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Does progesterone inhibit bacteria-stimulated interleukin-8 production by lower genital tract epithelial cells?

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Abstract

Objective: Progesterone (P_4) has been clinically shown to prevent the recurrence of preterm birth. The mechanism(s) of action is unclear, but may involve modulation of the immunologic inflammatory response of the lower genital tract. We evaluated the effects of P_4 on interleukin-8 (IL-8) production by vaginal and cervical epithelial cells stimulated with bacterial species that are commonly associated with preterm birth.

Methods: Vaginal and endocervical epithelial cells were incubated with up to 10,000 ng/mL P_4 overnight and stimulated with heat-killed *Escherichia coli, Gardnerella vaginalis*, or *Ureaplasma urealyticum*. Concentrations of IL-8 in conditioned medium were quantified by ELISA and viability of the cell cultures was measured by the reduction of a tetrazolium salt.

Results: *E. coli*, *G. vaginalis* and *U. urealyticum*-stimulated IL-8 production for both cell lines. P_4 inhibited basal and bacteria-stimulated IL-8 production for vaginal epithelial cells but enhanced IL-8 production by endocervical cells. P_4 reduced the number of viable cells for both cell lines.

Conclusions: P_4 inhibits IL-8 production by vaginal epithelial cells stimulated with pathogens associated with preterm birth, possibly by reducing the number of viable cells or by inhibiting their proliferation. Although P_4 also reduces proliferation of endocervical cells it also increases their production of IL-8.

Keywords: Infection; innate immunity; preterm birth; progesterone; vaginal immunity.

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Introduction

Supplemental progesterone or progestin (P_4) has been shown to prevent recurrent preterm birth in several clinical trials [6, 19, 22], however, the mechanism by which P_4 functions to prevent preterm birth remains unclear. Two recent studies have suggested that supplemental P_4 via vaginal suppositories prevents preterm birth in women with a short cervix [7, 13] and may also improve neonatal outcomes by reducing both admissions and lengths of stay at the neonatal intensive care unit [7]. In fact, P_4 had no significant effect on preterm birth in women with cervical lengths >28 mm. These studies suggest that the lower genital tract may be a target tissue for this hormone.

Many spontaneous preterm births are thought to be the end result of infections that ascend from the vagina through the cervix and become established in the upper genital tract. Bacterial products such as lipopolysaccharide (LPS) increase interleukin (IL)-8 production by cervical cells [29]. Aberrantly high concentrations of IL-8 in cervical-vaginal fluids have previously been correlated with preterm birth and chorioamnionitis [17] and both IL-8 and LPS have been shown to induce cervical ripening in animal models [5, 8, 9]. Premature cervical ripening could potentially permit bacteria to ascend into the upper genital tract where they can stimulate a preterm labor response. We therefore hypothesized that P_4 may prevent preterm birth by inhibiting bacteria-stimulated IL-8 production by cells of the lower genital tract. To test the biologic principle underlying this hypothesis, we used an *in vitro* model to test whether P_4 can alter IL-8 production by vaginal and endocervical cells exposed to bacterial pathogens commonly associated with infection-mediated preterm birth.

Materials and methods

Cell lines and bacterial cultures

Vaginal (VK2E6E7) and endocervical epithelial cells (ENDE6E7) were purchased from ATCC (Manassas, VA) for utilization in this experiment. These cell lines were produced from primary cultures isolated from non-pregnant subjects and immortalized by *in vitro* infection with human papilloma virus 16 and retain many characteristics of primary cells in terms of keratin expression [10]. Therefore, the responses of these cells are probably more physiological than other cell lines. Cells were propagated in keratinocyte serum-free (KSM) medium and split at 70–90% confluence by trypsinization. Stock reference cultures of *Escherichia coli*, *Gardnerella vaginalis*, *Ureaplasma urealyticum* (*U. urealyticum* serotype 1-now reclassified as *U. parvum*) were also purchased from ATCC. *E. coli* and *G. vaginalis* were cultivated as directed. *U. urealyticum* was cultivated in beef heart infusion + 20% horse serum + 10% Yeast extract dialysate + 1 g/L urea + 20 mg/L phenol red. Organisms were harvested by centrifugation at 10,000 *g* and re-suspended in KSFM or RPMI 1640. The number of colony forming U/mL was then determined for *E. coli* or *G. vaginalis* by plating serial dilutions on the appropriate agar. Re-suspended cultures of *U. urealyticum* were quantified by measuring the color changing units (CCU), defined as the lowest 10-fold dilution that causes a color change (indicating growth) in the broth above. Bacteria were heat-killed by heating at 80°C for 1 h and aliquots of the heat-killed bacteria were stored at –70°C until use. This is a standard method of preparing bacteria for *in vitro* experiments to test cellular immune responses to bacterial components (live bacteria divide rapidly and would quickly overgrow the mammalian cells in culture).

Cell cultures

Freshly split cells (diluted 1:4 after trypsinization) were plated on 96-well plates in 160 μL medium and permitted to adhere overnight in a humidified incubator at 37°C in a 5% CO_2 (v/v) atmosphere. P_4 treatments were then added to final concentration (0–10,000 ng/mL) in a volume of 20 μL KSM + 10% fetal bovine serum + 1% Dimethyl sulfoxide and cultures were incubated overnight as described above. Cells were then stimulated by adding heat-killed bacteria or an equivalent volume (20 μL) of vehicle and returned to the incubator for a final overnight incubation. A harvest of 100 μL of the conditioned medium was stored at –70°C until assay for IL-8 by ELISA using reagents purchased from R and D systems (Minneapolis, MN). Potential effects of P_4 on number of viable cells was evaluated using the MTT assay as previously described [25]. Briefly, 20 μL tetrazolium salt (5 mg/mL) was incubated for 2 h at 37°C. Formosan crystals formed by live cells were then dissolved by adding 100 μL 10% SDS + 0.01 M HCl to each well and incubating the plate at 37°C overnight. Optical density at 570 and 690 nm was quantified on a microplate spectrophotometer and data are expressed as $OD_{570-690}$, which is directly proportional to the relative number of live cells.

Statistical analyses

Data were analyzed using the general linear models procedure of SAS (SAS Institute, Cary, NC) for a randomized block design. Effects due to experiment were considered random and all others fixed. Results are reported as least-square means with accompanying asymptotic standard errors. Data from each experiment were evaluated for deviations from the assumptions of least-squares techniques (normality, equality, and independence of errors). When violations of the assumptions of ANOVA were detected, data were transformed using the Box-Cox algorithm [23]. Analyses from the transformed data were used for hypothesis testing (P-values). All data presented are from the untransformed dataset for clarity of presentation. IL-8 concentrations were not adjusted for confounding by viability of the cell cultures due to the possibility that any observed changes in IL-8 production may be caused by changes in the number of viable cells and would, therefore, violate the assumptions of analysis of covariance.

Results

Vaginal epithelial cells

IL-8 production by vaginal epithelial cells was significantly increased upon stimulation with 10^8 CFU/mL *E. coli* (1418.47 ± 454.03 vs. 6031.53 ± 454.03 pg/mL; control vs. stimulated cells; $P = 0.0116$), 2×10^6 CFU *G. vaginalis* (1324.06 ± 149.80 vs. 2991.49 ± 149.80 pg/mL; $P = 0.0024$) or 11 CCU *U. urealyticum* (2270.02 ± 429.25 vs. 14028.89 ± 429.25 pg/mL; $P = 0.0036$). P_4 significantly decreased IL-8 production by cultures stimulated with these pathogens as well as unstimulated cells but only at the highest concentration (10,000 ng/mL) tested (Figure 1). At this concentration, P_4 also significantly reduced the number of viable cells for unstimulated cultures ($P < 0.001$) as well as those treated with *E. coli* ($P < 0.001$), *G. vaginalis* ($P < 0.001$) or *U. urealyticum* ($P < 0.001$).

Endocervical cells

IL-8 production by endocervical cells was slightly enhanced by stimulation with *E. coli* (2129.34 ± 88.10 vs. 2946.65 ± 88.10 pg/mL; control vs. treated; $P = 0.0286$), *G. vaginalis* (2296.85 ± 120.74 vs. 5378.50 ± 120.74 pg/mL; $P = 0.0154$), or *U. urealyticum* (1920.23 ± 166.43 vs. 3288.87 ± 166.43 pg/mL; $P = 0.0668$). Both basal and bacteria-stimulated IL-8 production was enhanced by P_4 in a dose-dependent manner, however, results were only significant for cultures treated with very high doses of 1000 and 10,000 pg/mL P_4 (Figure 2). As with vaginal epithelial cells, the number of viable cells was reduced by 1000–10,000 pg/mL P_4 (Figure 2).

Discussion

We found that *E. coli*, *G. vaginalis* and *U. urealyticum* increase the production of IL-8 by both vaginal and cervical epithelial cells. This is consistent with previous studies which reported that *Neisseria gonorrhoeae* induce the

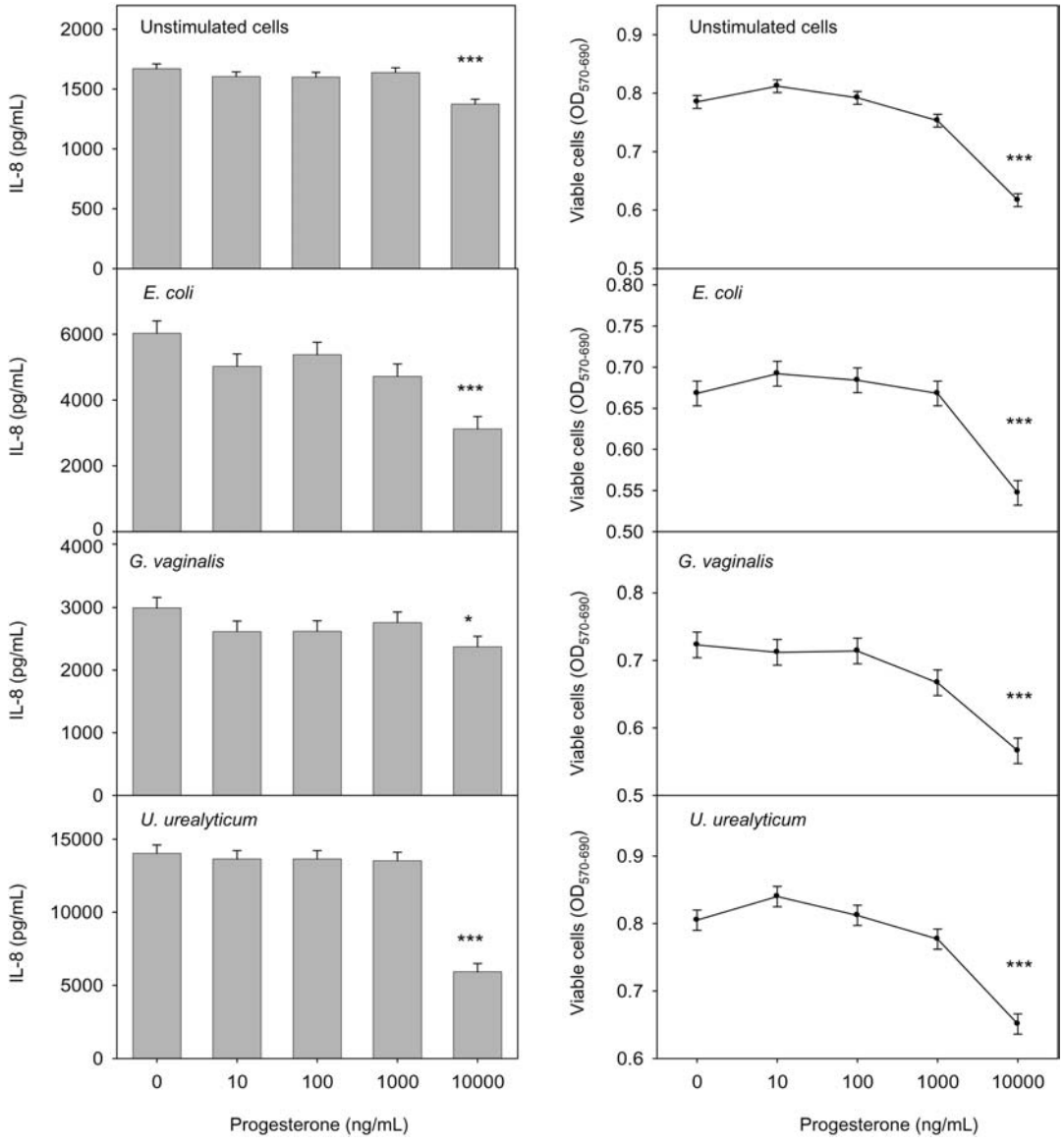

Figure 1 P_4 decreases bacteria-stimulated IL-8 production by vaginal epithelial cells. Vaginal epithelial cells were treated with 0–10,000 ng/mL P_4 overnight and then stimulated with 10^8 CFU/mL heat-killed *E. coli* for, 2×10^6 CFU/mL *G. vaginalis* or 11 CCU of *U. urealyticum* or an equivalent volume of sterile medium (unstimulated cells) in separate experiments. Shown are least-squares means ± SEM for IL-8 production (left panels) and relative number of viable cells as determined by the MTT assay (right panels). Points marked with an asterisk(s) are significantly different from 0 ng/mL P_4 for that bacterial treatment (*$P<0.05$; **$P<0.01$; ***$P<0.0001$).

production of proinflammatory chemokines by these cell lines [12]. Clinical studies have also shown an association with increased IL-8 levels in vaginal lavage of women with genital mycoplasmas compared with normal flora [28]. IL-8 levels in vaginal fluid are also positively correlated with concentrations of IgA to *G. vaginalis* hemolysin [2] which is consistent with our results.

In general, we found that P_4 significantly decreased bacteria-stimulated IL-8 production by vaginal epithelial cells for all pathogens studied as well as for cultures not treated with bacteria. This immunosuppressive effect is consistent with the observation that progesterone enhances the establishment of infections with *Mycoplasma pulmonis* [15], *M. pneumoniae* [14, 15], *M. genitalium* [15], *Chlamydia* [20] and herpes simplex virus [16] in animal models. Part of the mechanism for P_4-immunomodulation of bacteria-stimulated IL-8 production may involve effects on viability or proliferation of the vaginal epithelial cells. High concentrations of P_4 consistently lowered the number of viable vaginal epithelial cells regardless of whether or not pathogens were present. This is consistent with previous studies that reported

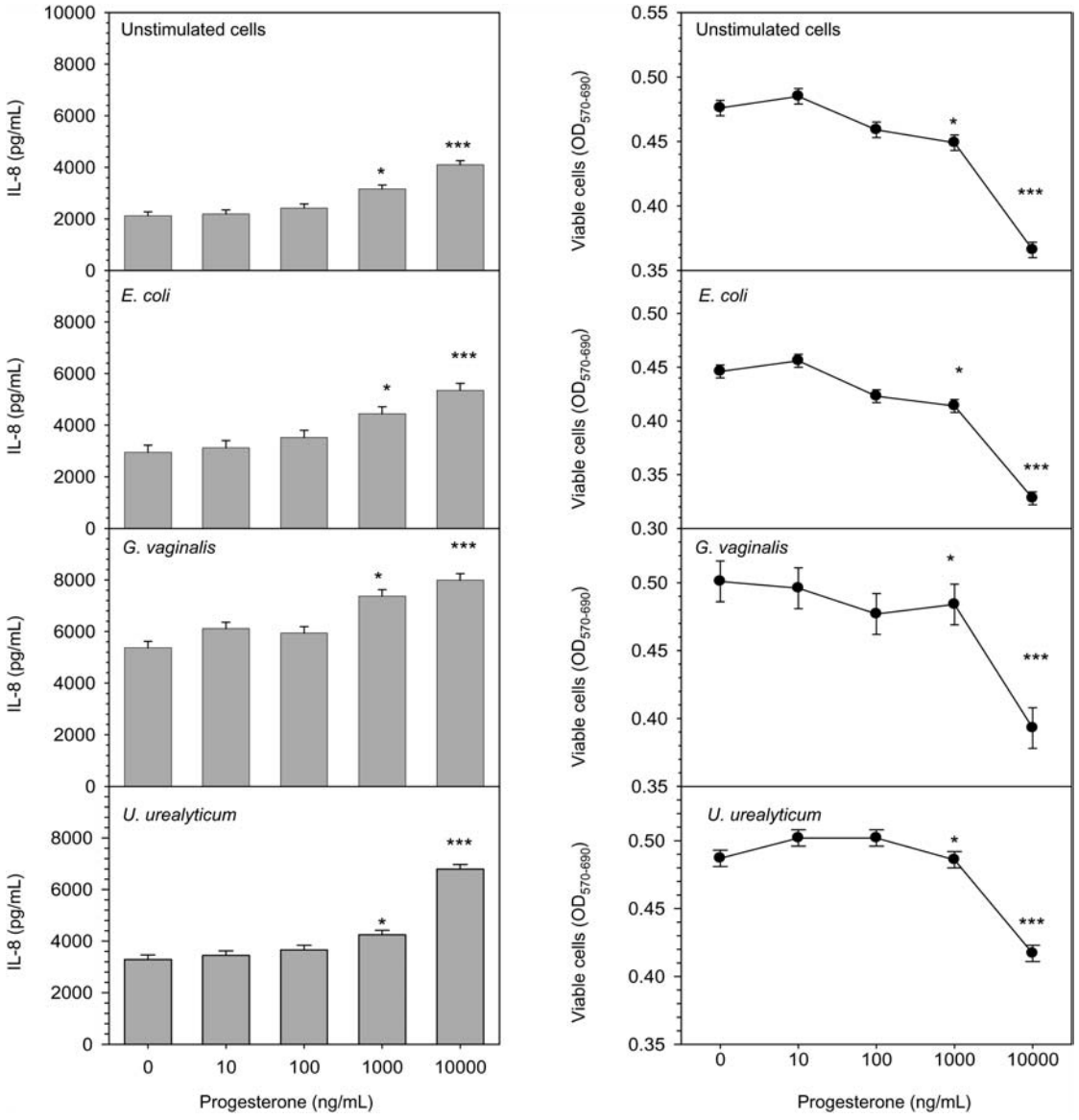

Figure 2 Effect of P_4 on bacteria-stimulated IL-8 production by endocervical epithelial cells. Cells were incubated with 0–10,000 ng/mL P_4 overnight and then left unstimulated or treated with heat-killed *E. coli*, *G. vaginalis* or *U. urealyticum*. Shown are results pooled for 3–9 separate experiments.

reductions of vaginal epithelium thickness *in vivo* by P_4 [24].

Previous studies demonstrated that P_4 can suppress IL-8 production in the rabbit cervix [18], therefore, we hypothesized that high concentrations of P_4 may block bacteria-stimulated increases in IL-8 production that, in turn, may prevent premature cervical ripening and its consequences, including preterm birth. Contrary to our expectation, however, we found that P_4 increases basal and bacteria-stimulated production of IL-8 by endocervical cells. These results differ from those of a previous report where P_4 had no effect on basal or phorbol ester-stimulated IL-8 production by cervical explants from non-pregnant women [1]. The difference between these results may be due to the concentrations of P_4 used to treat the cultures. Barclay et al. used 1 μM P_4 which is equivalent to 314.5 ng/mL. We tested P_4 at concentrations of up to 10,000 ng/mL which were supraphysiological because concentrations in the term placenta are only about 2000–3000 ng/g tissue [21]. It is likely however, that the concentration used in our *in vitro* study are still lower than what would be present in the lower genital tract after intravaginal placement of 90–200 mg P_4 as was done in the two protocols demonstrating P_4's effectiveness at preventing preterm birth in women with a short cervix [7, 13].

Although we found that high levels of P_4 enhance rather than inhibit IL-8 production in the cervix, recent evidence suggests that a small increase in IL-8 could be beneficial in preventing preterm birth. Asymptomatic women who later go on to develop intra-amniotic infection had lower levels of IL-8, IL-1β and IL-6 in the cervix compared to women who did not [27]. It is proposed that the lower levels of cytokines resulted in hypo-responsiveness to bacteria and enabled infections to become more easily established in the cervix and to ascend into the upper genital tract [27]. A slight enhancement of IL-8 production and perhaps other proinflammatory mediators by P_4 in the cervix may help to clear infections before they become established in the cervix and trigger a much more robust IL-8 response that causes a massive host response that results in cervical ripening and preterm birth.

Our finding that P_4 decreased the number of viable endocervical cells suggests that augmentation of IL-8 levels is not due to any proliferative effect of P_4 on the cells. Although fewer endocervical cells are present in P_4-treated cultures, it is likely that they could still produce more IL-8 upon stimulation with bacteria if IL-8 production in each cell was increased enough to compensate for the 20% fewer live cells that are present. Although it is possible that high concentrations of P_4 are toxic to cells, it is more likely that this hormone reduces their proliferation. A previous study reported that injection of medroxyprogesterone decreases DNA synthesis in the rabbit endocervix [3]. P_4 may do this by lowering the expression of the epithelial growth factor receptor on endocervical cells [4]. A small amount (0.1 ng/mL) of epidermal growth factor in the medium is required for the cultivation of these cells [11] and P_4-mediated reductions in the EGF receptor may inhibit the proliferation of these cells as well.

Although a number of other cytokines are increased during cervical ripening, we chose to focus on IL-8 for several reasons. First, our preliminary studies demonstrated that the cell lines responded most robustly to bacterial stimulation with this cytokine. Second, IL-8 is a chemoattractant and activator of neutrophils which are recruited to the cervix during ripening. IL-8 levels are positively correlated with granulocyte elastase levels in the cervix [26]. Third, placement of IL-8 into the cervix causes ripening in animal models [5, 9]. Although other proinflammatory cytokines such as TNF-α and IL-1β are increased in the cervix during cervical ripening, these cytokines also induce the production of IL-8 by endocervical cells [9]. Therefore, their biological activity for promoting cervical ripening may be mediated, at least in part, through IL-8 production. Genetic knock-out mice for many proinflammatory cytokines such as IL-1β, TNF-α, and MCP-1 are available and we are unaware of any defects in cervical ripening in such animals. Therefore, we chose to study IL-8 as a model cytokine for the host-immune response, and to test variety of species of bacteria containing different virulence factors rather than a variety of cytokines and chemokines that would be highly correlated with one another.

Our results are also limited by several factors that are inherent to *in vitro* experiments with transformed cell lines. For example, we are unable to account for the impact that other maternal or fetal hormones or cell types in the lower genital tract may have had on the ability for P_4 to modulate IL-8 concentrations in the cervix or the vagina. We are also unable to determine what exposure to P_4 over the course of many weeks may have on the responsiveness of cells to bacteria. Furthermore, immune responses to a live, growing culture of bacteria *in vivo* may be markedly different from those generated by stimulation of cells with heat-killed organisms. These limitations could partially be overcome by quantifying cervical levels of IL-8, and bacteria in clinical samples from women taking supplemental progesterone. It would be very difficult now, however, to get samples from a control group with similar reproductive histories since the use of this hormone for preventing preterm birth is now widespread. It would also be difficult to interpret how cervical cytokines respond to bacterial pathogens in women *in vivo* when the vaginal flora is complex and consists of many different species. In contrast, our study with transformed cells enabled to study many different treatments under carefully controlled conditions with human cells which would not be possible in a clinical setting.

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