EXPERIMENTAL STUDY

Thyroid hormone and retinoic acid induce the synthesis of insulin-like growth factor-binding protein-4 in prepubertal pig Sertoli cells

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Abstract

A large body of evidence suggests the existence of an intratesticular IGF system complete with ligands, receptors and binding proteins (IGFBPs); the aim of the present study was to evaluate tri-iodothyronine (T_3) and retinoic acid (RA) effects on IGFBP production by Sertoli cells. A significant dose-dependent increase in IGFBP-4 mRNA levels was observed in Sertoli cells cultured in the presence of physiological concentrations of T_3 or RA. This response was inhibited by cycloheximide, indicating that *de novo* protein synthesis is required, as well as by actinomycin D, suggesting that the increase in mRNA levels requires transcriptional activation. As shown by ligand blot assays the stimulatory effects of both agents on IGFBP-4 mRNA expression appears to be consistent with an enhanced synthesis and secretion of IGFBP-4, thus suggesting that the transcriptional response is transduced to the protein level. Our data establish an important direct role for T_3 and RA in regulating IGFBP-4 expression and consequently IGF activity at the testis level.

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Introduction

Thyroid hormone plays an important role in the regulation of normal testis development (for review see 1). An early and critical influence of thyroid status on Sertoli cell morphofunctional maturation and proliferation (2–5) as well as on testis growth (6–8) has been established. The exact role of thyroid hormone in the control of testis function, however, is not fully understood.

Specific cellular effects of thyroid hormone on target tissues are mediated by the interaction of the hormone with nuclear receptors that bind to regulatory regions of genes and modify their expression.

The presence of thyroid hormone receptors in Sertoli cells has been proved (9, 10) and, as regards genomic actions, a direct regulation of thyroid hormone on the Sertoli cell expression of androgen binding protein (11), glucose transporter isoform 1 (12), Mullerian-inhibiting substance (13), clusterin/inhibin (14) and collagen/entactin (15) mRNAs has been reported.

The aim of our previous and present research work was to evaluate the possible involvement of the insulin-like growth factor (IGF) system in mediating thyroid hormone effects at the Sertoli cell level. Actually the

testis harbours a complete IGF system replete with ligand, receptor and IGF-binding proteins (IGFBPs), and Sertoli cells are known to produce IGF-I, a powerful mitogenic and differentiating factor, which may play a fundamental role in the paracrine/autocrine regulation of testis development and function (16).

The effects of IGF-I are mediated by specific IGF receptors and modulated by IGFBPs. Currently, six IGFBPs have been cloned and sequenced. IGFBPs have been proposed to act as modulators of IGF by either potentiating or inhibiting the actions of the growth factor (for reviews see 17, 18).

The aim of the present study was to establish whether tri-iodothyronine (T_3) can directly affect IGFBP expression in cultured Sertoli cells. We also tested the possible involvement of retinoic acid (RA), which is known to have an impact on Sertoli cell physiology (19) and to act via nuclear receptors belonging to the steroid/thyroid hormone receptor superfamily.

In our experiments we utilised primary cultures of porcine Sertoli cells, which have been previously demonstrated to be a suitable and useful model for the study of thyroid hormone effects at the testis level. Actually, nuclear T_3 receptors have been characterised in Sertoli cells isolated from prepubertal piglet testes

(20); moreover, in these cells thyroid hormone has been shown to significantly increase the production of transforming growth factor β -like protein (21) and the rate of protein synthesis (22).

Materials and methods

Cell isolation and culture

Sertoli cells, obtained from 2- to 3-week-old piglet testes, were prepared and purified as previously described (23). Cells were plated at a density of $10^7/10$ ml per dish (diameter 100 mm) and cultured at 32 °C in a humidified atmosphere of 5% CO₂, in (1:1) Dulbecco's modified Eagle's/Ham's F12 medium (Grand Island Biological Co., Grand Island, NY, USA) containing 1.2 mg sodium bicarbonate/ml, 15 mmol/l Hepes and 20 μ g gentamicin/ml; this medium was supplemented with transferrin (5 μ g/ml) and vitamin E (10 μ g/ml). During the first 24 h in culture Sertoli cells were maintained in the presence of 0.5% (v/v) foetal calf serum. Therewith Sertoli cells were cultured for 1 day in control medium and for an additional 1–2 days in the presence or absence of physiological concentrations of T₃ or RA.

T₃ and RA were added to the culture medium at the indicated concentrations. The protein synthesis inhibitor cycloheximide (CEX) and the transcription inhibitor actinomycin D (ActD) were added to the cultures at concentrations of 0.1 and 5 μ g/ml respectively. To minimise side-effects by translational or transcriptional inhibition, the incubation time was reduced to 6 h. T₃, RA, CEX, ActD, transferrin and vitamin E were supplied by Sigma Chemical Co., St Louis, MO, USA.

RNA isolation

Total RNA was prepared using acid guanidinium thiocyanate–phenol–chloroform extraction (24). RNA was quantified by measuring the absorbance at 260 nm. Samples were stored at –80 °C until used.

cDNA probes

The rat IGFBP-1 (25), -2 (26), -3 (27), -4 (28), -5 (29) and -6 (30) cDNA probe was kindly provided by Dr S Shimasaki, Department of Cell Biology, The Scripps Research Institute, La Jolla, CA, USA. The rat 18S rDNA probe (BamHI–HindIII fragment) (31) was generously provided by Dr I G Wool, Department of Biochemistry and Molecular Biology, The University of Chicago, Chicago, IL, USA. The probes were labelled to high specific activity by multiprime DNA labelling systems (Amersham International plc, Amersham, Bucks, UK) using [³²P]dCTP (NEN Du Pont, Du Pont De Nemours GmbH, Bad Homburg, Germany; specific radioactivity 3000 Ci/mmol).

Northern blot analysis

Total RNA (20 μ g) was fractionated by loading on 1% agarose gel containing 2.2 mol/l formaldehyde in 20 mmol/l 3-[N-morpholino]propanesulfonic acid (pH 7.0), 5 mmol/l sodium acetate and 1 mmol/l Na₂EDTA. RNA was stained with ethidium bromide to check for the integrity of the samples and for equal loading in each lane. After transfer to nylon membranes by capillary blotting, prehybridisation was performed for 2 h at 42 °C in a solution containing 5 $\times$ SSPE (1 $\times$ SSPE = 0.15 mol/l NaCl, 0.01 mol/l NaH₂PO₄, 1 mmol/l EDTA), 50% formamide, 5 $\times$ Denhardt's solution, 0.5% SDS and 100 μ g/ml denatured salmon sperm DNA (32). Hybridisation was carried out for 20 h at 42 °C in fresh buffer containing the ³²P-labelled probe. After hybridisation, the filters were washed sequentially in 2 $\times$, 1 $\times$ and 0.1 $\times$ SSPE, 0.1% SDS at 42 °C, each wash lasting for 20 min. Filters were then exposed to Amersham Hyperfilm-MP films at –80 °C using intensifying screens.

To ensure even loading, the membranes were stripped and rehybridised with a rat 18S rDNA probe.

The signal intensity of the autoradiograms was quantified by scanning densitometric analysis using the Scion Image software package (Scion Corporation, USA). Arbitrary densitometric units were normalised to 18S and expressed as a percentage of the control values.

Ligand blotting

Sodium [¹²⁵I]iodide (carrier-free) was purchased from Amersham. Recombinant human IGF-I (lot no. 810288) was a gift from Novartis Pharma AG (Basel, Switzerland). IGF-I was iodinated by the chloramine-T method to a specific activity of approximately 60–80 μ Ci/ μ g.

For ligand blotting, samples were analysed according to Hossenlopp *et al.* (33) with slight modifications. Briefly 150 μ g protein from supernatant medium were separated on 10% SDS-PAGE under non-reducing conditions and transferred to nitrocellulose. Membranes were blocked overnight at 4 °C in 0.9% NaCl, 50 mmol/l Tris–HCl (pH 7.4), 0.5% fish gelatine, followed by incubation with ¹²⁵I-IGF-I (5–10 $\times$ 10⁶ c.p.m.) at 4 °C for 24 h in 0.9% NaCl, 50 mmol/l Tris–HCl (pH 7.4), 0.5% fish gelatine, 0.2% NP-40. After washing, membranes were air-dried and exposed for 24 h at –80 °C to X-ray films. The signal intensity of the autoradiograms was quantified by scanning densitometric analysis using the Scion Image software package.

Data analysis

The experiments reported here were repeated at least two or three times with independent cell preparations. A representative experiment of each series is presented; data are given as mean $\pm$ S.E.M. Statistical significance

was analysed using ANOVA with the multiple group comparison test of Scheffé. Differences are accepted as significant at $P \leq 0.05$.

Results

Preliminary analyses demonstrated that in cultured Sertoli cells IGFBP-2, -3, -4 and -5 messages were clearly expressed whereas IGFBP-1 and -6 mRNAs were not detected or barely detectable.

A significant increase in IGFBP-4 mRNA levels was observed in Sertoli cells cultured in the presence of physiological concentrations of T_3 or RA.

Figure 1 shows the result of Northern blotting experiments. Compared with the respective controls, the induction of IGFBP-4 mRNA was detectable at all time points studied. As observed in the time course assays IGFBP-4 mRNA levels in Sertoli cells cultured in the presence of T_3 reached a maximum between 6 and 12 h, while in RA-exposed cells the highest amount of the specific transcript was detectable between 12 and 24 h.

The dose-response curves of the effects of T_3 and RA on IGFBP-4 mRNA expression are illustrated in Fig. 2; the maximum response to thyroid hormone occurred at concentrations ranging from 10^{-8} to 10^{-10} mol/l, whereas the maximum increase in IGFBP-4 mRNA level was elicited by RA at a concentration of 10^{-7} / 10^{-8} mol/l. No synergistic or additive effects of the two agents on IGFBP-4 expression were observed (data not shown).

To establish whether increased levels of IGFBP-4 mRNA depend on transcriptional activation or to a greater stability of the messages, the effects of the transcriptional inhibitor ActD were tested. Moreover, to further investigate whether the stimulation of IGFBP-4 mRNA requires *de novo* protein synthesis, CEX was used as an inhibitor. As shown in Fig. 3, ActD and CEX both prevented the stimulation of IGFBP-4 mRNA expression by T_3 and RA.

IGFBP levels in conditioned medium of hormonally treated Sertoli cells were evaluated by ligand blotting. As shown in Fig. 4, the addition of T_3 and RA resulted in a dramatic increase in the production of an IGF-binding protein with a molecular mass of about 24 kDa corresponding to IGFBP-4 (34–36); a second major band of 29 kDa, probably representing the glycosylated form of IGFBP-4 (37), was also detected.

Discussion

A large body of evidence demonstrates the existence of an intratesticular IGF system complete with ligands, receptors and binding proteins (38). Within the testis the Sertoli cell has been proposed to be a site of IGF-I production (39), reception (40) and action (41, 42) as well as of IGFBP generation (43). The intratesticular

Figure 1 Time course of T_3 and RA effects on IGFBP-4 mRNA levels in immature pig Sertoli cells cultured for different times in the presence or absence of hormones. (A) Representative autoradiograms of Northern blots showing IGFBP-4 mRNAs. As a control to ensure even loading, hybridisation using an 18S probe was performed. (B) Densitometric quantification of IGFBP-4 Northern blots after normalisation to 18S. Data are given as a percentage of the control value. Values are the mean $\pm$ S.E.M. of three separate experiments. * $P \leq 0.05$, ** $P \leq 0.01$ (treated vs control).

IGF system appears to be under a complex regulation by endocrine and paracrine factors (16) not fully understood. On the one hand, previous evidence has suggested that the IGF system is a target for thyroid hormone in both the new-born and adult rat (44–47); on the other hand thyroid hormone as well as RA is known to be essential for normal testis development (1, 19) and T_3 has been proved to significantly increase IGF-I production by immature rat Sertoli cells (48).

IGF action is mediated by specific receptors and modulated by IGFBPs (17); despite many similarities, the different IGFBPs have distinct structural and functional properties, which together with differences in their tissue and developmental expression, determine the individual regulatory roles of the different IGFBPs (18).

Figure 2 Dose dependence of T_3 and RA effects on IGFBP-4 mRNA levels in immature pig Sertoli cells cultured in the presence or absence of different concentrations of hormones. (A) Representative autoradiograms of Northern blots showing IGFBP-4 mRNAs. As a control to ensure even loading, hybridisation using an 18S probe was performed. (B) Densitometric quantification of Northern blots after normalisation to 18S. Data are given as a percentage of the control value. Values are the mean $\pm$ S.E.M. of three separate experiments. * $P \leq 0.05$, ** $P \leq 0.01$ (treated vs control).

In this study we employed Sertoli cell primary cultures isolated from immature piglet testes to analyse the possible direct effect of T_3 and/or RA on IGFBP gene expression.

Our data establish a direct role for T_3 and RA in regulating IGFBP-4 expression at the Sertoli cell level. Actually, both agents induced a significant dose-dependent increase in IGFBP-4 mRNA levels over all time points tested. Such an increase requires new transcription, since it was abolished by the presence of ActD in the culture medium along with hormones. On the other hand, the CEX inhibition of T_3 and RA stimulation indicates that *de novo* protein synthesis is required, suggesting the involvement of a hormone-induced regulatory protein to enhance IGFBP-4 mRNA expression.

As shown by ligand blot assays the hormone-dependent increase in IGFBP-4 mRNA levels appears to be consistent with an enhanced synthesis and secretion of IGFBP-4, thus suggesting that the transcriptional response is transduced to the protein level.

A similar T_3 -increased IGFBP-4 expression has been recently reported in cultured rat hepatocytes and is consistent with *in vivo* experiments demonstrating an increase in serum IGFBP-4 levels in T_3 -treated rats (36). In addition, previous studies showed the specific stimulation of IGFBP-4 expression by T_3 and/or RA in human breast cancer cell lines (49) and mouse osteoblasts (35).

Taken together these results suggest a possible direct regulation of the IGFBP-4 gene by both T_3 and RA via their nuclear receptors, probably through a common receptor-binding site within the IGFBP-4 promoter.

Figure 3 Translational (CEX) and transcriptional (ActD) inhibition of T_3 and RA effects on IGFBP-4 mRNA levels in immature pig Sertoli cells cultured under different experimental conditions. (A) Representative autoradiograms of Northern blots showing IGFBP-4 mRNAs. As a control to ensure even loading, hybridisation using an 18S probe was performed. (B) Densitometric quantification of Northern blots after normalisation to 18S. Data are given as a percentage of the control value. Values are the mean $\pm$ S.E.M. of three separate experiments. * $P \leq 0.05$, ** $P \leq 0.01$ (treated vs control).

Actually, the thyroid hormone receptor and RA receptor are structurally similar and can bind as homodimers or heterodimers to a single synthetic DNA response element, acting as ligand-dependent transcription factors (50, 51). Both receptors belong to the steroid/thyroid hormone receptor superfamily, and interestingly one progesterone receptor-binding site has been localised in the rat IGFBP-4 gene 5'-flanking region (52).

The IGFBPs are known to act as modulators of the IGF system by either potentiating or inhibiting IGF action. Although its physiological role is still unclear, IGFBP-4 appears to inhibit IGF mitogenic actions under most experimental conditions, suggesting that it is an important negative regulator of cellular proliferation (17, 53). A major factor regulating IGFBP action is its

Figure 4 Effects of T_3 and RA on IGFBP-4 production by immature pig Sertoli cells cultured for 24 h in the presence or absence of hormones. (A) Representative autoradiograms of ligand blots showing IGFBP-4 levels in the conditioned medium of Sertoli cells cultured under different experimental conditions. (B) Densitometric quantification of ligand blots after normalisation for protein loading. Data are given as a percentage of the control value. Values are the mean $\pm$ S.E.M. of two separate experiments. (treated vs control: $P \leq 0.05$).

distribution between the soluble phase (interstitial fluid) and the extracellular matrix (ECM) or cell surface; inhibitory effects of IGFBP on IGF action are associated typically with high-affinity soluble forms. IGFBP-4 acts exclusively as an inhibitor of IGF action, because it does not associate with ECM components or the cell surface (18).

Our previous study suggested the involvement of IGF-I in mediating testicular effects of thyroid hormone (48). The present results strongly suggest a direct role for thyroid hormone in regulating IGFBP-4 expression and consequently in modulating IGF activity at the gonadal level.

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