and placenta. No specific signals for activin beta A- and activin beta B-mRNA were detectable in epididymis, liver, lymphoid tissue, muscle and intestine, making FS-mRNA the predominant RNA in those tissues; FS also dominated in heart, cerebellum, ovary and uterus.

Figure 1 (A) Steady state levels of FS-, activin beta A- and activin beta B-mRNA in 2.5–20 μg total RNA from cultured endothelial cells of the porcine aorta (AEC) (lanes 1–4, 2.5 μg; lanes 5–8, 5 μg; lanes 9–12, 10 μg; lanes 13–16, 20 μg), cultured endothelial cells of porcine brain microvessels (BMVEC) (lanes 17–20, 2.5 μg; lanes 21–24, 5 μg; lanes 25–27, 10 μg; lanes 28–31, 20 μg) and porcine ovary (lanes 32–35, 2.5 μg; lanes 36–39, 5 μg; lanes 40–43, 10 μg; lanes 44–47, 20 μg) as determined by multiplex S1 nuclease analysis. On the right side of the autoradiograph 2 μl of the undigested probes are shown. The sizes (bp) are indicated by arrows. Arrows on the left side indicate the sizes (bp) of the protected fragments. (B, C, D) Autoradiographs were scanned and integration values are shown as arbitrary units for AEC (B), BMVEC (C) and ovary (D). The columns represent the means ± s.d. of 3–4 separately assayed samples.

Figure 2 (A) FS-, (B) activin beta A- and (C) activin beta B-mRNA expression in 20 μ g total RNA from various porcine tissues measured by multiplex S1 nuclease analysis (AG: adrenal gland; LT: lymphoid tissue). Values are expressed in relation to the expression of the respective mRNA in the ovary (100%). The values are representative of two further experiments. Blood cells and stomach did not display signals above background levels and are not included in the graphs.

Table 1 Relative expressions of FS-, activin beta A- and activin beta B-mRNA in 20 µg total RNA from porcine tissues as measured by multiplex S1 nuclease analysis. The values are representative of two similar experiments and are given as percentage of the total densitometric units of the respective tissue. Blood cells and stomach did not contribute signals above background levels and are not included in the table.

Porcine tissues	FS	beta A	beta B
Adrenal gland	15.3	84.7	b.l.d.
Artery	8.2	52.3	39.5
Cerebellum	58.2	9.9	31.9
Cerebrum	18.5	42.8	38.7
Colon	45.6	54.4	b.l.d.
Epididymis	100	b.l.d.	b.l.d.
Fat	21.3	78.7	b.l.d.
Heart	66.1	33.9	b.l.d.
Intestine	100	b.l.d.	b.l.d.
Kidney	53.1	46.9	b.l.d.
Liver	100	b.l.d.	b.l.d.
Lung	55.5	b.l.d.	44.5
Lymphoid tissue	100	b.l.d.	b.l.d.
Muscle	100	b.l.d.	b.l.d.
Ovary	50.9	17.7	31.4
Pituitary	b.l.d.	b.l.d.	100
Placenta	39.2	14.0	46.8
Spinal cord	b.l.d.	59.9	40.1
Testis	b.l.d.	43.5	56.5
Uterus	50.6	25.7	23.7
Vein	29.5	48.9	21.6

b.l.d., below the limit of detection.

Discussion

Activins and FS play an essential role in cell differentiation and proliferation in multiple tissues throughout the organism (2–12). While earlier work on activins and FS concentrated mainly on the effects of both proteins in endocrine and especially gonadal tissues, recent research has shown that both factors participate in the regulation of non-reproductive functions such as tissue repair and inflammation (28, 29). Since the actions of activin are neutralized by binding to FS, the physiological balance of both polypeptides is most important in activin-responsive tissues. Until now, no study has examined the relative amounts of mRNA steady state levels of all three polypeptides in multiple tissues of one species. For this reason, we developed a multiplex S1 nuclease assay to investigate the steady state levels of FS-, activin beta A- and beta B-mRNA in a variety of porcine tissues. The assay is based on an earlier published method (36), but has several advantages; it avoids poor dissolvability of RNA in high salt solutions, long hybridization times and loss of pellets during the precipitating step.

For evaluation of the assay, we performed an S1 nuclease analysis of FS-, activin beta A- and activin beta B-mRNA expression in 2.5, 5, 10 and 20 µg total RNA from tissue (porcine ovary) and cell culture (AEC and BMVEC). The small variation of the quadruplicate samples in the standard curves demonstrates the

reproducibility of the assay procedures. No signals for activin beta B-mRNA in AEC were detected. This was in contrast to earlier results performed with a single S1 nuclease analysis (34), where we could demonstrate activin beta B expression in AEC at a very low level. The difference is most likely caused by a slight decrease in sensitivity of the multiplex assay in comparison to the analysis of a single RNA. After long exposure times of the X-ray films, activin beta B-mRNA in AEC was visible. This, however, caused a massive overexpression of the stronger signals and an unacceptable increase of background signals. Therefore, the lack of detection of a certain RNA in a given tissue with our assay does not necessarily imply a complete lack of expression of the respective RNA, but that its expression is negligible in comparison to the detected subunits.

Meunier *et al.* (25) compared the expression of inhibin subunits in rat tissues and detected the highest level of beta A-mRNA in placenta and the ovary, followed by bone marrow, brain and spinal cord. Moderate signals were detected in spleen, adrenal and testis, while no expression of beta A-mRNA was found in the kidney, pancreas, liver and pituitary. Beta B-mRNA was detected in the ovary, pituitary, placenta, testis and brain. Our experiments yielded similar results in porcine tissues. Activin beta A-mRNA showed the highest expression in the ovary. It also predominated in the adrenal gland, fat tissue, vena cava, thoracic aorta and uterus. No specific signals were obtained in epididymis, pituitary, lymphoid tissue, lung, liver, muscle and intestine (Fig. 2B). The expression of beta B-mRNA was almost identical in rat (25) and porcine tissues (our study, Fig. 2C). Although the expression of a mRNA is the premise for the expression of the respective protein, it must be remembered that the occurrence of a specific mRNA does not necessarily imply its translation (37).

The tissue distribution of FS-mRNA in rat tissue was examined by Michel *et al.* (36): high levels of FS-mRNA were found in the ovary, kidney, muscle, uterus and cortex, while liver, spleen, blood cells and cerebellum did not display signals above background levels. The results of our study in porcine tissues showed a similar pattern, but we also found some significant exceptions. In contrast to earlier work with rat (36) and porcine tissues (38), the decreased sensitivity of our multiplex assay, in comparison to a single mRNA analysis (34), did not allow the detection of FS mRNA in porcine pituitaries. Nevertheless, the data clearly demonstrate the predominance of the beta B subunit mRNA in porcine pituitaries. Furthermore, we did not detect FS-mRNA in testis, and the signals of the kidney and muscle were unexpectedly low (Fig. 2A). As it is known that steady state levels of FS vary during development (26), this discrepancy might be due to age differences. In an earlier study, 10- to 12-fold differences in the steady state levels of FS-mRNA were seen in the rat kidney during postnatal rat development (26). This

study also demonstrated a high variation of FS-mRNA levels in the kidney. The high variation in kidney FS-mRNA levels was also seen in kidneys of adult mice, where RNA extracted from whole kidneys was used for analysis (O Schneider & U Michel, unpublished observations).

The physiological role of the FS/activin system *in vivo* is still controversial. Circulating activin A in serum increases with age, but is not correlated with the increase in follicle-stimulating hormone (24, 39). The source of FS and activin in serum is still unknown and a variety of studies suggest that the gonads are not the major source of both peptides in serum (40). Besides other effects (see Introduction), activin participates, for example, in the regulation of inflammation (29) and neuroprotection (41, 42) and the relative amounts of FS and activin are likely to be crucial for the correct adjustment of the processes. Another factor which might influence the availability of beta subunits is the possible concomitant expression of the inhibin alpha subunit in a given tissue (see Meunier *et al.* (25)).

In summary, we developed a multiplex S1 nuclease assay for FS-, activin beta A- and beta B-mRNA and analyzed the steady state levels of these mRNAs in different porcine tissues. With this assay we demonstrated a high variation in the relation of activin subunits- and FS-mRNA in the tissues examined. The physiological ratios of FS-/activin-mRNAs are an important prerequisite for studies of the expression of these RNAs under pathophysiological conditions. Although the expression of a mRNA does not necessarily provide evidence for the synthesis of the respective polypeptide, these data might facilitate the localization of tissues or/and organs which contribute to systemic FS and activin peptide concentrations.

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