Differential expression and regulation of progesterone receptor isoforms in rat and mouse pituitary cells and LβT2 gonadotropes

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Abstract

Manipulation of endogenous progesterone receptor (PR) does not produce equivalent physiological effects in mouse and rat pituitary cells. To test whether this may be due in part to difference in PR isoform expression, we examined hormonally regulated pituitary PR-A and PR-B mRNA levels using quantitative real-time PCR. The LβT2 mouse gonadotrope line or pituitary cells from adult, ovariectomized rats or mice were cultured with or without 0·2 nM 17β-estradiol (E_2) for 3 days. PR-A was the predominant form expressed for all groups. For mouse cells, E_2 led to an increase in both isoforms without a change in the A:B ratio; for rat cells, the PR-B response to E_2 was more robust resulting in a decrease in the A:B ratio. Exposure of E_2-treated pituitary cells to 200 nM progesterone for 6 h decreased both PR-A and PR-B levels in rat cells, but had no effect on PR isoform expression in mouse cells even when exposure was extended to 12 h. The low level of PR expression found in LβT2 gonadotropes was unaffected by E_2, alone or with progesterone. The weak PR expression and lack of responsiveness of LβT2 cells cannot be explained by a male phenotype as was shown by the more than tenfold higher PR mRNA level in primary cultures of male mouse pituitary cells, which responded to E_2 stimulation with a proportional increase in PR isoforms similar to female cells. Functionally, E_2-stimulated changes in PR mRNA isoform ratios in rat, mouse or LβT2 cells correlated with the degree of progesterone augmentation of GnRH-stimulated LH secretion in these models. These results are consistent with the hypothesis that robust GnRH priming and progesterone augmentation of LH secretion in the rat compared to these events in the mouse are a consequence, in part, of differences in the E_2-modulated ratio of PR isoforms.

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Introduction

Progesterone can augment or inhibit gonadotropin-releasing hormone (GnRH)-stimulated luteinizing hormone (LH) secretion in a time-dependent manner (reviewed in Levine *et al.* (2001)). The acute, stimulatory action of progesterone contributes to the dimensions of the preovulatory LH surge in rats and humans (Odell & Swerdloff 1968, Chang & Jaffe 1978, Liu & Yen 1983, Fink 1988, Turgeon & Waring 1990, Ringstrom *et al.* 1997). Also serving as an amplification signal during the LH surge is GnRH self-priming, and we have shown for rat gonadotropes that there is overlap of the self-priming pathway with activation of the progesterone receptor (PR; Waring & Turgeon 1992, Turgeon & Waring 1994). For mouse pituitary cells, progesterone augmentation of GnRH-stimulated LH secretion can be demonstrated, but it is about half of that found for the rat and is only partially prevented by PR antagonists (Turgeon & Waring 2001). Additionally in mouse gonadotropes, progesterone has no effect on depolarization-induced LH secretion; whereas in rat gonadotropes, the same progesterone treatment effects a several fold augmentation (Turgeon & Waring 1983, 1999, 2001, Ortmann *et al.* 1994). These functional differences may be due in part to differences in the expression of PR isoforms.

PR is expressed in multiple target tissues in at least two isoforms generated from a single gene under differential promoter regulation. The PR-A protein isoform is an N-terminally truncated variant of full-length PR-B isoform (Schott *et al.* 1991, Kraus *et al.* 1993). PR-A and PR-B have distinct transactivation properties when expressed individually, and PR-A can modulate the activity of PR-B (Giangrande & McDonnell 1999, Giangrande *et al.* 2000). Additionally, the ratio of PR-A:PR-B varies among target tissues, suggesting that their differential expression contributes to cell-specific responses. This translates into discrete physiological functions shown in the studies of PR isoform-specific knockout mice or mice with altered A:B ratios (Shyamala *et al.* 1998, Mulac-Jericevic & Conneely 2004). Taken together, this suggests that the relative levels of the PR isoforms have a critical role in context-specific responses.

For the PR in the pituitary gland, relatively little is known of specific isoform expression or targets. In rat pituitaries, total PR mRNA is upregulated by 17β-estradiol (E_2; Turgeon *et al.* 1999, Sánchez-Criado *et al.* 2004), and Szabo

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et al. (2000) report that both PR-A and PR-B are expressed. There are no data available for PR isoform expression in mouse pituitary cells. Some differences have been noted for pituitary PR protein between the two models: for rat pituitary cells in the presence of E_2, the PR localizes exclusively to gonadotropes, and for mouse cells under the same conditions, the receptor is found in lactotropes as well as in gonadotropes (Turgeon & Waring 2000, Turgeon *et al.* 2001). Regarding regulation, estrogen increases PR protein in both rat and mouse pituitary cells (Evans *et al.* 1978, Attardi & Palumbo 1981, Krey *et al.* 1990, Turgeon & Waring 2000), while progesterone leads to a dramatic decrease in PR immunoreactivity in the rat, but only a modest reduction in mouse gonadotropes in a similar time frame (Turgeon & Waring 2000, Turgeon *et al.* 2001).

The gonadotrope-derived LβT2 cell line was generated by targeted oncogenesis in mice and has been useful in studies of GnRH-signaling pathways. Although the expression and regulation of estrogen receptor isoforms have been well characterized for LβT2 cells (Schreihofer *et al.* 2000), little is known of the pattern of PR expression in these cells, either basal or following steroid hormone manipulation. Because of the reported cross-talk between the GnRH self-priming pathway and the PR in primary gonadotropes (Turgeon & Waring 1994), information on the PR in LβT2 cells is of particular relevance as this cell line does not exhibit GnRH priming (Turgeon *et al.* 1996).

We hypothesize that the divergence between rat and mouse LH secretion modulated by either GnRH self-priming or progesterone augmentation is due in part to differences in the ratio of PR isoforms in gonadotropes. Therefore, the aims of this study were to (1) determine PR-A and PR-B mRNA levels in cultured pituitary cells from rats and mice and in the LβT2 cell line and (2) establish roles for E_2 and progesterone in PR isoform expression in these models.

Materials and Methods

Pituitary cell culture and experimental protocols

Protocols employed in these experiments were reviewed and approved by the University of California Davis Institutional Animal Care and Use Committee. Media and sera for cell culture were as described previously (Turgeon & Waring 1990). GnRH was from Sigma-Aldrich. Steroids (progesterone from Calbiochem (La Jolla, CA, USA), E_2 and dexamethasone from Sigma-Aldrich) were prepared as stock solutions in ethanol. The anesthetic tribromoethanol (Sigma-Aldrich) was prepared as a stock solution in amyl alcohol and diluted in PBS on the day of use.

Adult female Sprague–Dawley rats (Simonsen Laboratories, Gilroy, CA, USA) and adult female and male wild type mice (C57/6/129sv hybrid as described in Lydon *et al.* (1995)) were maintained in controlled light conditions (12 h light:12 h darkness). All animals were gonadectomized under i.p. tribromoethanol (mice) or ether (rats) anesthesia and treated with s.c. buprenorphine as analgesic in the immediate post-operative period. Animals were maintained for 2 weeks postgonadectomy before use. Pituitary glands were removed following CO_2 narcosis and decapitation. Anterior pituitary tissue was enzymatically dispersed and prepared for cell culture as described (Turgeon & Waring 1990). Cells were plated at 3×10^5–5×10^5 in 22 mm multiwell plates and flooded with Minimum Essential Medium (MEM) supplemented with 200 μM kanamycin sulfate, 10% fetal bovine serum (FBS) that had been charcoal treated to remove endogenous steroids, and with or without 0·2 nM E_2. Residual steroid concentrations in the charcoal-treated serum were below the detection limits of the RIAs, and thus final media concentrations were less than 10 pM for progesterone and 1 pM for E_2. Pituitary cells were maintained in a humidified atmosphere (37 °C) of 5% CO_2 in air; on day 3 media were replenished (day of plating = day 1).

LβT2 cells from Dr Pamela Mellon (University California, San Diego, CA, USA) were passaged weekly and cultured in Dulbecco's Modified Eagle's Medium (DMEM; 25 mM glucose, 44 mM $NaHCO_3$, 200 μM kanamycin sulfate) and supplemented with 10% FBS as described (Turgeon *et al.* 1996). For experimental protocols, dispersed cells were used between passages 4 and 15 and plated at 3×10^6–5×10^6 in 60 mm dishes (RNA study) or 3×10^5–5×10^5 in 22 mm wells (secretion study) and flooded with DMEM containing 10% charcoal-treated FBS, with or without 0·2 nM E_2, and with or without 20 nM dexamethasone. LβT2 cells were maintained in a humidified atmosphere (37 °C) of 15% CO_2 in air and media replenished on day 3.

RNA protocol On day 4, media were replenished with serum-free MEM (pituitary cells) or serum-free DMEM (LβT2 cells) containing 1 mg BSA/ml and ± E_2 and dexamethasone as indicated. For some experimental groups, media also contained 200 nM progesterone. After 6- or 12-h incubation, cells were rinsed with ice-cold PBS, lysis buffer containing guanidinium thiocyanate was added, the cells scraped and triturated, and stored at −70 °C until processed for RNA.

Secretion protocol Cells were cultured in E_2-containing medium. On day 4, the cells were changed to serum-free medium containing 0·2 nM E_2, 1 mg BSA/ml, and with or without 200 nM progesterone. For rat and mouse pituitary cells, successive 15-min incubations were collected before, during, and after a 15-min pulse of 1 nM GnRH administered 90 min after progesterone was initiated. For LβT2 cultures, on days 1–3 the cells were treated as described by Turgeon *et al.* (1996) with GnRH (10 nM, 15-min pulses × 4 with a 90-min interpulse interval) to increase GnRH responsiveness. On day 4, LβT2 cells were treated with or without 200 nM progesterone and challenged at 90 min with a single 10 nM GnRH 15-min pulse as described above for primary pituitary cultures. Samples were stored at −70 °C until assayed for LH by RIA as described previously (Turgeon & Waring 1990, 2001, Turgeon *et al.* 1996).

RNA isolation and real-time PCR

Cell lysates were processed for total RNA using the RNeasy Mini Kit (Qiagen) and included an on-column DNase I digestion step. Total RNA (0·5–3 μg) was concentrated, resuspended in RNase-free water, and converted to cDNA using SuperScript II reverse transcriptase (Invitrogen Life Technologies) according to kit instructions. To assess the quality of first-strand cDNA synthesis, samples were subjected to PCR amplification using β-actin primers and agarose gel electrophoresis.

Quantitative real-time PCR (QRTPCR) assays were performed using ABI SYBR Green master mix (Applied Biosystems, Foster City, CA, USA) and an ABI Prism 7900 Sequence Detection System (Applied Biosystems; Yuen *et al.* 2002). PCR amplification conditions were: 50 °C for 2 min, DNA polymerase activation at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Cycle threshold was set at a level at which the exponential increase was approximately parallel between samples.

For the analysis of total PR (A+B) transcripts, primers were directed at a region downstream of the second ATG translation initiation site. For analysis of PR-B transcripts, primers were used to amplify a region between the first and second ATG codons. None of the primer set sequences corresponded to sequences in other steroid hormone receptors. For the housekeeping gene, β-actin, primers were directed at a region common to the mouse and rat. Primers were selected using Primer Express, and optimal experimental conditions were established for each set. Specific primers are summarized in Table 1. As template, replicate samples of approximately 500 or 1000 pg of first-strand cDNA from the experimental material were used.

To determine transcript copy number, standard curves were generated for each primer pair using tenfold serial dilutions of linearized plasmids containing known number of copies of the gene. The threshold cycle of each dilution was determined and plotted against the log value of the cDNA copy number. Transcript number for each experimental sample was interpolated by its detection threshold value using the respective standard curve. For rat PR, a plasmid containing full-length rat PR (rPR6Bp from Dr B Katzenellenbogen, University of Illinois at Urbana-Champagne, IL, USA) was used to generate standard curves; for mouse, a plasmid containing full-length mouse PR (mPR3 from Dr G Shyamala, University of California, Berkeley, CA, USA) was utilized. A mouse β-actin plasmid (from Dr L Huang, University of California, Davis, CA, USA) was used to generate standard curves for assessing the reference gene. Table 2 shows the resulting standard curve coordinates and ranges. As the PCR results for total PR and PR-B were quantitated using the same plasmid as standard, the amount of PR-A was determined by subtracting the standardized value for PR-B from total PR. Expression levels were normalized to that for the housekeeping gene, β-actin, to control for input RNA.

Statistical analysis

Data are presented as the mean ± S.E.M. Each experiment represents a separate pool of dispersed pituitary cells or a different passage; *n* refers to the number of separate experiments and reflects a minimum of three separate cell dispersions or passages per experimental group. All statistical analyses were done using SigmaStat (Systat Software, Inc., Point Richmand, CA, USA). For multiple comparisons, differences among groups were determined by ANOVA and the Tukey's test. Where appropriate, differences between two groups were determined by paired *t*-test. Where differences are indicated as being significant, $P<0·05$.

Results

Female rat or mouse pituitary cells

The aim of the initial experiments was to determine the effect of E_2 on expression of PR isoforms in cultured pituitary cells from ovariectomized rats or mice. As shown for rat cells in Fig. 1, the presence of 0·2 nM E_2 for 3 days resulted in significant increases in both PR-A (~fivefold) and PR-B (~16-fold) expression normalized to β-actin as determined by QRTPCR. The increase in total PR expression following E_2 stimulation is consistent with our previous report based on

Table 1 Primer sequences for quantitative real-time PCR

	Primer sequence	Accession no.
Transcript		
Rat PR-A+B	F:5′-TGGTTCCGCCACTCATCA-3′ R:5′-TGGTCAGCAAAGAGCTGGAAG-3′	NM022847
Rat PR-B	F:5′-CCAATACCGATCTCCCTGGAC-3′ R:5′-CTTCCACTCCAGAGAAAGCTCC-3′	NM022847
β-actin	F:5′-TCATGAAGTGTGACGTTGACATCCGT-3′ R:5′-CCTAGAAGCATTTGCGGTGCACGATG-3′	NM03114
Mouse PR-A+B	F:5′-GGTGGGCCTTCCTAACGAG-3′ R:5′-GACCACATCAGGCTCAATGCT-3′	AK036862
Mouse PR-B	F:5′-GGTCCCCCTTGCTTGCA-3′ R:5′-CAGGACCGAGGAAAAAGCAG-3′	AK036862

Table 2 Quantitative real-time PCR standard curves

	Primer set	Curve range (copy #)	Standard curves			
			Slope	Y-intercept	r^2	*n*
Plasmid						
Mouse PR	mPR-A+B	$10–10^6$	−3·06±0·02	34·31±0·27	0·9957±0·0020	6
	mPR-B	$10–10^6$	−3·11±0·01	35·22±0·20	0·9977±0·0003	6
Rat PR	rPR-A+B	$10–10^6$	−3·43±0·26	38·97±1·17	0·9362±0·02	3
	rPR-B	$10–10^6$	−3·52±0·27	40·72±0·9	0·9526±0·0194	3
β-actin	m/rβ-actin	$10^3–10^8$	−3·56±0·01	39·01±0·26	0·9983±0·0004	9[a]

[a]A standard curve for β-actin was included in mouse PR assays and rat PR assays. Results were pooled for presentation as there were no significant differences between actin curves in the two assay systems.

Northern blot analysis of rat pituitary cells (Turgeon *et al.* 1999). Figure 1 also shows the response for female mouse pituitary cells; E_2 led to significant increases of four- to sevenfold for PR-A and PR-B respectively.

We next asked whether there was a differential change in isoform type in response to E_2 stimulation. As determined by the PR-A:PR-B ratios shown in Fig. 2, PR-A is the predominant isoform in the absence of E_2 in either rat or mouse pituitary cells. Although the expression levels for both PR isoforms increased in the presence of E_2 (Fig. 1), in the rat pituitary cells the A:B ratio significantly decreased (Fig. 2) thus providing evidence for a more robust response of PR-B to E_2 stimulation. For mouse pituitary cells, the tendency for a greater increase in PR-B than PR-A in the presence of E_2 did not reach statistical significance in the reflected ratio (Fig. 2).

To examine the effect of progesterone on the expression of PR isoforms, pituitary cells cultured in the presence of E_2 were exposed to 200 nM progesterone for 6 or 12 h. As shown in Fig. 3 in female rat pituitary cells, the expression of PR-A and PR-B were significantly decreased at 6 h progesterone exposure. The progesterone-induced decrease in rat PR was not isoform-specific as indicated by the lack of effect of progesterone treatment on PR-A:PR-B ratios (Fig. 4).

In contrast to rat cells, acute progesterone treatment of female mouse pituitary cells had no significant effect on the expression of PR isoforms or the A:B ratio as determined by

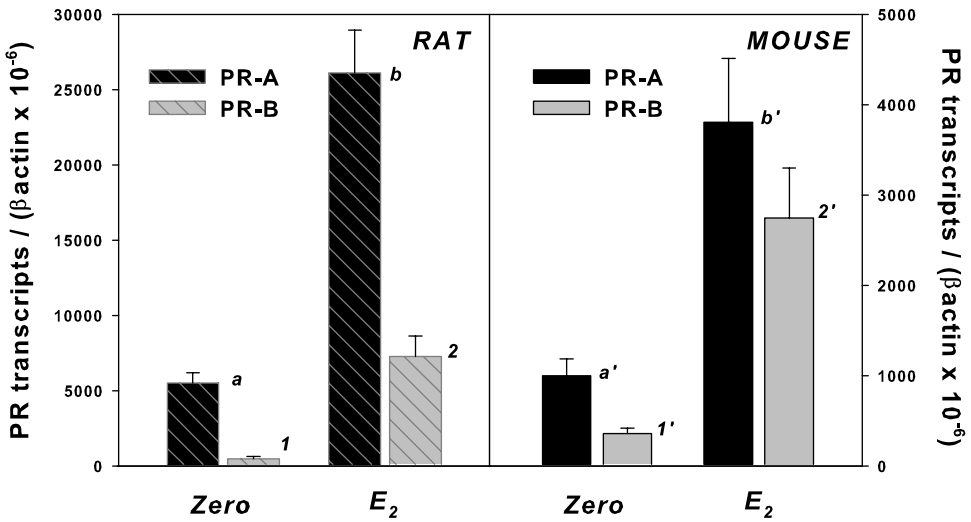

Figure 1 PR mRNA isoform expression is upregulated by E_2 in rat and in mouse pituitary cells in culture. Anterior pituitary cells from ovariectomized rats or mice were cultured with or without 0·2 nM E_2 for 3 days. RNA isolated from these cells was analyzed for PR-A+PR-B and PR-B mRNA by QRTPCR using standard curves against rat or mouse PR plasmids; levels of PR-A mRNA were determined by subtraction. Estimates of PR isoform transcript number were normalized to β-actin mRNA for each sample. Data are the means±S.E.M. of independent experiments/treatment: for rat cells, $n=3$ (zero) and 5 (E_2); for mouse cells, $n=5$ (zero) and 7 (E_2). For PR-A, bars not sharing the same letter (rat) or letter′ (mouse) are significantly different from each other ($P<0·05$). For PR-B, bars not sharing the same number (rat) or number′ (mouse) are significantly different from each other ($P<0·05$).

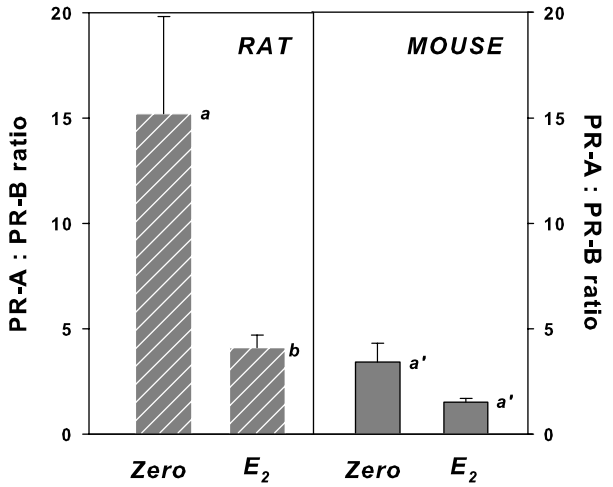

Figure 2 E_2 affects the ratio of PR-A:PR-B mRNA in rat but not mouse pituitary cells. Ratios were calculated from the data in Fig. 1. Within a panel, bars not sharing the same letter are significantly different from each other ($P<0{\cdot}05$).

QRTPCR, even when progesterone exposure was extended to 12 h (Figs 3 and 4).

LβT2 cells

The aim of these experiments was to determine the PR-A and PR-B isoform expression levels in LβT2 cells and to investigate whether PR is upregulated by E_2, investigate or affected by progesterone treatment. As presented in Table 3 and Fig. 5, PR-A is the predominant isoform expressed in LβT2 cells. The results in Fig. 5 clearly show that the presence of 0·2 nM E_2 for 3 days had no significant effect on the level of either PR isoform. Acute progesterone treatment had also no effect on PR expression levels (data not shown) or PR-A:PR-B ratios (Table 3).

The low level of PR expression, particularly PR-B, in LβT2 cells is consistent with the inability of progesterone to augment GnRH-stimulated LH secretion. Figure 6 shows that when E_2-treated LβT2 cells are presented with a GnRH pulse, the LH-secretory response is the same whether or not the cells had been exposed to 200 nM progesterone for 90 min preceding the GnRH challenges. The same treatment in mouse pituitary cells resulted in a 1·5-fold augmentation of GnRH-stimulated LH secretion; the augmentation due to progesterone in rat pituitary cells was threefold (Fig. 6).

The unresponsiveness of the PR in LβT2 cells to E_2 stimulation was somewhat surprising. The LβT2 cell line is mouse-derived and, compared to heterogeneous cultures of female mouse pituitary cells, PR expression in homogeneous LβT2 cultures was extremely low (compare normalized data in Figs 1 and 5). One prominent difference between the cell line and primary cultures is the extensive mitotic activity in the former, which is low to absent in the latter. In earlier work, we noted that treatment of LβT2 cultures with a glucocorticoid resulted in a reversible dampening of proliferation (Turgeon *et al.* 1996). Therefore, we tested whether PR expression levels in LβT2 cells would be affected

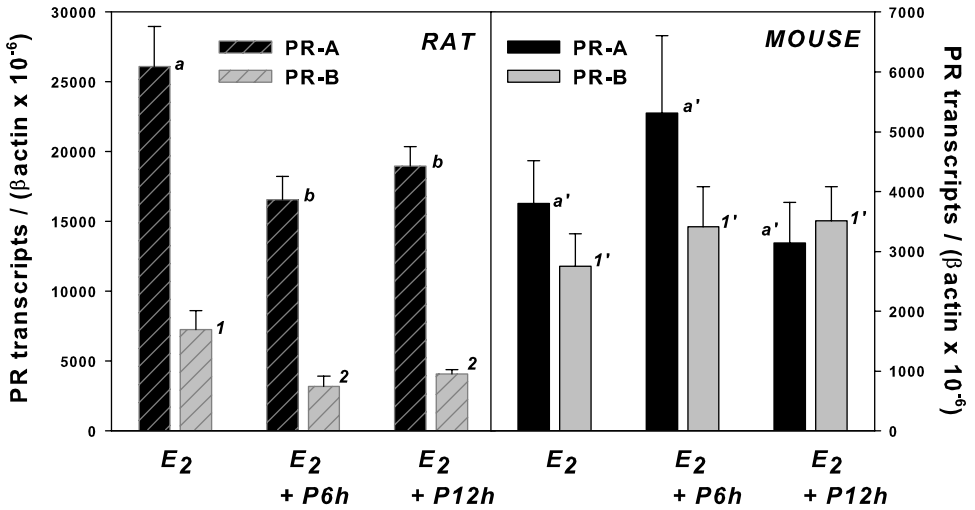

Figure 3 Progesterone downregulates PR mRNA isoform expression in rat but not mouse pituitary cells. Anterior pituitary cells from ovariectomized rats or mice were cultured in 0·2 nM E_2 for 3 days. At 6 or 12 h before RNA isolation, 200 nM progesterone (P) was added to the culture. Analysis of PR-A and PR-B mRNA by QRTPCR was as described for Fig. 1. Data are the means±S.E.M. of independent experiments/treatment: for rat cells, $n=4$ (E_2+P6h) and 3 (E_2+P12); for mouse cells, $n=7$ (E_2+P6h) and 7 (E_2+P12h). Data for E_2-treated cells are repeated from Fig. 1 for comparison. For PR-A, bars not sharing the same letter (rat) or letter′ (mouse) are significantly different from each other ($P<0{\cdot}05$). For PR-B, bars not sharing the same number (rat) or number′ (mouse) are significantly different from each other ($P<0{\cdot}05$).

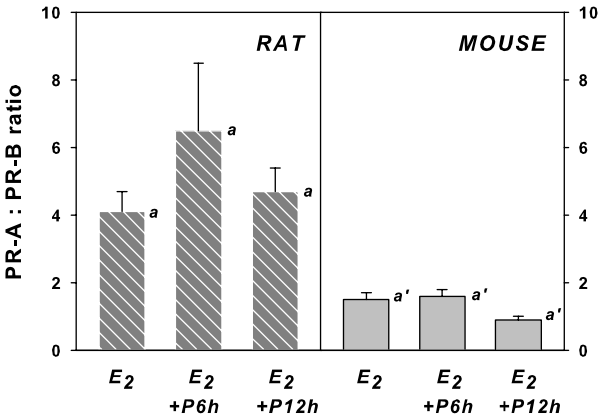

Figure 4 The ratio of PR-A:PR-B mRNA is not affected by progesterone treatment in mouse or rat pituitary cells. Ratios were calculated from the data in Fig. 3. Within a panel, bars sharing the same letter are not significantly different from each other.

by including 20 nM dexamethasone in the culture media in the presence and absence of chronic E_2 and acute progesterone exposure. Although PR isoform expression tended to be higher in the presence of the glucocorticoid, the difference did not reach statistical significance (e.g. in E_2-treated LβT2 cells, normalized total PR (A+B) expression was 121±22 in the presence and 74±16 in the absence of dexamethasone).

Male mouse pituitary cultures

We next asked whether the low expression level for PR in LβT2 cells and the unresponsiveness to E_2 in these cells were related to a male genotype. Figure 5 shows the results for pituitary cultures from orchidectomized mice. In the absence of E_2 in the culture medium, the levels of PR isoform expression were significantly higher than in LβT2 cells and not significantly different from the female mouse pituitary cells (compare Figs 1 and 5). The presence of E_2 for 3 days led to significant increases in expression of both PR isoforms in the male pituitary cells (Fig. 5) that were slightly less robust than female mouse pituitary cells (Fig. 1). The ratio of PR-A:PR-B was similar to the female mice and, as for females, E_2 did not have a significant effect on the ratio in male pituitary cells (Table 3).

Discussion

The difference between rat and mouse LH-secretory responses modulated by progesterone led us to hypothesize that the ratio of PR-A:PR-B diverges between the two rodent species. This study provides evidence that the regulation of the expression of these two PR isoforms is different between rat and mouse pituitary cells.

The rationale for comparing the regulation of pituitary PR expression comes from the observed differences in LH-secretory responses between rats and mice following progesterone augmentation or GnRH self-priming. Both of these events are estrogen- and transcription-dependent (Turgeon & Waring 1991). In the rat, acute progesterone has stimulatory actions at the hypothalamus and pituitary in facilitating the preovulatory LH surge (reviewed in Levine *et al.* (2001)). For rat pituitary cells cultured in the presence of E_2, progesterone treatment for 1–2 h leads to a tripling of GnRH- or depolarization-stimulated LH secretion, and the augmentation can be completely blocked by PR antagonists (Turgeon & Waring 1990, 1991, 1999). In contrast, identical progesterone treatment in mouse pituitary cells leads to a more modest augmentation of GnRH-stimulated secretion (Fig. 6) and has no effect on depolarization-stimulated LH secretion (Turgeon & Waring 2001). Another component of the preovulatory LH surge is GnRH self-priming, which contributes to signal amplification. In the rat, activation of the PR has been shown to have a role in the expression of the GnRH-priming response, and this occurs even in the absence of progesterone (Turgeon & Waring 1994). PR antagonists prevent the potentiation of the LH-secretory response to multiple GnRH pulses, the hallmark of GnRH priming (Waring & Turgeon 1992, Sánchez-Criado *et al.* 2004). For the mouse, GnRH priming has been reported *in vivo* and *in vitro*, and a potential role for the PR in this pathway is suggested by the demonstration that the priming response is absent or blunted in PR-knockout mice (Chappell *et al.* 1999, Turgeon & Waring 2001). However, unlike the rat, blockade of the PR with two different PR antagonists failed to prevent

Table 3 Comparison of PR isoform ratios for mouse pituitary and LβT2 cells

	PR-A:PR-B ratio*		
	Zero	E_2	E_2+P 6 h
Cell source			
LβT2 cells	10·9±2·3 (7)	14·2±2·9 (8)	14·0±1·5 (3)
Male mouse pituitary cells	2·5±0·2 (3)	1·6±0·1 (3)	ND

*Mean±S.E.M.; (*n*)=number of separate experiments. Cell source: LβT2 gonadotropes or pituitary cells from orchidectomized mice were cultured with or without 0·2 nM E_2 for 3 days. For LβT2 cells, with or without 200 nM progesterone (P) was included 6 h before termination. Statistical analysis: for each cell source group, there were no statistical differences among treatments. ND, not determined.

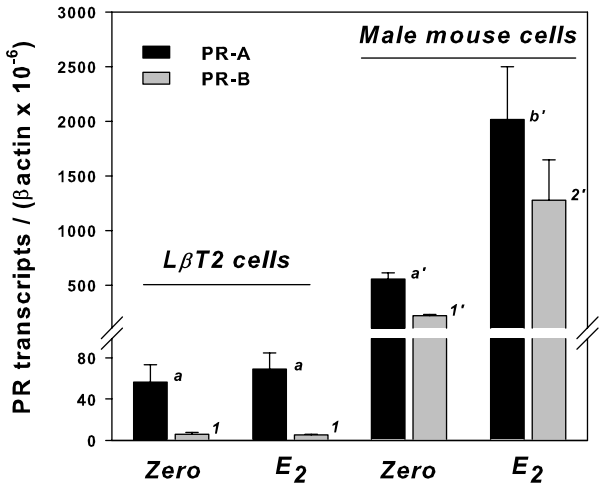

Figure 5 PR-A and PR-B expression is upregulated by E_2 in male mouse pituitary cells but not in LβT2 gonadotropes. Anterior pituitary cells from orchidectomized mice were cultured with or without 0·2 nM E_2 for 3 days. The analysis of PR-A and PR-B mRNA by QRTPCR was described in Fig. 1. Data are the means ± S.E.M. of independent experiments/treatment: for LβT2 cells, $n=7$ (zero) and 8 (E_2); for male mouse cells, $n=3$ (zero) and 3 (E_2). For PR-A, bars not sharing the same letter (LβT2) or letter′ (male mouse) are significantly different from each other ($P<0·05$). For PR-B, bars not sharing the same number (LβT2) or number′ (male mouse) are significantly different from each other ($P<0·05$).

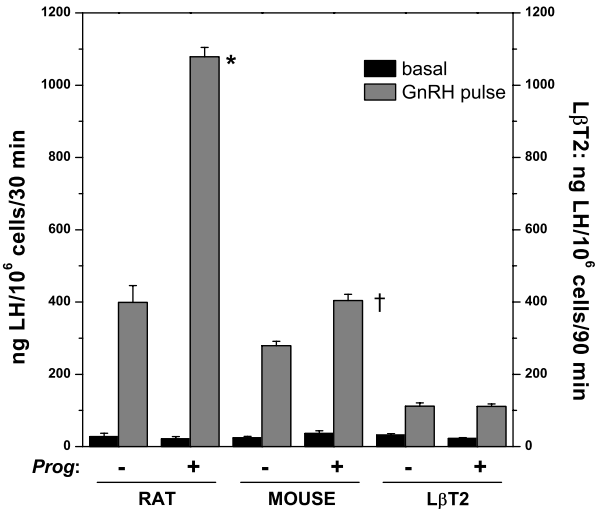

Figure 6 GnRH-stimulated LH secretion is augmented by progesterone in rat or mouse pituitary cells but not in LβT2 gonadotropes. Anterior pituitary cells from ovariectomized rats or mice or gonadotropes from the LβT2 line were cultured in 0·2 nM E_2 for 3 days and on day 4 were challenged with a 15-min pulse of either 1 nM (rats and mice) or 10 nM (LβT2 cells) GnRH. For the medium change at 90 min before the pulse, 200 nM progesterone was included for some groups (+prog). Note that LH is presented as integrated secretion over 30 min for rat or mouse cells and 90 min for LβT2 cells before (basal) and in response to the GnRH pulse. Mouse secretion data are fromTurgeon & Waring (2001) and presented here as normalized to time and total pituitary cell count for comparison. The symbols indicate a significant difference ($P<0·05$) between GnRH-stimulated groups (− or + prog) for rats (*) or for mice (†). For each group, $n=3$.

GnRH priming in wild type mouse gonadotropes *in vitro* (Turgeon & Waring 2001).

While there are likely multiple components contributing to these differences between rat and mouse responses, altered expression of pituitary PR isoforms is a strong candidate. In other cell systems, PR-A and PR-B have been reported to exhibit differential interactions with coregulators and to have different transcriptional activities when expressed separately (Mulac-Jericevic *et al.* 2000, Richer *et al.* 2002, An *et al.* 2005). *In vitro*, PR-B generally has been found to be a stronger transactivator than PR-A, and PR-A can modulate the transcriptional activity of PR-B (reviewed in Giangrande & McDonnell (1999)). In physiological settings, the ratio of PR-A:PR-B varies among target tissues, and these variations have been invoked to explain, in part, cell-specific differences in response to progesterone within complex tissues, for example, human uterus and mammary gland (Mote *et al.* 2000, 2002, Mesiano *et al.* 2002, Vienonen *et al.* 2002, Graham *et al.* 2005). As model systems to examine both cell-specific expression and functional contributions for each isoform, the use of mouse gene knockouts selective for either PR-A or PR-B have been particularly informative (reviewed in Mulac-Jericevic & Conneely (2004)).

The norm, however, for most PR-positive cells *in vivo* is the co-expression of A and B, and the ratio can change in response to physiological regulation and in pathological situations. As an example of the latter, PR-A and PR-B are equally expressed in normal human breast tissue, but the ratio is disrupted in breast cancer (Mote *et al.* 2002, Hopp *et al.* 2004). In a recent study, the functional consequence of a PR-transcriptional activity change in the balance of A to B expression was examined in an inducible model of PR-A predominance in breast cancer lines. While most PR-target genes were not sensitive to the ratio change, progestin responsiveness was acquired in a subpopulation of gene targets involved in a distinct functional role: cell shape and adhesion (Graham *et al.* 2005). Similar findings have been reported for ductal growth and epithelial transformations in mammary glands of transgenic mice carrying a change in the balance of the native ratio of A:B due to overexpression of PR-A (Shyamala *et al.* 2000, Chou *et al.* 2003).

Our study addresses the physiological regulation of the ratio of PR-A:PR-B in terminally differentiated pituitary cells, thus a change in the balance of isoforms would be expected to elicit more subtle alterations in cell function, such as a modified secretory response. E_2 treatment led to a several fold increase in the total pituitary PR for rats and mice. Although PR-A remained the predominant isoform in both species, only in the rat pituitary was the ratio of A:B dramatically decreased (Fig. 2). We speculate that such a modification in the balance of PR isoforms could affect transcriptional activity mediating the early response to

progesterone, such as augmentation of GnRH- or depolarization-induced LH secretion. Further studies are necessary to define specific pathways and mechanisms, but possible areas for focus would address the consequences of a change in the proportion of hetero- and homodimers (increase in A/B with associated decrease in A/A) or the effect of an alteration in PR-A on coregulator availability (Giangrande *et al.* 2000, Smith & O'Malley 2004). In this scenario, the shift in the balance of PR isoforms would be sufficient to enhance the transcriptional efficiency required, for example, for a downstream event in the secretory pathway. Consistent with this, the increase in isoform expression in the mouse without a change in the ratio correlates with the less robust or absent early response to progesterone observed in secretion studies (Fig. 6; Turgeon & Waring 2001).

Further suggestion that the absence of an E_2-induced change in the balance of PR isoforms in the mouse pituitary may impact the response to progesterone comes from the observation that progesterone treatment was unable to downregulate PR expression. This is in contrast to the response of rat pituitary cells in which the expression of both isoforms was reduced by ~40% at 6 h progesterone exposure (Fig. 3), which is in agreement with our earlier report that progesterone reduced total PR mRNA levels by half within this same time frame as determined by Northern blot analysis (Turgeon *et al.* 1999).

We and other researchers have hypothesized that the loss of gonadotrope responsiveness to progesterone through receptor downregulation contributes to the termination of the LH surge (Banks & Freeman 1978, Turgeon & Waring 2000). Pituitary PR protein has been shown in rats *in vivo* to fall from a high level on proestrus to a low on estrus (Thrower & Lim 1980, Rainbow *et al.* 1982). We reported in rat pituitary cells *in vitro* that progesterone led to a profound loss in PR protein (>75% by 9–12 h progesterone exposure) that was due, at least in part, to a proteasome-mediated pathway (Turgeon & Waring 2000). In mouse pituitary cells under similar conditions, the fall in pituitary PR protein following progesterone treatment is much less (30–40% decrease; Turgeon *et al.* 2001). One explanation derived from the present study for the difference between the two species is that the dramatic loss in PR in rat gonadotropes is a composite of decreased PR mRNA and increased proteosome-mediated degradation of PR protein; whereas in the mouse, without the change in mRNA, the more modest decrease in available PR protein is due entirely to downregulation of the protein. It remains to be established whether there might be differences in isoform-specific degradation induced by the proteasome pathway in gonadotropes. However, given that both A and B isoforms contain the consensus sequence ('destruction box') implicated as a target in the ubiquitin–proteasome pathway (Qiu & Lange 2003) and both isoforms have been shown to be similarly regulated by this pathway in a breast cancer cell line *in vitro* (Lange *et al.* 2000) and in the brain *in vivo* (Camacho-Arroyo *et al.* 2002), a divergence in the regulation of PR-isoform degradation in the gonadotrope would seem less likely.

Mouse pituitary dynamics are unusual in that the PR is found in lactotropes as well as in gonadotropes (Turgeon *et al.* 2001), unlike the rat (Fox *et al.* 1990, Turgeon & Waring 2000, Turgeon *et al.* 2001) or the monkey (Sprangers *et al.* 1989, 1990) in which PR localizes exclusively to gonadotropes. In the present study, PR-isoform expression was determined in anterior pituitary cell extracts, and therefore the mouse results reflect activity in both cell types. We are not aware of any reports describing cell type-specific expression of the isoforms of PR in mouse pituitaries. However, for total PR protein, we reported earlier that the overall pattern of regulation is similar in mouse lactotropes and gonadotropes; for both cell types, upregulation by E_2 and downregulation by progesterone are comparable except that the level of PR protein tended to be less in lactotropes (Turgeon *et al.* 2001). Although a divergence in isoform expression between mouse gonadotropes and lactotropes is formally possible, the similarity in steroid regulation of PR protein would support the simple interpretation that the two cell types share similarities in isoform expression.

The mouse gonadotrope-derived LβT2 cell line has been a valuable tool for dissecting signaling pathways in GnRH action and gonadotropin production. The expression of estrogen receptor isoforms and their regulation in LβT2 cells have been well characterized (Schreihofer *et al.* 2000), but little information is available for PR. We report here that PR-A is the predominant isoform expressed in this cell line, but, unlike that found for primary cultures of either mouse or rat pituitary cells, E_2 treatment did not lead to the upregulation of either isoform. Another divergence was the very low level of total PR mRNA in LβT2 cells, which is remarkable given that LβT2 cultures represent a homogeneous cell type compared to primary mouse pituitary cultures. That this weak expression of PR cannot be explained by a male phenotype is demonstrated by PR mRNA expression in primary cultures of male mouse pituitary cells, which is greater than tenfold higher than in LβT2 cells. Also, distinct from LβT2 cells, primary male pituitary cells showed the same balance of PR-A to PR-B and a proportional increase to E_2 stimulation as was also seen in female cells.

Whether the pronounced dissimilarities in PR expression and regulation between LβT2 cells and their non-immortalized counterparts are a function of the cell line's arrested development or transformation remains to be determined. Relative to our work, however, the observation that LβT2 cells express very low levels of PR primarily composed of the A isoform, with no demonstrable regulation by E_2, is of interest for interpretation of our functional studies of LH secretion. We show here that progesterone does not augment GnRH-stimulated LH secretion in LβT2 cells (Fig. 6) and, significantly, this cell line does not exhibit GnRH priming (Turgeon *et al.* 1996). This is consistent with our hypothesis that PR has a role in GnRH priming.

In summary, PR-A is the predominant isoform expressed in rat or mouse pituitary cells and LβT2 gonadotropes. While PR mRNA levels in LβT2 cells were unresponsive to E_2, rat or mouse pituitary cultures respond to E_2 stimulation with an increase in expression of both A and B isoforms. However, in the rat, but not the mouse, the PR-B response to E_2 is more robust, and the result is a decrease in the A:B ratio. Another point of departure is the progesterone-induced decrease in PR-A and PR-B mRNA in rat but not mouse pituitary cells. These results provide correlative support for our hypothesis that the divergence between rat and mouse LH secretion modulated by either GnRH self-priming or progesterone augmentation, both of which are E_2- and transcription-dependent events, is due in part to differences in the balance of PR isoforms modulated by E_2 in gonadotropes.

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