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# Steroid Regulation of Parathyroid Hormone-related Protein Expression and Action in the Rat Uterus

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**The gene encoding parathyroid hormone-related protein (PTHrP), an autocrine/paracrine inhibitor of vascular and nonvascular smooth muscle contractility, is regulated by hormonal steroids including estrogens ($E_2$), 1,25-dihydroxy vitamin D (Vit $D_3$) and glucocorticoids. While $E_2$ increases PTHrP gene expression, Vit $D_3$ and glucocorticoids inhibit transcriptional activity of this gene. In the uterus of ovariectomized rats, $E_2$-treatment increases both PTHrP mRNA levels and smooth muscle sensitivity to the action of PTHrP(1–34). To examine the action(s) of Vit $D_3$ and glucocorticoids on these parameters, OVX rats were treated with $E_2$, Vit $D_3$ or the synthetic glucocorticoid, dexamethasone (Dex), alone, or with $E_2$ following a 1 h pretreatment with Vit $D_3$ or Dex. PTHrP and PTH/PTHrP receptor mRNA were measured by blot hybridization analysis of RNA prepared from uteri collected 2, 4 and 24 h after treatment. Uterine horns were used to measure the effect of the steroids on the ability of PTHrP(1–34) to inhibit spontaneous myometrial contraction. When $E_2$, Vit $D_3$ and Dex were given alone, only $E_2$ altered PTHrP mRNA levels in the uterus, however, a 1 h pretreatment with Dex but not Vit $D_3$ markedly diminished this effect of $E_2$. The temporal decline in uterine PTH/PTHrP receptor mRNA levels measured 2 and 4 h after $E_2$ treatment inversely correlated to changes in sensitivity of the tissue to PTHrP(1–34) measured at 24 h after $E_2$ administration. In comparison to $E_2$ alone, treatment with Vit $D_3$ and $E_2$ augmented the uterine responsiveness to PTHrP(1–34) while pretreatment with Dex (1 mg/kg) and $E_2$ decreased this response. These data indicate that in the uterus, Dex opposes the positive effect of $E_2$ on PTHrP gene activity and differentially modulates the action of PTHrP on myometrial tone. Moreover, elevations in the circulating levels of cortisol at term may serve to decrease both the uterine expression of PTHrP and the local action of PTHrP on the myometrium prior to parturition, therefore promoting myometrial contraction associated with labor.**

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## INTRODUCTION

Parathyroid hormone-related protein (PTHrP) was originally identified in cancers derived from patients with humoral hypercalcemia of malignancy [1–3]. The production of PTHrP in normal tissues as diverse as the skin [4], mammary gland [5], chicken oviduct shell gland [6], areas of the central nervous system [7], the urinary bladder [8], and the gravid and non-gravid rat uterus [9–11], suggest that it may be a locally acting protein. Furthermore, the localization of PTHrP mRNA and immunoreactive PTHrP in smooth muscle, such as vascular smooth muscle, the detrusor and the myometrium, and the potent relaxation of the smooth muscle in these tissues by amino-terminal fragments of PTHrP [6, 8, 11, 12] suggest that PTHrP may be an important paracrine regulator of smooth muscle activity.

A common property of PTHrP in the smooth muscle of visceral tissues, such as the bladder, stomach and uterus, is the up-regulation of PTHrP gene expression in response to tissue distension [8, 9, 13]. In the immature and cycling rat uterus, however, regulation of PTHrP gene expression is also under the influence of the ovarian hormone estrogen ($E_2$) [10, 11]. Furthermore, $E_2$ dominance in the estrus cycle of the female rat is associated with an increased relaxing action of PTHrP on isolated rat myometrium [11]. Similarly, pretreatment of immature female rats with $E_2$ markedly

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increased the responsiveness of the uterus to synthetic PTHrP(1–34) [11]. Together these findings suggest that PTHrP expression and action in the uterus is linked to both tissue stretch and circulating $E_2$ levels.

*In vitro*, $E_2$ has also been shown to stimulate the expression of the PTHrP gene in cultures of rat pituitary [14] and human endometrial [15] cells. In addition, the expression of the PTHrP gene is regulated by other steroids, such as glucocorticoids and 1,25-dihydroxy vitamin $D_3$ (Vit $D_3$). Dexamethasone (Dex) decreases PTHrP gene transcription in COLO16 epidermal squamous cancer cells [16], H-500 rat Leydig tumour cells [17], and TT human carcinoma cells [18]. Similarly, Vit $D_3$ has been shown to decrease levels of PTHrP mRNA in H-500 rat Leydig tumour cells [17], in TT human carcinoma cells [18] and in a human T cell lymphotrophic virus type I-infected T cell line (MT-2) [19] also via the inhibition of transcription. Although these data support a role for $E_2$, Vit $D_3$ and glucocorticoids in the expression of PTHrP, little is known about the effect of Vit $D_3$ and Dex on PTHrP gene expression, *in vivo*. In addition, the affect(s) of these steroids on the expression of the PTH/PTHrP receptor gene [20, 21], *in vivo*, is not known. In this study, we investigated the levels of PTH/PTHrP receptor mRNA, and PTHrP mRNA and protein in the ovariectomized (OVX) rat uterus following administration of Dex and Vit $D_3$ alone or in combination with $E_2$. Furthermore, we also explored whether Dex or Vit $D_3$ affect the $E_2$-induced enhancement of the myometrial response to PTHrP(1–34).

## MATERIALS AND METHODS

### *Ovariectomy and steroid administration*

The guidelines for animal experimentation used in this study were approved by this institution. 6-week-old Sprague–Dawley rats (Charles River, Boston, MA) were ovariectomized (OVX) and allowed to recover for 10 days following surgery. OVX rats were treated with 17$\beta$-estradiol benzoate ($E_2$), and Dex (Sigma, St Louis, MO) or 1,25-dihydroxy vitamin $D_3$ (Vit $D_3$) (Biomol, Plymouth Meeting, PA). Steroids were dissolved in a vehicle (10% ethanol in phosphate buffered saline) and administered subcutaneously in a total volume of 200 $\mu$l. For tissue harvests, rats were killed by $CO_2$ asphyxiation and uteri were dissected free of fat and connective tissue. For the preparation of RNA, uteri were immediately frozen in liquid nitrogen. For the 24 h samples, one uterine horn was frozen for RNA preparation while the other horn was used for tissue bath analysis.

### *RNA preparation and blot hybridization analysis*

Uterine horns ($n = 3$–4) were homogenized in a solution containing 4 M guanidium isothiocyanate, 0.03 sodium acetate and 0.4 gm/ml CsCl using a Polytron (Brinkman, Littau, Switzerland) homogenizer. Total RNA was then pelleted through a CsCl (5.7 M)/sodium acetate (0.03 M) cushion by overnight centrifugation at 100,000 *g*. Total RNA for each pooled sample was determined by reading absorbance at 260 nm. For Northern blot hybridization analysis 20 $\mu$g of total RNA from each sample was separated by electrophoresis through a 0.9% formaldehyde-agarose gel, transferred to a supported Nitrocellulose membrane (Schleider & Schuell, Keene, NH) and cross-linked to membranes using a Stratalinker (Stratagene Inc., La Jolla, CA). The membranes were prehybridized in a solution containing 50% deionized formamide, 6 × SSC (1 × SSC = 0.5 M NaCl and 0.05 M Na Acetate), 7.5 × Denhardt's solution (1 × Denhardt's solution = 0.02% Ficoll, polyvinyl-polypyrrolidone and bovine serum albumin), and 0.1 mg/ml heat-denatured sonicated salmon sperm DNA for 8 h at 42°C. Rat-specific PTHrP, PTH/PTHrP receptor and 18S rRNA cDNA probes were labeled with $^{32}$P[$\alpha$CTP] using a random oligonucleotide priming kit (Pharmacia, Piscataway, NJ). Membranes were hybridized at 42°C for 20 h in hybridization solution containing $1.5 \times 10^6$ cpm/ml of $^{32}$P labeled cDNA. Following hybridization, the membranes were finally washed in a solution of 0.3 × SSC/0.1% SDS at 55°C. Membranes were exposed to Hyperfilm MP (Amersham, Chicago, IL) for up to 7 days. For sequential hybridizations, membranes were incubated in prehybridization buffer at 52°C for at least 5 h to remove excess probe.

### *Rat uterine horn tissue-bath assay and data analysis*

The mid-portion (2–3 cm long) of the uterine horns of each rat were removed and mounted in 20 ml organ baths in physiological salt solution (PSS) with 95% $O_2$ and 5% $CO_2$ at 37°C, pH 7.4. The composition of the PSS (mM) was as follows: NaCl (117), KCl (4.7), $MgSO_4$ (1.2), $NaH_2PO_4$ (1.2), $NaHCO_3$ (25), $CaCl_2$ (2.5), D-(+)-glucose (11) and sodium ethylenediamine tetraacetic acid (0.67). The isometric tension was recorded using GRASS FT.03 tension–displacement transducers and displayed on a GRASS polygraph. A resting tension of 1 g was applied to uterine horns removed from OVX rats, which were allowed to equilibrate for at least 30 min. All horns from different treatments exhibited spontaneous contractions within the time of equilibration. PTHrP(1–34) (Bachem California, Torrance, CA) was diluted in 0.1% (w/v) bovine serum albumin and 10 mM acetic acid and added to the tissue bath in a cumulative fashion of half-log increments where the final concentrations ranging from 0.01 to 1000 nM. The decrease in the amplitude of spontaneous contractions 2 min after each addition of PTHrP(1–34) was taken as the index of inhibition. Zero percent inhibition was taken as the average amplitude of spontaneous contractions during the resting phase, 5 min prior to the addition of PTHrP(1–34). Relaxation of spontaneous contractions

was expressed as a percentage of the maximal response obtained in the absence of PTHrP(1–34). Data was pooled for each uterine horn of individual experiments. The molar concentrations producing 50% of the maximal response ($EC_{50}$) were calculated, together with 95% confidence limits by linear regression analysis. All results are expressed as the mean ± SEM, except where noted. Comparison of the potency of PTHrP(1–34) on uteri taken from different steroid treatment groups were made by means of Student's unpaired $t$-test. $P < 0.05$ was taken as an index of statistical significance.

## RESULTS

*Effect of $E_2$, Dex and Vit $D_3$ on the levels of PTHrP and PTH/PTHrP receptor mRNA in the rat uterus*

As seen in Fig. 1, the administration of $E_2$ to OVX rats rapidly increased uterine levels of PTHrP mRNA which peak between 2 and 4 h and return to basal levels by 24 h after treatment. In contrast, levels of PTH/PTHrP receptor mRNA in the uterus rapidly fell following $E_2$-treatment with levels at 24 h being only about 50% of basal levels. 2 h following treatment with Vit $D_3$ (20 μg/kg) or Dex (1 mg/kg) levels of PTHrP mRNA were unchanged, however, steady state levels of PTH/PTHrP receptor mRNA were reduced 60% by Dex.

To examine the influence of Vit $D_3$ and Dex on $E_2$-stimulated changes in PTHrP and PTH/PTHrP receptor mRNA levels in the uterus, rats were treated with either steroid hormone 1 h prior to administration of $E_2$. While Vit $D_3$-pretreatment did not alter $E_2$-stimulation of PTHrP mRNA, pretreatment with Dex completely blocked the $E_2$-stimulated increase in PTHrP mRNA seen after 2 h (Fig. 2). This potent effect of Dex on $E_2$-stimulation of PTHrP mRNA was somewhat transient since levels of PTHrP mRNA after 4 h were approx. 50% of those measured in rats treated with $E_2$ alone. Although Vit $D_3$ did not appear to influence the effect of $E_2$ on PTH/PTHrP receptor mRNA levels. Pretreatment with Dex attenuated the reduction in this mRNA associated with $E_2$ treatment. While $E_2$-treatment was generally associated with lower levels of PTH/PTHrP receptor mRNA in the uterus after 24 h, there were no notable differences in the levels of either PTHrP or PTH/PTHrP receptor mRNA in rats treated for 24 h with vehicle or with combinations of steroid hormones (See Fig. 3).

*Effect of Dex or Vit $D_3$ alone or in combination with $E_2$ on the myometrial response to PTHrP(1–34) in the OVX rat*

PTHrP(1–34) concentration-dependently inhibited spontaneous contractions of the myometrium in OVX rats (Fig. 4). Administration of $E_2$ (20 μg/kg) to OVX rats increased the potency of PTHrP(1–34) by approx. 5000-fold, 24 h following administration. Furthermore, Vit $D_3$ (20 μg/kg) or Dex (1 mg/kg) treatment alone increased the potency of PTHrP(1–34) inhibition of myometrial contractions by approx. 20- and 40-fold respectively, 24 h after administration. When $E_2$ was

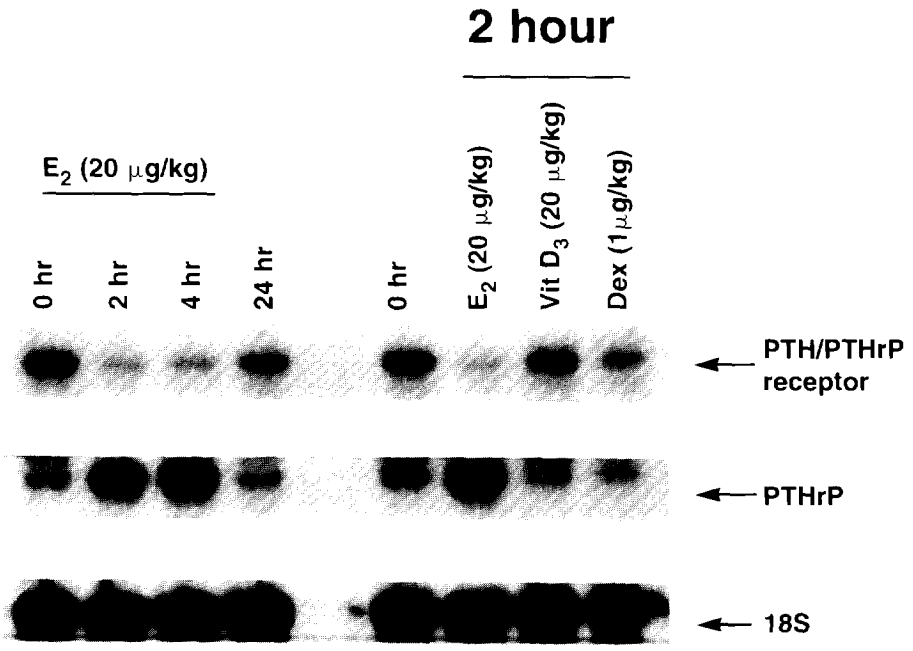


Fig. 1. Effect of $E_2$, Dex and Vit $D_3$ on the levels of PTHrP and PTH/PTHrP receptor mRNA levels in the rat uterus. Uteri were collected from rats treated with $E_2$ (20 μg/kg) for 2, 4 or 24 h or with Vit $D_3$ or Dex for 2 h. Total RNA (20 μg/lane) was prepared and hybridized sequentially to cDNAs encoding rat PTHrP, PTH/PTHrP receptor and 18S rRNA, as described in Materials and Methods. mRNA sizes are PTHrP mRNA ~1.5 kb, PTH/PTHrP receptor mRNA ~2.4 kb and 18S rRNA ~1.8 kb.

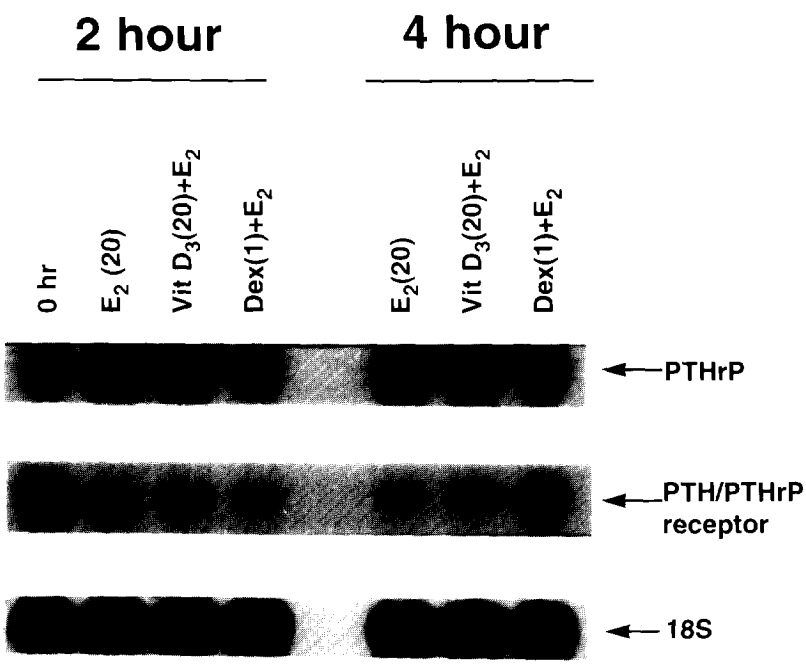


Fig. 2. Effect of Dex and Vit $D_3$ pretreatment on the $E_2$-stimulated changes in levels of PTHrP and PTH/PTHrP receptor mRNA levels in the rat uterus. Rats were injected with either vehicle (10% ethanol). Vit $D_3$ (20 $\mu$g/kg) or Dex (1 mg/kg) and 1 h later they were given $E_2$ (20 $\mu$g/kg). RNA was prepared from uteri collected from rats 2 and 4 h after administration of $E_2$. Total RNA (20 $\mu$g/lane) was hybridized sequentially to cDNAs encoding rat PTHrP, PTH/PTHrP receptor and 18S rRNA, as described in Materials and Methods.

administered to OVX rats 1 h after 2 or 20 $\mu$g/kg of Vit $D_3$ treatment, only the higher dose of Vit $D_3$ potentiated the $E_2$-induced increase in the potency of PTHrP(1–34) by approx. 10-fold, 24 h after $E_2$ administration, whereas the smaller dose was without effect. In contrast, when rats were pretreated with Dex (0.1 or 1 mg/kg) the 1 mg/kg dose of Dex inhibited the $E_2$-enhanced potency of PTHrP(1–34) on myometrial contractions by approx. 11-fold while the effect of pretreatment with 0.1 mg/kg Dex was not significant. The calculated $EC_{50}$ and relative potency values of PTHrP(1–34) in inhibiting myometrial contractions in rats treated with different steroids and steroid combination treatments are shown in Table 1.

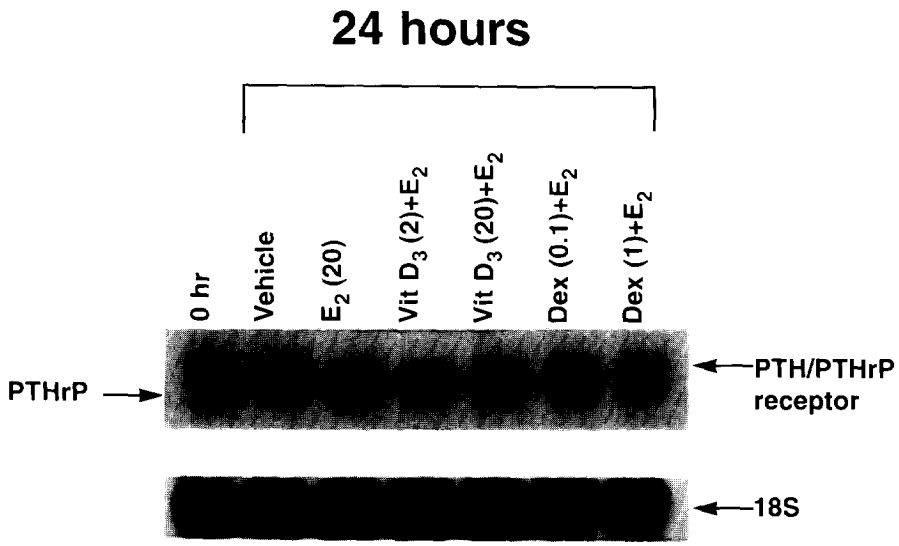


Fig. 3. Effect of Dex and Vit $D_3$ pretreatment on the $E_2$-stimulated changes in levels of PTHrP and PTH/PTHrP receptor mRNA levels in the rat uterus. Rats were injected with vehicle (10% ethanol/90% phosphate buffered saline), Vit $D_3$ (2 or 20 $\mu$g/kg) or Dex (0.1 or 1 mg/kg) and 1 h later they were given a dose of vehicle or $E_2$ (20 $\mu$g/kg). Total RNA was prepared from uteri collected 24 h after vehicle or $E_2$ administration and 20 $\mu$g total RNA/lane was hybridized sequentially to cDNAs encoding rat PTHrP, PTH/PTHrP receptor and 18S rRNA, as described in Materials and Methods.

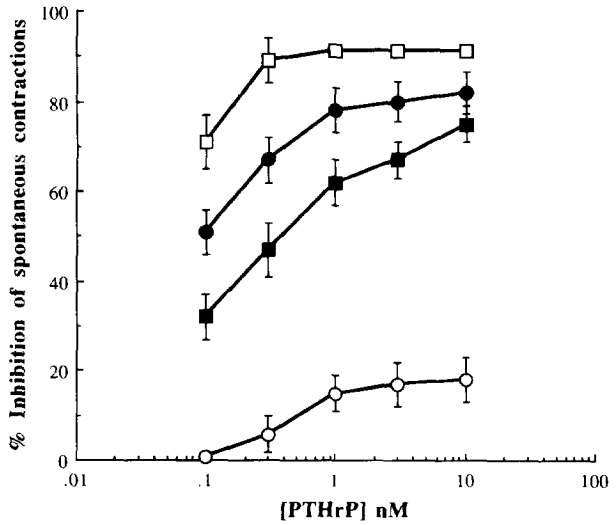


**Fig. 4. Effect of cumulative additions of PTHrP(1–34) on spontaneous contractions of uteri taken from OVX rats treated for 24 h with vehicle (○), $E_2$ (20 μg/kg) (●), Vit $D_3$ (20 μg/kg) and $E_2$ (20 μg/kg) (□), or Dex (1 mg/kg) and $E_2$ (20 μg/kg) (■). Points are means ± SEM ($n = 4$).**

## DISCUSSION

The rat uterus is a widely used *in vivo* model for investigating the actions of estrogenic steroids on gene expression, cell proliferation and smooth muscle tone. The response of the uterus to $E_2$ can be measured by the changes in the levels of mRNAs encoding protooncogenes [22–25] and growth factors [26–28] which may, in turn, drive the proliferative activity of the hormone, while other gene products, such as the calciotropic protein PTHrP appear, in part, to regulate the contractile activity of the myometium [11, 29, 30]. *In vitro* [14, 15] and *in vivo* [10, 11], $E_2$ stimulates a rapid, yet transient increase in PTHrP mRNA via mechanisms involving *de novo* transcription and mRNA turnover, responses indicative of an early response gene [31]. In the rat uterus, $E_2$-treatment also results in a marked enhancement of the responsiveness of the myometrium to PTHrP(1–34) [11], suggesting that $E_2$ modulates levels of endogenous PTH/PTHrP receptors in this tissue. Other hormonal steroids, such as glucocorticoids and Vit $D_3$, have been shown to decrease the transcriptional rate of the PTHrP gene *in vitro* [17–19], however, nothing is known about the role of glucocorticoids and Vit $D_3$ on the expression or activity of the PTHrP gene in the rat uterus.

*Table 1. $EC_{50}$ values and relative potency of PTHrP(1–34) inhibition of spontaneous contractions of uterine horns taken from OVX rats treated for 24 h with vehicle, 20 μg/kg $E_2$, 2 or 20 μg/kg Vit $D_3$ and $E_2$ (Vit $D_3$ (2 or 20) + $E_2$), 0.1 or 1 mg/kg Dex and $E_2$ (Dex(0.1 or 1) + $E_2$), 20 μg/kg Vit $D_3$(20)), or 1 mg/kg Dex (Dex(1)) alone*

| Treatment | $EC_{50}$(pM) | (95% c.l.) | Relative potency |
|---|---|---|---|
| $E_2$ | 66.9 | (7.53–243) | 1 |
| Vehicle | 11900 | (503–28300)* | 4930 |
| Vit $D_3$(2) + $E_2$ | 49.6 | (8.11–115) | 1.24 |
| Vit $D_3$(20) + $E_2$ | 4.21 | (0.457–38.0) | 0.0936 |
| Dex(0.1) + $E_2$ | 309 | (210–453) | 4.34 |
| Dex(1) + $E_2$ | 466 | (154–1110)* | 11.4 |
| Vit $D_3$(20) | 6630 | (3130–14000)* | 259 |
| Dex(1) | 4130 | (2550–8550)* | 126 |

*Significant difference from $E_2$-treated group ($P < 0.05$)
$EC_{50}$ values are represented with their corresponding 95% confidence limits (95% c.l.).

In this study, we used the $E_2$-deficient, OVX rat to examine the effect of $E_2$, the synthetic glucocorticoid, Dex, and Vit $D_3$ on the expression of the PTHrP and PTH/PTHrP receptor genes in the uterus. We found that unlike $E_2$, neither Dex nor Vit $D_3$, when administered alone, affected early changes in the levels of PTHrP mRNA in the uterus. However, when rats were pretreated with Dex, there was a complete blockade of the early (2 h) increase in levels of PTHrP mRNA stimulated by $E_2$, indicating that glucocorticoids may oppose the activity of $E_2$ at the level of the PTHrP gene. Vit $D_3$ alone or when administered prior to $E_2$ did not affect the steady state levels of PTHrP mRNA in the uterus within the range of doses and time points used in this study. Therefore, these data indicate that in the uterus, glucocorticoids may selectively oppose the action of $E_2$ on the expression of both the PTHrP and PTH/PTHrP receptor gene.

Adminstration of $E_2$ to either sexually immature [11] or OVX (present data) rats causes a marked enhancement in the responsiveness of the myometrium to the inhibitory action of N-terminal synthetic PTHrP peptides, indicating that $E_2$ likely increases the levels of functional PTH/PTHrP receptors in the myometrium. Interestingly, we found that $E_2$ caused a rapid decline in steady state levels of PTH/PTHrP receptor mRNA, and that neither Dex nor Vit $D_3$ pretreatment appeared to alter this decline, except at the 4 h time point when uteri from rats pretreated with Dex showed a higher level of this message relative to those seen in uteri of $E_2$-treated animals. This negative effect of $E_2$ extended to 24 h where the levels of PTH/PTHrP receptor mRNA were approx. 50% of those seen in the tissue at time of injection. We consider this rapid decline in PTH/PTHrP receptor mRNA associated with $E_2$-treatment to result from an increase in the translation of PTH/PTHrP receptor mRNA in this tissue, which may increase the response of the myometrium to PTHrP. Alternatively, the increased responsiveness of the tissue to PTHrP in $E_2$-treated animals may result from an effect of $E_2$ on intracellular signalling pathways, such as adenylyl cyclase [32, 33]. Additional experimentation will be needed to clarify the molecular mechanisms behind the action of $E_2$ on the PTH/PTHrP receptor mRNA and myometrial responsiveness to PTHrP.

Although little is known about the possible actions of Vit $D_3$ on the uterus, the effect of glucocorticoids on this tissue are well-documented. Functional glucocorti-

coid receptors have been demonstrated in both human [34] and rat [35] uteri. Administration of Dex to OVX rats blocked both the $E_2$-stimulated increases in uterine wet weight and ornithine decarboxylase activity [36]. Furthermore, administration of Dex to $E_2$-treated, hypophysectomized rats reduced the uterine phopholipase $A_2$ activity induced by $E_2$ [37]. As previously seen in human endometrial cells [15], Dex did not alter levels of PTHrP mRNA in the rat uterus, however, like $E_2$, Dex alone lowered the levels of PTH/PTHrP receptor mRNA in the uterus within 2 h of treatment. In $E_2$-treated animals, pretreatment with either Vit $D_3$ or Dex did not alter the reduction in PTH/PTHrP receptor mRNA seen after 2 h. After 4 h, levels of this message were unaffected by Vit $D_3$-pretreatment but were relatively higher in rats pretreated with Dex than in rats treated with $E_2$ alone, suggesting that Dex may oppose the effect of $E_2$ on the transient lowering of PTH/PTHrP receptor mRNA. Therefore, glucocorticoids modulate the expression of PTH/PTHrP receptor mRNA and possibly the subsequent responsiveness of the myometrium to the inhibitory action of PTHrP.

In support of the possibility that glucocorticoids may alter the responsiveness of the myometrium to the action of PTHrP, we found that uteri from rats treated with Dex alone displayed an increase in sensitivity to PTHrP(1–34) as compared to tissues from vehicle treated animals. These data suggest that the rapid decline in steady state levels of PTH/PTHrP receptor mRNA observed 2 h after treatment with Dex treatment may be due to an increase in translation of the PTH/PTHrP receptor mRNA which results in higher levels of surface receptors measured after 24 h. While Dex-pretreatment appeared to attenuate the negative effect of $E_2$ on levels of PTH/PTHrP receptor mRNA measured after 4 h, this treatment also decreased the responsiveness of the uterus to the action of PTHrP compared to animals treated with $E_2$ alone. Although the increased responsiveness of the myometrium to PTHrP in Dex-treated animals may result from an effect of Dex on intracellular signalling pathways, the effect of Dex on the $E_2$-induced increase in the responsiveness to PTHrP may also result from an inhibition of $E_2$-induced translation. The present data suggests that Dex may differentially affect the responsiveness (i.e. levels of PTH/PTHrP receptor) of the uterus to PTHrP depending upon the availability of endogenous $E_2$. Further experimentation, however, is required to clearly resolve the mechanism by which Dex modulates the expression of both PTHrP and PTH/PTHrP receptor gene in the uterus.

During the later stages of pregnancy, circulating levels of glucocorticoids such as cortisol and corticosterone, are markedly increased with highest levels seen at parturition [38]. Elevations in these steroids have been shown to have profound effects on uterine activity and may function to promote the onset of labor [39, 40]. In the rat uterus, the expression of PTHrP, a potent inhibitor of myometrial contraction, is markedly increased during the last days of pregnancy [9], indicating a potential, albeit, unproven role for the protein in opposing the contractile activity of this tissue. In the present study we show that the synthetic glucocorticoid, Dex can decrease the responsiveness of the $E_2$-primed myometrium to PTHrP. Therefore, we propose that the elevations in glucocorticoids seen at term may function in the promotion of labor by decreasing the endogenous relaxing activity of PTHrP on the myometrium.

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