Thyroid hormone effects on androgen receptor messenger RNA expression in rat Sertoli and peritubular cells

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Abstract

Postnatal Sertoli cell maturation is characterized by a pronounced rise in androgen receptor (AR) expression, which increases several fold between birth and adulthood. Since both 3,3′,5-triiodothyronine (T3) and FSH regulate Sertoli cell proliferation and differentiation, we have determined the effects of T3 and FSH on AR mRNA expression in cultured Sertoli cells from 5-day-old rats. These cultures contain 5–9% peritubular cells, which also express AR mRNA. To insure that the observed T3 responses did not result from peritubular cells, we examined T3 effects on AR mRNA expression in cultured 20-day-old Sertoli cells (which contain minimal peritubular contamination) and peritubular cells, and measured thyroid hormone receptor (TR) mRNA expression in both of these cell types. Sertoli cells from 5- and 20-day-old rat testes were grown in serum-free medium alone (controls) or with ovine FSH (100 ng/ml) and/or T3 (100 nm) for 4 days. Peritubular cells purified from 20-day-old rat testes were grown in serum-containing medium for 8 days. These cells were split 1:4, and grown an additional 8 days, the last 4 days in serum-free medium with or without T3. TR and AR mRNA levels in all cultures were determined by Northern blotting. AR mRNA levels in 5- and 20-day-old cultured Sertoli cells were significantly (P<0.05) increased by both T3 and FSH alone. Furthermore, AR mRNA levels in Sertoli cells treated with T3 and FSH were greater than with either alone. TR mRNA expression was detected in cultured peritubular cells, but TR mRNA levels in these cells were only approximately 30% of that seen in 20-day-old cultured Sertoli cells. In contrast to Sertoli cells, T3 did not affect peritubular AR mRNA expression. These results indicate that T3 is an important regulator of the postnatal Sertoli cell AR mRNA increase. The additive effects of maximally stimulatory doses of FSH and T3 suggest these hormones work through different mechanisms to increase AR mRNA. TR mRNA expression in peritubular cells indicates these cells may be direct T3 targets, though the function of T3 in these cells is unknown.

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Introduction

Postnatal Sertoli cell maturation is characterized by steadily increasing androgen receptor (AR) expression, which is barely detectable in these cells at 5 days postnatal but reaches maximal levels by 90 days of age (Bremner et al. 1994, Shan et al. 1995, 1997). Androgens are essential for the initiation of normal spermatogenesis and adult Sertoli cell function (Griswold 1995). This rise in Sertoli cell AR expression during neonatal and juvenile life may be necessary for the Sertoli cell to achieve full differentiation and androgen responsiveness, resulting in a cell competent to support complete spermatogenesis. The factors which regulate the increase in AR expression during Sertoli cell development are therefore of great importance. Follicle-stimulating hormone (FSH), the major tropic hormone for Sertoli cells (Griswold 1993), increases expression of AR and its mRNA in juvenile Sertoli cells (Sanborn et al. 1984, 1991, Verhoeven & Cailleau 1988), and androgens also appear to regulate Sertoli cell AR (Shan et al. 1997). However, the overall hormonal mechanisms regulating Sertoli cell AR expression during development are not well understood.

Extensive work in the past few years has indicated that thyroid hormones are major regulators of Sertoli cell development. Transient neonatal hypothyroidism in rats produces unprecedented increases of 80 and 140% in adult testis weight and sperm production respectively (Cooke & Meisami 1991, Cooke et al. 1991). This increase in testis size results primarily from increased Sertoli cell proliferation during neonatal and juvenile life (van Haaster et al. 1992) and consequent larger populations of adult Sertoli and germ cells (van Haaster et al. 1992, Hess et al. 1993). Sertoli cells express thyroid hormone receptor (TR) and its mRNA during the neonatal period (Jannini et al. 1990, 1994) and 3,3′,5-triiodothyronine (T3), the biologically active thyroid hormone, directly inhibits Sertoli cell proliferation in vitro (Cooke et al. 1994). T3 also appears to stimulate maturation of Sertoli cells (van Haaster et al.
1993), and has been shown to directly increase production of Sertoli cell secretory proteins (Cooke et al. 1994, Janninni et al. 1995) and decrease aromatase activity (Panno et al. 1994, Ulisse et al. 1994).

The aim of the present study was to use cultured neonatal Sertoli cells to determine if T₃ might be involved in the Sertoli cell AR increase during development. These experiments are complicated by the presence of peritubular cells in the cultures, which express high levels of AR neonatally and could make interpretation of the results difficult. Therefore, we also directly evaluated the expression of TR mRNA by cultured peritubular cells and the ability of T₃ to regulate AR mRNA in these cells in vitro to determine if these cells had the capacity to directly respond to T₃ and whether T₃ might also modulate AR mRNA expression in peritubular cells.

Materials and Methods

Animal purchase, care and breeding were as described previously (Cooke & Meisami 1991). All experiments described here involving animals were approved by the Laboratory Animal Care Advisory Committee of the University of Illinois, and were conducted in accordance with the Guiding Principles for the Care and Use of Research Animals.

Sertoli cell culture from day-old rats

Sertoli cells from day-old Sprague–Dawley rats (day of birth=day 0) were isolated using a sequential enzymatic procedure that we (Cooke et al. 1994) and others (Rong-Xi et al. 1987, Ort & Boehm 1990) have described previously. Briefly, for each culture, pools of Sertoli cells were obtained from eight to ten males from two litters. Sertoli cells were grown in 24-well plates coated with Matrigel (Collaborative Research, Waltham, MA, USA) diluted 1:5 with Hank’s balanced salt solution. Cells were plated at a density of 4 × 10⁵ cells/well. The nutritive media was Dulbecco’s minimum essential medium (DMEM) supplemented with sodium pyruvate (1 mM), non-essential amino acids (0.1 mM) and an antimicrobial solution (Rong-Xi et al. 1987, Ort & Boehm 1990). Cells were grown for 4 days in a humidified atmosphere of 95% air/5% CO₂ at 34 °C on 24-well plates coated with Matrigel, as described for 5-day-old Sertoli cells. The nutritive medium was the same as used for Sertoli cells, but supplemented with 10% fetal calf serum (Sigma); medium was changed every 2 days. Cells were grown for 8 days, then removed from their substrate using trypsin (0.25%), split 1:4 and replated into 24-well plates as above. Cells were grown an additional 4 days in serum-containing medium, then cultured for 4 days without serum and either with or without T₃ (100 nM). The data shown are based on five separate isolation procedures.

Viability and purity of cultured cells

For all cell cultures described, the percentage of viable cells was determined by trypan blue exclusion before plating. To assess contamination with other cell types, cultures were examined daily using an inverted microscope. In addition, at the end of the culture, wells of all cell types were stained for alkaline phosphatase or 3β-hydroxysteroid dehydrogenase (3β-HSD), which are markers for peritubular and Leydig cells respectively (Chapin et al. 1987, Ort & Boehm. 1990, Cooke et al. 1994). Since Leydig
cells differentiate during early postnatal life and may not
stain as intensely for 3β-HSD during this period as fully
mature adult Leydig cells, both intensely and weakly
stained cells were scored as positive.

Northern analysis

Total RNA was prepared from all cell cultures using the
RNaseasy Mini Kit (Qiagen, Chatsworth, CA, USA). Total
RNA was also isolated from 20-day-old testis and adult
spleen, which served as positive and negative controls
respectively, for the various probes. Purified total RNA
was dissolved in diethylpyrocarbonate (DEPC)-treated
water. Purity and concentration of the RNA were deter-
mined by UV (260/280 nm) absorbance in a spectropho-
tometer. Equal amounts of total RNA (8 µg) from the
various treatment groups were electrophoresed on a 1·5%
agarose formaldehyde gel. The gel was blotted to nylon
membrane and the RNA fixed to the membrane by UV
cross linking.

The following probes were used in this study: 1) c-erbAα, a probe which detects both TRα1 and
α2 mRNA (Murry et al. 1988), 2) rat antigen binding
protein (ABP) (Joseph et al. 1987), 3) rat AR (Chang et al.
1988) and 4) human 28S rRNA (Erickson & Schimickel
1985). Initial Northern studies of AR mRNA expression
were performed with the full-length 2·83kb AR cDNA
construct, while later experiments were performed using a
1·15 kb AR cDNA fragment (Chang et al. 1988). Both
probes recognized the 9·5 kb AR mRNA transcript
(Chang et al. 1988). Insert for each cDNA probe was
isolated from the plasmid vector by restriction digestion
and gel purification. Each insert was labeled with
32P-dCTP using the Multiprime DNA labeling system
(Amersham, Arlington Heights, IL, USA) and used to
probe the membrane.

All hybridizations were carried out in QuikHyb
(Stratagene, La Jolla, CA, USA) according to manu-
facturer’s recommendations at 68 °C in a Robbins
scientific hybridization oven (Sunnyvale, CA, USA). The
hybridized membrane was washed, covered with plastic
wrap and exposed to Kodak X-omat X-ray film with
intensifying screens. After hybridization with the TRα
cDNA probe, membranes were stripped of probe by
incubation in 50% formamide at 65 °C for 1 h and then
rehybridized with the AR cDNA probe. These Northern
blots were also rehybridized with the ABP cDNA probe.
For normalization of RNA load between lanes, the
membrane was reprobed for a final time with labeled 28S
rRNA cDNA probe.

The mRNA bands on the autoradiograms were scanned
and quantitated using a computer-linked scanning laser
densitometer and RFLPrint software (Pdi, Huntington
Station, NY, USA). Relative levels of mRNA transcripts
were adjusted to compensate for differences in total RNA
loaded per gel lane as determined by densitometry of 28S

rRNA hybridization signals (Bunick et al. 1994). All
statistical analyses were performed using the SYSTAT
statistical package (Wilkinson 1990). The optical densities
(OD) of the bands in the various treatment groups were
compared by two-way ANOVA, and differences between
various treatment groups were compared using Tukey’s
HSD test. Differences were considered significant when
P<0·05.

Results

Characterization of Sertoli and peritubular cell cultures

Trypan blue exclusion experiments indicated that more
than 95% of the isolated peritubular and Sertoli cells were
viable. The Sertoli cell cultures from the 5-day-old rats
had a morphology typical of Sertoli cells in vitro (Cooke
et al. 1994). Approximately 5% of the cells were peri-
tubular, as judged by their morphology and positive
alkaline phosphatase staining, while Leydig cell contami-
nation was minimal (<1%). The Sertoli cell cultures from
the 20-day-old rats also showed a very low level of Leydig
and peritubular cell contamination (<1%). Primary
peritubular cell cultures consisted predominately of fibro-
blastic cells; small numbers of Sertoli cells could also be
identified by their nuclear morphology and cell shape.
However, after the peritubular cells were passaged, Sertoli
cells were absent and cultures consisted exclusively of
fibroblastic cells. Alkaline phosphatase staining was very
strong in peritubular cell cultures, while less than 1% of the
cells stained for 3β-HSD, indicating minimal Leydig cell
contamination.

Expression of AR and TR mRNA in Sertoli cell cultures
from 5-day-old rats

In Northern blots for AR (Fig. 1), a 9·5 kb band which has
previously been shown to correspond to AR mRNA
detected in cultured Sertoli cells from 5–day-old rats. AR
mRNA was also detected in 20–day-old rat testis, a
positive control, but not in spleen, a negative control
which lacks detectable AR mRNA (Shan et al. 1995).
Densitometric analysis of hybridization results (Fig. 2)
indicated that steady-state levels of AR mRNA expression in cells treated with T3 or FSH alone were both slightly under 2-fold that of the untreated controls (\(P<0.05\) for both compared with controls). AR mRNA expression in cells treated with both of these hormones was approximately 2.5-fold that of control cultures, indicating that T3 and FSH acted additively to increase AR mRNA expression to a level greater than with either hormone alone. Cultured 5-day-old Sertoli cells also expressed relatively high levels of TR\(\alpha1\) and \(\alpha_2\) mRNA (data not shown).

Figure 2  Normalized densitometric data of AR mRNA expression by 5-day-old Sertoli cells cultured 4 days with or without hormonal supplementation, as in Fig. 1. Signals were normalized against a 28S rRNA cDNA probe to compensate for interlane differences in RNA loading. The values for each of the treatment groups were expressed as a fold increase relative to the control, and the data are presented as means ± S.E.M. of values from 10 separate experiments. The mean values for all treatment groups were different from each other (\(P<0.05\)) with the exception of the groups treated with FSH or T3 alone, which did not differ.

Expression of AR, TR and ABP mRNA in Sertoli and peritubular cell cultures from 20-day-old rats

Hormonal regulation of AR mRNA expression in 20-day-old Sertoli cells (Fig. 3) was similar to that in 5-day-old Sertoli cells. Normalized densitometric data (Fig. 4) indicated that T3, FSH, or T3 and FSH significantly (\(P<0.05\)) stimulated steady-state AR mRNA expression above control levels. The T3 and FSH response was greater than that seen with either hormone alone, and the T3 and FSH groups were not significantly different. In some cases, RNA from both 5- and 20-day-old Sertoli cell cultures were run on the same Northern blot, which allowed a direct comparison of mRNA levels of AR in the various groups. These results indicated that 20-day-old Sertoli cells expressed approximately 2-fold more AR mRNA than 5-day-old cells from the same treatment group (not shown).

Although AR mRNA could be clearly detected in peritubular cell cultures, T3 treatment did not have a significant effect on the steady-state AR mRNA level (Fig. 3), a conclusion confirmed by analysis of the normalized densitometric data (not shown). Thus, T3 does not appear to stimulate AR mRNA in cultured peritubular cells as it does in Sertoli cells.

Northern blotting for TR mRNA in Sertoli cell cultures from 20-day-old rats indicated that they strongly
expressed TRα1 and α2 mRNA (Fig. 5). However, TRα1 and α2 mRNA expression was not significantly different in the 5- or 20-day-old Sertoli cells or amongst the different treatment groups at these ages. Therefore, in contrast to the results obtained with AR mRNA, neither FSH nor T3 treatment nor age of the Sertoli cells used had a significant effect on the steady-state levels of TRα1 and α2 mRNA.

TRα1 mRNA was also detected in freshly isolated peritubular cells from 20-day-old rats (data not shown) and in these peritubular cells following 16 days of culture (Fig. 5). Both TRα1 and α2 mRNA were detected in cultured peritubular cells and the relative abundance of the TRα2 mRNA was approximately 2-fold greater than that for TRα1 in both the peritubular and Sertoli cells. TRα mRNA expression in peritubular cells was less than in Sertoli cells; in the control cultures, the relative abundance of TRα1 and α2 mRNA per µg of RNA was about 3-fold greater in cultured 20-day-old Sertoli cells compared with 20-day-old peritubular cells (data not shown).

Expression of ABP mRNA in peritubular cell cultures was not detectable, in stark contrast to the 20-day-old Sertoli cell cultures, in which ABP mRNA was strongly expressed (Fig. 6). Therefore, Sertoli cell contamination is minimal in the peritubular cell cultures.

**Discussion**

Sertoli cell AR is barely detectable by immunohistochemistry in 5-day-old neonatal rats (Bremner et al. 1994). AR concentration per Sertoli cell then increases during juvenile life, reaching maximal levels during adulthood (Buzek et al. 1987, Buzek & Sanborn 1988, Shan et al. 1997). The rise in Sertoli cell AR expression closely parallels that in Sertoli cell AR mRNA expression. For example, Sertoli cells show a 2-fold rise in both AR and AR mRNA between day 21 and adulthood (Shan et al. 1995, 1997).

The present results indicate that T3 treatment results in increased AR mRNA expression in cultured Sertoli cells from 5-day-old rats and may be an important endocrine regulator of the developmental increase in Sertoli cell AR in vivo. Recent work has also indicated that T3 increases androgen binding in cultured juvenile rat Sertoli cells (Panno et al. 1996). Our results suggest that the increased AR levels observed in this study in response to T3 result from an increase in AR mRNA steady-state levels rather than changes in translation.

The mechanism by which T3 treatment increases AR mRNA expression is not clear. T3 has previously been shown to cause a number of changes which are associated with increasing maturation in the neonatal Sertoli cell, such as decreased proliferation, increased production of secretory proteins, and active pro luminal secretion of fluid (Francavilla et al. 1991, van Haaster et al. 1993, Cooke et al. 1994). It therefore seems likely that the increases in steady-state AR mRNA levels may reflect general stimulatory effects of T3 on Sertoli cell maturation rather than a specific, direct effect of T3 on AR mRNA production, though the latter possibility can not be totally excluded.

The present results are the first demonstration that FSH stimulates AR mRNA expression in Sertoli cells of neonatal rats. In addition, although the stimulatory effects of FSH on AR mRNA in juvenile Sertoli cells have been shown (Sanborn et al. 1991), the present results are the first to show that the combined effects of T3 and FSH on AR mRNA in neonatal and juvenile Sertoli cells are greater than with either alone. The additive effects of T3 and FSH suggest that these hormones are not causing their effects through the same pathway, a conclusion consistent with...
previous observations that T₃ and FSH effects on other Sertoli cell parameters, such as production of mRNA for an inhibin subunit, are also additive (Cooke et al. 1994).

Testosterone also affects AR expression in postnatal Sertoli cells (Verhoeven & Cailleau 1988, Sanborn et al. 1991). Therefore, it appears that a number of hormones, including T₃, FSH and testosterone, and possibly other hormones as well, are necessary for the normal postnatal rise in Sertoli cell AR expression. Additional studies on the role of all of these hormones, alone and especially in combination, will be necessary to obtain a complete understanding of the factors that control Sertoli cell AR mRNA expression, the overall developmental sequence of postnatal Sertoli cell maturation, and the onset of functional activity in these cells.

One possible complication of using Sertoli cell cultures from early postnatal rats is that they contain significant peritubular cell contamination. Schlatt et al. (1996) showed that Sertoli cell cultures from 7-day-old rats contain approximately 6% peritubular cells after 3 days of culture, and 9% peritubular cells by the seventh day of culture. Peritubular cell AR expression exceeds that of Sertoli cells in 5-day-old rats (Bremner et al. 1994), indicating that even low levels of contaminating peritubular cells in these cultures could significantly affect total AR measurements. Therefore, data showing effects of T₃ on AR or its mRNA, such as our Northern results with Sertoli cells from 5-day-old rats and previous androgen binding data obtained from Sertoli cells derived from 2-week-old rats (Panno et al. 1996), must be interpreted cautiously.

Sertoli cell cultures from 20-day-old rats have been reported to contain less than 1% peritubular cell contamination (Raychoudhury et al. 1993), in contrast to the 5–9% peritubular cell contamination seen in neonatal Sertoli cell preparations (Cooke et al. 1994, Schlatt et al. 1996). The ability to obtain purer cultures with juvenile rat Sertoli cells allows the potentially confounding effects of peritubular cells to be minimized. Therefore, to obtain unequivocal information on regulation of AR mRNA in Sertoli cells, we also examined the effects of T₃ and FSH on AR mRNA expression in 20-day-old Sertoli cells.

The effects of T₃ and FSH, alone and in combination, on AR mRNA expression in the 20-day-old Sertoli cell cultures were similar to that seen in the 5-day-old cultures. These results, obtained in cultures where peritubular cell contamination is minimal, strongly suggest that the stimulatory effects of T₃ and T₃ and FSH seen in the 5-day-old Sertoli cell cultures were due to the effects of these hormones on the Sertoli cells themselves, rather than effects on the contaminating peritubular cells in these cultures. In addition, the 20-day-old Sertoli cells expressed approximately twice the AR mRNA of their 5-day-old counterparts, consistent with previous data suggesting a rise in AR between the neonatal and juvenile periods (Buzek et al. 1987, Bremner et al. 1994).

Previous work has indicated that TRα1 and α2 mRNA are produced at high levels in the testis and Sertoli cells neonatally. This expression declines with advancing age, reaching relatively low levels during adulthood (Jannini et al. 1990, 1994, Bunick et al. 1994). The major functional form of TR in the developing testes and Sertoli cells is TRα1. The other major functional TR, TRβ1, can only be detected in Sertoli cells by PCR techniques (Jannini et al. 1994, Palmero et al. 1995), indicating very low abundance. TRα2 (also called c-erbAα2) is a splicing variant of the TRα locus, and does not bind T₃ despite its very high homology to TRα1. Thus, TRα2 is not a receptor in the strict sense. The function of TRα2 is unknown, though it may have an inhibitory effect on thyroid hormone action (Williams 1994).

If peritubular cells were indeed contributory to the increase in AR mRNA in response to T₃ seen in Sertoli cell-enriched cultures from 5-day-old rats, peritubular cells would be expected to express TR mRNA and show increased AR mRNA expression when exposed to T₃ in vitro. We therefore directly evaluated TR mRNA expression and T₃ effects on AR mRNA production in 20-day-old peritubular cell cultures. Peritubular cells from 20-day-old rats were used because the small size of the 5-day-old rat testis makes it difficult to obtain sufficient peritubular cells to perform Northern blotting experiments. In addition, well-defined protocols for the purification, culture and assessment of 20-day-old peritubular cells are available (Tung & Fritz 1977, Sanborn et al. 1991).

Our results indicate that cultured 20-day-old peritubular cells expressed both TRα1 and TRα2 mRNA. However, overall levels of TRα1 and α2 mRNA per µg of RNA in cultured peritubular cells were only about 30% of the levels in Sertoli cells cultured from 20-day-old rats. Although initial reports indicated that TR was present only in Sertoli cells of the testis and could not be detected in other cell types by classical T₃ binding assays (Jannini et al. 1990) or in situ hybridization for TR mRNA (Jannini et al. 1994), Hardy et al. (1996) recently reported that TRα1 and α2 mRNA could be detected in immature and adult Leydig cells, as well as their juvenile mesenchymal precursors. These data and the present results with peritubular cells suggest that TR may be ubiquitous in the cell types of the developing testis, although levels in these other cell types during development are less than in Sertoli cells (Hardy et al. 1996). Thus, a complete determination of the role of thyroid hormones in the testis will require an understanding of the effects of thyroid hormone on all of these cell types both during development and adulthood.

One potential complication of measuring TR mRNA in peritubular cell cultures is that the strong expression of TR and its mRNA by juvenile rat Sertoli cells (present results and Jannini et al. 1990, 1994) suggests that even low-level Sertoli cell contamination in peritubular cell
cultures would result in detectable TR mRNA expression. Sertoli cells produce large amounts of ABP, while Leydig and peritubular cells do not produce this protein (Pelliniemi et al. 1981). ABP therefore functions as a specific and sensitive marker for Sertoli cell contamination in peritubular cell cultures. The lack of detectable ABP mRNA in the cultured peritubular cells indicated that Sertoli cell contamination was minimal in the peritubular cell cultures. Therefore, peritubular cells themselves, rather than contaminating Sertoli cells, produce the TR mRNA detected in these cultures.

The expression of TR mRNA in peritubular cell cultures indicates that thyroid hormones could have direct effects on these cells, but T₃ did not increase AR mRNA in cultured peritubular cells. Thus, T₃ regulates AR mRNA in Sertoli, but not peritubular cells, a finding similar to what has been previously reported for the effects of FSH on AR mRNA expression in these two cell types (Sanborn et al. 1991). The role of T₃ in peritubular cells is presently unknown. Mendis-Handagama & Sharma (1994) reported an increase of approximately 50% in connective cells (which included peritubular myoid cells as well as other cells such as pericytes and fibroblasts) in adult testes of rats that had been made hypothyroid during neonatal life compared with age-matched untreated controls. These results suggest that T₃ may have an effect on the proliferation of peritubular cells, as it does in Sertoli cells (Cooke et al. 1994). Likewise, an effect of T₃ on peritubular cell differentiation is also plausible, based on similar effects in Sertoli cells. Peritubular cells communicate extensively with other testicular cell types such as Sertoli and Leydig cells (Skinner 1991) suggesting that in addition to possible direct T₃ effects, T₃ binding to TR in peritubular cells could also result in effects on other cell types in the testis through the production of paracrine factors by peritubular cells.

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