Regulation of cytochrome P450 aromatase gene expression in adult rat Leydig cells: comparison with estradiol production

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Abstract

Regulation of aromatase gene expression in purified rat Leydig cells has not yet been investigated. Therefore, using a highly specific quantitative RT-PCR method, we have measured the amount of cytochrome P450 aromatase (P450arom) mRNA and aromatase activity in mature rat Leydig cells submitted to various treatments during 24 h. Estradiol production was enhanced in a dose-related manner in the presence of testosterone, the maximum (28% increase) being obtained with 200 ng/ml. Related to the P450arom mRNA levels, a decrease was observed in the presence of low concentrations (50 and 100 ng/ml) of testosterone, then a 20% increase of the amount of transcripts was recorded for the higher concentrations (200–500 ng/ml). The same result was obtained in the presence of 5 α -dihydrotestosterone (an androgen resistant to aromatase activity). The addition of ovine LH (oLH; 0.1–50 ng/ml) to the Leydig cell culture medium induced a dose-related augmentation of estradiol output up to 10 ng/ml oLH, although a decrease was observed with 50 ng/ml when compared with maximal values. mRNA levels slightly decreased in the presence of low

concentrations (0.1–1 ng/ml) of oLH, an effect that was abolished by the addition of testosterone; mRNA levels were increased by oLH (5–10 ng/ml) 35 and 75% respectively in the absence and presence of testosterone (when compared with Leydig cells incubated without treatment). With 50 ng/ml oLH, a large augmentation (twofold) of the P450arom mRNA level either without or with testosterone was observed. Dibutyl cyclic AMP (1 mM) mimicked the effect of oLH. The half-life of the P450arom mRNAs was twofold increased in the presence of testosterone and oLH when compared with the half-life in the absence of treatment (5.8 ± 0.6 h). Taken together, our data have demonstrated that, in freshly isolated Leydig cells from mature rat testes, the regulation of aromatase expression and enzymatic activity is under LH (through cyclic AMP) and steroid control; moreover seminiferous tubule-secreted factor(s) are also involved. Therefore, rat Leydig cell aromatase is controlled at both transcriptional and post-transcriptional steps by endocrine and/or locally produced modulators.

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Introduction

Gonadotropins and testosterone, together with numerous intratesticular factors, play a crucial role in the development and maintenance of spermatogenesis in the mammalian testis (for reviews see Sharpe 1993, Saez 1994). Even though estrogens are considered to be a specific female hormone, the source of these hormones in the male genital tract has been well known for several decades (for review see Carreau *et al.* 1999). The ability of the testis to transform androgens into estrogens is related to the presence of a microsomal enzymatic complex named aromatase, which is composed of a specific glycoprotein, the cytochrome P450 aromatase (P450arom) and an ubiquitous flavoprotein, the NADPH-cytochrome P450 reductase (Simpson *et al.* 1994). In the rat testis, there is an age-related change in the cellular localization of the aromatase activity, which is mainly in Sertoli cells in

immature animals whereas it is located in Leydig cells in adults (Papadopoulos *et al.* 1986). Indeed, the aromatase has been immunolocalized in the Leydig cells of vertebrates (Carreau *et al.* 1999). However, striking species differences exist, since in mouse (Nitta *et al.* 1993), rat (Levallet *et al.* 1998a, Janulis *et al.* 1998), bank vole (Bilinska *et al.* 2000), brown bear (Tsubota *et al.* 1993) and rooster (Kwon *et al.* 1995) the aromatase is present both in Leydig cells and in seminiferous tubules, predominantly in spermatids (Carreau & Levallet 2000). It is known that Leydig cell aromatase activity is under luteinizing hormone (LH) control (Valladares & Payne 1979a,b) and that paracrine factors of Sertoli cell origin are also involved (Papadopoulos *et al.* 1987, Carreau *et al.* 1988). Nevertheless, despite much work focused on aromatase, especially in the male gonad, regulation of P450arom gene expression has not been studied extensively in purified testicular cells, except in cultured rat Sertoli cells (Levallet

& Carreau 1997) and in rat Leydig tumor cell lines (Young & McPhaul 1998). No one has explored the regulation of aromatase gene expression in freshly purified Leydig cells from mature rats. Therefore, using a highly specific RT-PCR method (Levallet & Carreau 1997), we have studied the effects of various hormonal treatments on the amount and half-life of P450arom transcripts and, in parallel, measured estradiol output.

Materials and Methods

Materials

Collagenase-dispase was purchased from Roche Diagnostics (Meylan, France). Soybean trypsin inhibitor, deoxyribonuclease, testosterone, 17β -estradiol, 5α -dihydrotestosterone (DHT), dibutyl cyclic AMP ((Bu)₂cAMP), actinomycin D, Trypan blue, nitro-blue tetrazolium, nicotinamide adenosine dinucleotide, niacinamide and dehydroepiandrosterone were purchased from Sigma Chemicals Co. (St Louis, MO, USA). Ham's F-12/DME medium was from Seromed (Berlin, Germany). Percoll and [2,4,6,7-³H]estradiol were obtained from Amersham Pharmacia Biotech (Orsay, France). Anti-estradiol antibody was purchased from Biosys (Compiègne, France). Moloney-murine leukemia virus-reverse transcriptase (M-MLV-RT), deoxy-NTP, oligo d-T(12–18 mer), *Taq* polymerase and RNasin were from Promega (Charbonnières, France). Primer sets (5'ARO–3'ARO) and (5'STD–3'STD) were obtained from Gibco-BRL (Cergy Pontoise, France). Ovine LH (oLH; NIH-oLH-26; specific activity, 2.3 U/mg) was provided by the NIDDK (Bethesda, MD, USA).

Purification of Leydig cells and incubation procedures

Testes from mature rats (>80 days) were decapsulated and submitted to an enzymatic treatment with collagenase-dispase (0.05%), soybean trypsin inhibitor (0.005%) and deoxyribonuclease (0.001%) in Ham's F-12/DME medium (1/1, v/v) for 10 min at 32 °C, and the Leydig cells were then purified on discontinuous Percoll gradients (Papadopoulos *et al.* 1985). The cells were incubated (either 4×10^6 cells/5 ml for total RNA extraction or 3×10^5 cells/1 ml for estradiol measurement) in Ham's F-12/DME medium at 32 °C for various times under an atmosphere of air/CO₂ (95/5, v/v) with or without testosterone, DHT, 17β -estradiol, seminiferous tubule conditioned medium (STM), oLH or (Bu)₂cAMP.

Cell viability and histochemical staining for 3β -hydroxysteroid dehydrogenase (3β -HSD)

The viability of testicular cells was evaluated using the Trypan blue exclusion test; the stained cells were considered to be dead. The enriched cell fractions col-

lected after Percoll sedimentation were characterized for the presence of Leydig cells by histochemical staining for 3β -HSD; colored cells were identified as Leydig cells (3β -HSD positive).

Preparation of STM

Isolated segments of 10–20 mm (total length 200 mm) of seminiferous tubules were dissected by transillumination (Parvinen & Vanha-Perthulla 1972). After several washes with Ham's F-12/DME medium (1/1, v/v), they were incubated in 1 ml medium for 20 h at 32 °C under an atmosphere of air/CO₂ (95/5, v/v). After centrifugation (2000 g, 10 min, 4 °C), the STMs were charcoal-treated to remove endogenous steroids and stored at –20 °C before measurement of their biological effects on Percoll-purified Leydig cells.

Reverse transcription-polymerase chain reaction (RT-PCR) assay

The mRNA present in the total RNA extracted by the guanidium thiocyanate-derived method (Chomczynski & Sacchi 1987) was reverse transcribed to cDNA as follows: 1 h at 37 °C with 200 IU M-MLV-RT, 500 µM deoxy NTP, 0.2 µg oligo-dT(12–18 mer) and 24 IU RNasin. The cDNAs obtained were further amplified by PCR using selected oligonucleotides. PCR was performed for 35 cycles (30 s at 94 °C, 30 s at 60 °C and 1 min at 72 °C with 2-s delay at 72 °C for each cycle) in the presence of 200 µM deoxy NTP, 1 µM primer set and 1.5 IU *Taq* polymerase in a final volume of 50 µl. The P450arom primers (5'ARO and 3'ARO) have been selected (Levallet *et al.* 1998a) to amplify a highly conserved sequence (289 bp length), including helical and aromatic regions of the P450arom gene (Table 1).

RNA standard preparation and quantitative competitive RT-PCR

The RNA standard was prepared by an internal nucleotide deletion of 29 bp from the 289 bp amplified product as described elsewhere (Levallet *et al.* 1998a). Briefly, total RNA from rat ovary was subjected to RT and PCR with the appropriated primers (Table 1). The 5'STD primer contained three specific sequences including: (i) the complementary sequence for the cDNA-binding region of T7 RNA polymerase, (ii) the primer 5'ARO (underlined) and (iii) a 27 bp sequence (position 1607–1634) located 29 bp downstream from the 5'ARO primer (in bold). The 3'STD primer resulted from the association of oligo dT with the specific 3'ARO sequence (underlined).

Validation of the quantitative competitive RT-PCR method

In order to verify the quality and size of the RNA standard, RT-PCR was performed with different

Table 1 Primers used for RT-PCR analysis

Primer	Sequence	Orientation
5' ARO	GCTTCTCATCGCAGAGTATCCGG	Sense (1555—1577)*
3' ARO	CAAGGGTAAATTCATTGGGCTTGG	Antisense (1821—1844)*
5' STD	TAATAGCACTCACTATAGGGAGAGCTTCTCATCGCAGAGTATCCGG	Sense
3' STD	CACACTGTTGTTGGTGACAGAGACATA TTTTTTTTTTTTTTTCAAGGGTAAATTCATTGGGCTTGG	Antisense

*Numbered as described by Hickey *et al.* (1990) (GenBank, accession no. M33986).

For definition of underlining and bold see section on 'RNA standard preparation and quantitative competitive RT-PCR'.

concentrations of standard (10 to 10^{-5} amol; Fig. 1A). The lower limit of detection was 10^{-5} amol and the expected band size of the standard was 260 bp. The area of the band (Fig. 1B) was proportional to the quantity of standard

added whereas, for the intensity, a plateau was observed from 1 amol standard (Fig. 1C). Up to 1 amol standard, good correlations ($r=0.99$) were recorded between intensity and standard concentration and between surface

Figure 1 RNA standard amplification by RT-PCR. (A) A 1:10 serial dilution (10 to 10^{-5} amol) of RNA standard was reverse transcribed and amplified with the 5'ARO–3'ARO set of primers. The amplified products were subjected to electrophoresis on 2% agarose gel containing ethidium bromide and then photographed. Lanes 8 and 9 correspond respectively to positive (O, ovary RNA) and negative (W, RNA was replaced by water) controls. The surface (B) and intensity (C) values for each band are reported as a function of the standard concentration. (D) Correlation between intensity and surface values used for quantification of cytochrome P450arom mRNA.

Figure 2 Quantitative competitive RT-PCR of total RNA from adult rat Leydig cells. P450arom mRNA was co-amplified with serial dilutions of RNA standard. The amplified products were subjected to electrophoresis on 4% agarose gel, the gel was photographed and then subjected to scanning densitometry. The P450arom mRNA content was calculated by determination of the equivalence point according to the equation established by Menzo *et al.* (1992). Lanes 8 and 9 correspond respectively to negative (RNA was replaced by water) and positive (ovary RNA) controls.

and intensity of the spots (Fig. 1D). Therefore, for quantitative RT-PCR experiments, increasing dilutions of RNA standard were added to a known amount of total RNA. After RT and PCR with the P450arom-specific set of primers, the amplified products were run on a 4% agarose gel stained with ethidium bromide (Fig. 2), then photographed and submitted to densitometry scanning analysis (Bio-Profil System, Vibert Lourmat, France). The intensity and surface of each band was calculated and the sample/standard ratio was plotted against standard concentration, and then the quantity of P450arom mRNA present in the total RNA was determined.

Estradiol determinations

The steroids were first extracted from the cell culture media by 5 volumes of diethyl ether. The estradiol outputs were determined by radioimmunoassay using a highly specific antibody (the cross-reaction of testosterone with the antibody was 0.001 when 50% of labeled ligand was bound; Papadopoulos *et al.* 1986). The inter- and

intra-assay coefficients of variation were 5 and 8% respectively and the sensitivity was 6 pg/tube.

Half-life of the P450arom mRNA

In order to study the stability of the Leydig cell transcripts, increasing concentrations of actinomycin D were added to the cell incubation medium, and then the amount of P450arom mRNA was measured by quantitative competitive RT-PCR for each treatment performed during 24 h. The amounts of specific P450arom mRNAs are expressed as percentages when compared with the level determined at time zero (referred to as 100%). For each treatment (three separate cultures were used) we obtained three values from which the half-lives were calculated assuming that the decrease of the mRNA followed a stochastic process, according to the equation: $C = C_0 e^{-k_d t}$ (Ross 1995). In the equation, k_d represents the mRNA decay and for $c = c_0/2$ we obtained the half-life ($t_{1/2}$). The statistical evaluations were performed on $t_{1/2}$, k_d and slopes using the ANOVA Tukey–Kramer test.

Statistical analysis

Experiments were repeated three times either in triplicate for P450arom mRNA measurements or in duplicate for estradiol determinations; results are means $\pm$ S.E. Statistical analyses were performed using ANOVA followed by the Tukey–Kramer test; statistical significance was accepted at $P < 0.05$ (Staworks; Brain Power Inc., Calabasas, CA, USA).

Results

Influence of incubation time on rat Leydig cell parameters

The optimal conditions for both P450arom gene expression and estradiol production have been determined. According to the duration of incubation, the cell viability

Table 2 Influence of incubation time on several rat Leydig cell parameters. Values are expressed as percentage (time 0 as 100%) $\pm$ S.E.M. of triplicate determinations from three different experiments

Time (h)	Cell viability	3β-HSD-positive cells	Quantity of P450arom mRNA (expressed as %)	
			Basal conditions	In the presence of testosterone (200 ng/ml) and oLH (5 ng/ml)
0	98%	85%	100 $\pm$ 3	100 $\pm$ 3
6	98%	85%	108 $\pm$ 3 NS(a)	90 $\pm$ 6 NS(a,b)
24	95%	83%	110 $\pm$ 2 NS(a)	197 $\pm$ 3 *** (a,b)
48	65%	60%	61 $\pm$ 1 ** (a)	62 $\pm$ 6 * (a) NS(b)

* $P < 0.05$; ** $P < 0.01$, *** $P < 0.001$; NS, not significant; (a) when compared to time zero, (b) when compared to cells incubated without treatment and for the same time.

Figure 3 Dose-related effects of testosterone on the production of estradiol by Percoll-purified Leydig cells from mature rat testes. This production is expressed in percentages; control cells are considered to be 100%. Values are means $\pm$ S.E. of duplicate determinations from three separate experiments. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ when compared with control cells.

and the percentage of 3 β -HSD-positive cells were not significantly modified during the first 24 h (Table 2). Conversely, after 48 h of incubation, both cell viability and percentage of 3 β -HSD-positive cells were decreased. The quantity of P450arom mRNA was not affected after 6 h of incubation, either in the absence or the presence of hormonal treatment, when compared with the initial time. Thereafter a twofold increase of P450arom mRNA level was observed when Leydig cells were incubated for 24 h in the presence of testosterone and oLH, although there was no significant change in basal conditions. By contrast, after 48 h of incubation the quantity of P450arom mRNA decreased (Table 2). Therefore a 24-h incubation period was chosen for all further experiments except when stated otherwise.

Dose-related effects of various treatments on P450arom mRNA level and aromatase activity

Whatever the concentration of testosterone used, a dose-dependent increase in Leydig cell estradiol production was observed when compared with control (referred to as 100%). The estradiol output was maximally increased (28%; $P < 0.01$) in the presence of 200 ng/ml testosterone; for the higher concentrations (400 and 500 ng/ml), estradiol production was in the same order of magnitude (Fig. 3).

The amount of P450arom mRNA followed the same pattern and was maximal when Leydig cells were incubated with 200 ng/ml testosterone; the increase was 20% ($P < 0.01$) when compared with Leydig cells without substrate (referred to as 100%). For the higher doses of testosterone, the P450arom mRNA levels were in the

same range as for 200 ng/ml; conversely, the level of specific transcripts decreased slightly (10 and 14% respectively for 50 and 100 ng/ml testosterone; Fig. 4). In order to find out whether these observations were or were not related to an androgen effect, we incubated the Leydig cells with 5 α -DHT (androgen not metabolized by the aromatase). As shown in Table 3, 5 α -DHT (200 ng/ml) induced a 25% increase in P450arom mRNA levels ($P < 0.05$).

In addition, when the cells were treated with various concentrations of 17 β -estradiol, a dose-related increase of the amount of P450arom mRNA was recorded with a maximum (23% augmentation) obtained for 0.5 ng/ml estradiol (Table 3). It is noteworthy that in the presence of 5 ng/ml estradiol the amount of specific P450arom transcript decreased.

Moreover, when increasing concentrations of STM (20–80%, v/v) were added to the Leydig cell incubation medium, a dose-dependent enhancement of the amount of P450arom mRNA was observed, the maximal effect (34% increase; $P < 0.01$) was already achieved in the presence of 40% STM (Table 3).

Effects of oLH on the amounts of P450arom transcripts and estradiol

In order to analyse the specific effects of the gonadotropin, the Leydig cells were incubated with or without 200 ng/ml testosterone. Whatever the concentration of oLH used up to 50 ng/ml, an increase in estradiol production was observed when compared with control A (Leydig cells without exogenous testosterone) which was taken to be 100% (Fig. 5).

When testosterone was added to the Leydig cell incubation media, the pattern was similar but the production of estradiol was markedly enhanced when compared with Leydig cells incubated without testosterone. The maximal outputs of estradiol were reached for oLH concentrations ranging from 1 to 10 ng/ml: 38% and 300% increases (when compared with control A) respectively in the absence and the presence of testosterone. With a dose of 50 ng/ml oLH, a decrease in estradiol production was noticed.

The amounts of P450arom mRNA (Fig. 6) were diminished by 25% ($P < 0.05$) and 15% (NS) respectively for 0.5 and 0.1–1 ng/ml oLH when compared with control A; indeed the addition of testosterone reversed this negative effect. The addition of oLH (5–10 ng/ml) to the Leydig cell incubation media induced an increase in the amount of P450arom mRNA, both in the absence and the presence of testosterone; testosterone further improved the effect of oLH. In the presence of 5 ng/ml oLH the level of P450arom transcripts was enhanced by 35% ($P < 0.01$) and 81% ($P < 0.001$) respectively in the absence and the presence of testosterone (when compared with

Figure 4 Dose-related effects of testosterone on P450arom mRNA levels determined by quantitative competitive RT-PCR in Percoll-purified Leydig cells from mature rat testes. (A) Ethidium bromide-stained gel (representative pattern). Lanes W and O correspond to negative (W, RNA was replaced by water) and positive (O, ovary RNA) controls. (B) The P450arom mRNA levels are expressed in percentages when compared with control (no treatment). Values are means $\pm$ S.E. of triplicate determinations in three different experiments. ** $P < 0.01$; *** $P < 0.001$; NS, not significant when compared with control cells.

control A). These augmentations were respectively of 13% (NS) and 50% ($P < 0.01$) when compared with control B. The mean P450arom mRNA values were similar for 10 ng/ml oLH. When the larger concentration of oLH (50 ng/ml) was used, although a decrease in estradiol production was observed, the amount of P450arom mRNA was enhanced. The level of specific P450arom transcripts was maximally increased (twofold) when Leydig cells were incubated for 24 h with 200 ng/ml testosterone and 50 ng/ml oLH.

Effect of cyclic AMP on aromatase gene expression

A 1 mM concentration of the cyclic AMP analogue (Bu)₂cAMP was used since it mimicked the maximal effect registered with oLH on estradiol production (Fig. 5). With regard to the amount of P450arom mRNA, the results were of the same order of magnitude as those obtained with oLH concentrations ranging between 5 and 10 ng/ml: when compared with the amount of P450arom mRNA in Leydig cells (control A) the specific transcripts

Table 3 Effect of various treatment on the amount of P450arom transcripts. Values are expressed as percentage (cell incubated without treatment are considered to be 100%) of triplicate determinations from three different experiments

Treatment	P450arom mRNA (%)
Control	100
+Testosterone (200 ng/ml)	+20 **
+5 α -DHT (200 ng/ml)	+25 *
+17 β -estradiol (0.1 ng/ml)	+9 NS
+17 β -estradiol (0.5 ng/ml)	+23 *
+17 β -estradiol (1 ng/ml)	+17 *
+17 β -estradiol (5 ng/ml)	-15 *
+STM (20%)	+11 NS
+STM (40%)	+34 **
+STM (60%)	+35 **
+STM (80%)	+39 **

* $P<0.05$; ** $P<0.01$; *** $P<0.001$; NS, not significant.

were increased 57% ($P<0.01$) and 95% ($P<0.001$) respectively in the absence and the presence of androgen. These augmentations were of 30% ($P<0.01$) and 63% ($P<0.001$) in the absence and the presence of testosterone when compared with testosterone-treated cells (Fig. 6).

The half-life of the Leydig cell P450arom transcripts

We used actinomycin D to examine the effect of oLH and testosterone on the half-life of the cytochrome P450arom mRNA. Various concentrations of actinomycin D (1, 2.5, 5 and 7.5 μ g/ml) were added to the Leydig cell culture media and incubations were carried out over increasing time up to 24 h. A concentration of 5 μ g/ml actinomycin D was selected since lower concentrations were ineffective on transcription even after 24 h, although higher doses were toxic for Leydig cells (60% increase of dead cells recorded during the first 2 h; data not shown). In the presence of 5 μ g/ml actinomycin D, we did not observed any significant change during the first 2 h whatever the treatment, then the quantity of P450arom mRNA decreased until 12 h of incubation. For longer periods of incubation, the P450arom signal was undetectable by

Figure 5 Effects of increasing concentrations of oLH on the production of estradiol by purified Leydig cells from mature rat testes incubated either in the absence or the presence of testosterone (200 ng/ml). (Bu)₂cAMP (1 mM) was used to mimic the optimal effect of oLH. The results are expressed as percentages. Control A (no treatment is referred to as 100%). Values are means $\pm$ S.E. of duplicate determinations obtained from three separate experiments. * $P<0.05$; ** $P<0.01$; *** $P<0.001$; NS, not significant when compared with (a) control A (cells without treatment) or (b) control B (cells incubated with 200 ng/ml testosterone).

Figure 6 Effects of various doses of oLH on P450arom mRNA levels determined by quantitative competitive RT-PCR in Percoll-purified Leydig cells from mature rat testes incubated either in the absence or the presence of testosterone (200 ng/ml). Values are means $\pm$ S.E. of triplicate determinations from three experiments. (Bu)₂cAMP (1 mM) was used to mimic the optimal effect of oLH. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; NS, not significant when compared with (a) control A (cells without treatment) or (b) control B (cells incubated with 200 ng/ml testosterone).

RT-PCR, except when cells were treated with testosterone (200 ng/ml) and oLH (5 ng/ml), when a weak signal was still observed after 15 h (Fig. 7A). A 50% diminution of the initial quantity of P450arom mRNA was observed after 5.8 ± 0.6 h in basal conditions (i.e. without any hormonal treatment of Leydig cells). The addition of either testosterone (200 ng/ml) or oLH (5 ng/ml) alone did not modify the profile of the curves, the results were identical and the half-life was 7.2 ± 0.8 h. By contrast, when the Leydig cells were incubated with testosterone plus oLH, the half-life of the specific P450arom mRNA was significantly ($P < 0.05$) increased and reached 10.8 ± 1.2 h (Fig. 7B).

Discussion

From these *in vitro* studies we have shown, in freshly isolated Leydig cells from mature rat testes, that the regulation of aromatase gene expression is under LH (via cyclic AMP) and steroid control. These observations are corroborated by the estradiol output and our data concerning the positive regulation of the aromatase activity by LH in rat Leydig cells are in agreement with those of

Valladares & Payne (1979a,b) and Papadopoulos *et al.* (1986). When the regulation of the aromatase gene expression was analysed in terms of specific transcripts in mature rat Sertoli cells it was found that testosterone alone and follicle-stimulating hormone, either alone or with testosterone, induced an increase in the amount of P450arom mRNAs (Levallet & Carreau 1997). Whatever the somatic cells studied (either Leydig or Sertoli cells), gonadotropins provoke a dose-related increase in the P450arom mRNA levels but the nadirs are different: between 3 and 6 h in Sertoli cells (Rappaport & Smith 1996) whereas we found no changes until 6 h in Leydig cells, the maximum being observed after 24 h. Therefore LH (and cyclic AMP) controls the P450arom gene expression in the rat Leydig cells via the existence of cyclic AMP response motifs (CRE) localized in the proximal promoter PII of the aromatase gene (Bulun *et al.* 1993).

It is noteworthy that in both rat Sertoli cells (Levallet & Carreau 1997) and Leydig cells (data herein) we have been able to measure specific mRNA transcripts of aromatase without any hormonal treatment. Obviously, these observations on the constitutive expression of the aromatase

Figure 7 Determinations of cytochrome P450arom mRNA half-life after addition of actinomycin D (5 µg/ml) to the Leydig cell culture media using the quantitative competitive RT-PCR method. (A) Representative ethidium bromide-stained gel obtained after incubation of Leydig cells with testosterone (200 ng/ml) and oLH (5 ng/ml). Lanes W and O correspond respectively to negative (W, RNA was replaced by water) and positive (O, ovary RNA) controls. (B) P450arom mRNA levels (in percentages) measured in Leydig cells incubated either in the absence or the presence of testosterone (200 ng/ml) or oLH (5 ng/ml). Values are means $\pm$ S.E. of triplicate determinations from a representative experiment for each treatment. Three separate experiments for each treatment were performed.

gene in rat Leydig cells are in agreement with the reported data on aromatase activity (Papadopoulos *et al.* 1986). In the tumor Leydig cell lines R2C and H 540, aromatase is constitutively highly expressed and requires not only several CRE-like motifs but also the steroidogenic factor-1 within the proximal promoter of the aromatase gene (Young & McPhaul 1998). The differences in constitutive and regulated aromatase are likely relevant to the physiological status of the Leydig cells and the steroidogenic cells in general: acute controls via available mRNA and long-term regulation of specific genes.

The estradiol production of the Leydig cell is correlated with the amounts of P450arom mRNA and is maximum in the presence of either testosterone or DHT showing, therefore, that androgens are strong modulators of aromatase gene expression and activity. Similar observations dealing with the regulation of aromatase activity by androgens have been reported in the brain of the rat (Roselli & Resko 1984, Zwain *et al.* 1997, Resko *et al.* 2000). Below a threshold of 100 ng/ml testosterone the Leydig cell increase of estradiol output is the consequence of either post-transcriptional events and/or use of

available mRNAs without transcriptional effects (which may indeed explain the slight diminution of the amount of P450arom mRNA; Fig. 4A,B). Together with oLH, it is clear that testosterone further controls the aromatase both at the transcriptional level and in terms of biological activity. However, with a larger concentration of gonadotropin (50 ng) the Leydig cell estradiol output decreases whereas the amount of transcripts is higher suggesting, therefore, additional regulatory steps and maybe the expression of truncated P450arom mRNAs as shown previously in rat Sertoli and germ cells (Levallet *et al.* 1998b). Consequently, our data related to the positive effects of steroid hormones on the expression of the aromatase gene indicate that besides CRE, androgen responsive elements as well as estrogen responsive elements are necessary for a full expression of the aromatase in Leydig cells. In addition to promoter II, an exon I.6 has been demonstrated in the testicular aromatase gene (Shozu *et al.* 1998), therefore suggesting other putative regulatory factors such as insulin-like growth factor-I which increases the rat Leydig cell aromatase activity in the presence of human chorionic gonadotropin (hCG) (Rigaudière *et al.* 1989) or Sertoli cell-secreted factors which modulate Leydig cell aromatase (both in terms of mRNA and enzyme activity) not only in the rat (Papadopoulos *et al.* 1987, Carreau *et al.* 1988) but also in man (Lejeune *et al.* 1998). It is therefore important to take into account the fact that the regulation of the aromatase promoter(s) is likely to be related to the presence of other transcriptional factors, either repressor or activator (Utsumi *et al.* 1996).

In order to analyse the effects of LH and/or testosterone treatments on Leydig cell regulation of transcriptional activity, we have used actinomycin D and studied the half-life of the specific P450arom transcripts. After such a treatment the rates of degradation of the specific P450arom mRNAs are similar; conversely a twofold increase of the stability of the transcripts was noted when the Leydig cells were treated with oLH plus testosterone, although the mRNA of actin was unaltered in presence of actinomycin (data not shown). Therefore, the observed changes are not at the transcriptional level but rather the hormonal treatments would induce a protective effect on the mRNAs by as yet unidentified factors. Tang *et al.* (1998), using differential display analysis of Leydig cell mRNA obtained from rats treated with a desensitizing dose of hCG, reported that transcripts for β -HSD were down-regulated (five- to eightfold), whereas minor or no change was found at lower concentrations. However, they used 10 μ g/ml actinomycin D; in our reported experiments we were unable to quantify the specific mRNA since a dramatic increase in cell death was recorded in the presence of 10 μ g actinomycin D.

It is necessary to keep in mind that Leydig cells not only express a gonadotropin-regulated aromatase which leads to the synthesis of estrogens but they also contain estrogen receptors, allowing intracrine/autocrine/paracrine roles

for these female hormones. With regard to the roles of estrogen, it is essential to take into account the existence of Leydig cell populations as well as their maturational stages. In fact, Leydig cells contain estrogen receptors very early in life and the amount of estrogen receptor mRNA is much higher in precursor than in mature Leydig cells (Zhai *et al.* 1996). The Leydig cell steroidogenic pathway is in part negatively controlled by estrogens (for review see Abney 1999), and other data have shown that estrogens are deleterious for Leydig cell development via opposite effects on cyclin A2 and G1 (Ge & Hardy 1997). Therefore, estrogens seem to exert mostly a negative role on Leydig cells but our preliminary observations demonstrate a positive effect of estradiol on the regulation of the aromatase expression in Leydig cells. Moreover numerous data on estrogen receptor knockout mice (Couse & Korach 1999) and aromatase knockout mice (Robertson *et al.* 1999) have provided clear evidence for a positive role of estrogens in the male reproductive tract.

In conclusion, in testicular somatic cells it is clear that besides gonadotropins, steroid hormones are involved in the regulation of aromatase gene expression, although in germ cells our preliminary results demonstrate opposite effects of growth factors and cytokines on the amount of specific P450arom transcripts (Bourguiba *et al.* 2000). Thus, the rat Leydig cell cytochrome P450arom which catalyzes the final conversion of androgens into estrogens is transcriptionally regulated at least by LH (via cyclic AMP) and steroid hormones, demonstrating that the aromatase promoter contains besides CRE, androgen and estrogen responsive elements together with unknown sequences targeted by paracrine factors from a tubular origin.

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