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Stress-Induced versus Preovulatory and Pregnancy Hormonal Levels in Modulating Cytokine Production following Whole Blood Stimulation

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Key Words
Stress · Pregnancy · Prolactin · Estradiol · Cortisol · Progesterone · Interleukin-12 · Interferon-γ · Interleukin-10 · Tumor necrosis factor-α

Abstract
Estradiol, progesterone, prolactin and cortisol concentrations are substantially increased during pregnancy. Also, cortisol and prolactin levels are elevated during stress. In the present study, we exposed peripheral blood to estradiol, progesterone, prolactin and cortisol alone or in combination for 24 h before stimulation with T-dependent (phytohemagglutinin, PHA) and independent activators (lipopolysaccharide, LPS) to study their immunomodulatory role in interleukin-12 (IL-12), tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), and IL-10 production in a whole blood model. This should be similar to in vivo exposure conditions such as long-term stress, preovulatory or pregnancy periods. The present study showed that the stress-induced and preovulatory levels of prolactin and estradiol, respectively, increased the production of IFN-γ and IL-12 levels (and IL-10 in the case of estradiol) in PHA + LPS-stimulated whole blood, and inhibited a hydrocortisone (100 nmol/l) suppressive effect on IFN-γ, IL-12 and IL-10 productions. In LPS-stimulated whole blood, however, prolactin enhanced only IL-10 production levels in a non-concentration-dependent manner. Higher prolactin levels as in pregnancy did not modulate any of the cytokines, but pregnancy estradiol concentrations only induced higher IL-10 levels in PHA + LPS-stimulated whole blood. All progesterone levels tested revealed no effect on any of the cytokines following whole blood stimulation. Our results indicate that (1) a long exposure time of prolactin and estradiol to whole blood modulates the production of cytokines in a concentration- and stimulus-dependent manner; (2) stress-induced levels of prolactin and preovulatory estradiol concentrations can regulate cortisol-induced cytokine suppression, and (3) even though the cytokine pattern is different, pregnancy estradiol and cortisol levels decreased the IFN-γ/IL-10 ratio, thereby keeping the anti-inflammatory IL-10 levels favored during pregnancy, which could be useful in regulating inflammatory-mediated autoimmune diseases.

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Introduction

Cytokines are the main regulators of the immune response. For instance, interleukin-12 (IL-12), produced by activated monocytes/macrophages or other antigen-presenting cells, is a major inducer of T helper 1 (Th1) cytokines. Th1 cytokines such as interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) plus TNF-α from monocytes/macrophages stimulate the function of T cytotoxic cells, NK cells, and activated macrophages. In addition to IL-12, the latter cytokines are considered proinflam-

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matory cytokines because they stimulate the synthesis of nitric oxide and acute phase proteins, attract inflammatory cells, and upregulate the synthesis of secondary mediators and proinflammatory cytokines [1, 2]. On the other hand, the anti-inflammatory cytokine, IL-10, derived from Th2 lymphocytes and monocytes, promotes humoral immunity and suppresses monocytes/macrophages and Th1 lymphocyte activation by inhibiting IL-12, TNF-α and IFN-γ production [1, 2]. Thus, the distinct cytokine secretions have an important role in triggering and/or maintaining different physiological mechanisms such as stress-induced, pregnancy or pathological inflammatory or antibody-mediated autoimmune diseases.

One of the important modulators of cytokine production is endocrine hormones such as glucocorticoids, estrogen, progesterone and prolactin. During pregnancy, where all the above hormones are raised substantially, antibody-mediated autoimmune diseases such as systemic lupus erythematosus tend to develop or flare up but in inflammatory-mediated autoimmune diseases such as rheumatoid arthritis and multiple sclerosis exacerbation attacks remitted [3–5]. These results suggest that during pregnancy a shift towards a Th2 cytokine response occurs and this shift could be attributable to the increase in the levels of estrogen, progesterone and glucocorticoids [6–8]. Furthermore, estradiol and progesterone fluctuate for days in females during the menstrual cycle and cortisol and prolactin increase following stress [9–11]. Thus, it is expected that such phases, pregnancy, preovulatory and stress, should show different patterns of cytokine modulation and would be an important point to be addressed in hormonal immunoregulatory studies.

Several in vitro and in vivo studies have studied the effects of glucocorticoids, estrogen, progesterone and prolactin on cytokine production [8, 12–21]. However, these studies raised several important issues which the present study will address. First, 17β-estradiol, the biologically active form of estrogen, slightly suppressed IFN-γ production at pharmacological doses [15], enhanced antigen-stimulated IL-10, and to a lesser extent IFN-γ secretion in a dose-dependent manner from whole blood cultures and T cell clones [14, 16], but did not modulate the production of IL-12, TNF-α or IL-10 in lipopolysaccharide (LPS)-stimulated whole blood [8]. These results suggest that cytokine modulation by estradiol is dependent on the type of immune cells and on the immune stimulus. Similarly, progesterone stimulated Th2 cytokine production [17, 18] but failed to modulate IL-10 in LPS-stimulated whole blood [8]. Second, prolactin enhanced IFN-γ production from natural killer cells and T lymphocytes [19, 20] and is required for T cell activation by IL-2 [21]. These results indicate that prolactin modulates cytokine towards inflammation-induced responses. Other reports, however, showed that NK cell function, lymphocyte proliferation to mitogens and IL-2 production in hyperprolactinemia patients were all decreased [22, 23], and hyperprolactinemia was observed in patients with inflammatory and antibody-induced autoimmune diseases [24–26]. These results may suggest that prolactin has a dual effect on cytokine modulation. Third, stress, as a modulator of endocrine responses, has been shown to increase cortisol and prolactin levels. Matalka et al. [27] suggested that this increase in prolactin may have a regulatory role and counters the effect of cortisol. Fourth, different exposure times (minutes, hours, or days) of hormones such as glucocorticoids to immune cells have been shown to produce different cytokine patterns [13, 28, 29]. Therefore, in long-term stress, preovulatory and pregnancy conditions, immune cell exposure time to hormones is long enough to cause detrimental effects.

Therefore, in the present study, we exposed peripheral blood to estradiol, progesterone, prolactin and cortisol alone or in combination for 24 h before stimulation with T-dependent and independent activators to study their immunomodulatory role in IL-12, TNF-α, IFN-γ, and IL-10 production in a whole blood model. This should be similar to in vivo exposure conditions such as long-term stress, preovulatory or pregnancy periods. In addition, stimulating peripheral blood with two different immune cell activators (T-dependent vs. T-independent) might explain some of the discrepancies in estradiol and prolactin studies. Furthermore, this whole blood model is closer to in vivo conditions by keeping all the physiological cellular interactions and the natural microenvironment intact. The aim, however, is to establish the base of the effect of these hormones alone and in combination on cytokine induction in a model that is similar to in vivo conditions in order to understand the multicomplex nature of the hormonal balance in the immune system.

Materials and Methods

Subjects

Twenty-eight healthy volunteers (13 males and 15 females) with a mean age of 26.1 years (± 5.2) enrolled in the study and gave written informed consent prior to their participation. All female participants were in the early to mid-follicular phase (days 3–9) of their menstrual cycle. None of the volunteers had taken any medication for at least a week, had done any exercise, or eaten before the blood sample was drawn. All blood samples were drawn in the morning between 8 and 9:30 a.m.

Reagents

The following reagents, RPMI 1640, penicillin-streptomycin, *L*-glutamine, LPS (L-6143), phytohemagglutinin (PHA-L, L-4144), hydrocortisone, 17β-estradiol, and bovine serum albumin, were purchased from Sigma. Recombinant human prolactin was obtained from R&D systems, UK, and progesterone was a gift from the WHO (International Chemical Reference Substances; Soinc 3, Sweden). Endotoxin-free Dulbecco's phosphate buffer (without calcium and magnesium) was obtained from PAA Laboratories (Linz, Austria). Culture 6-well plates and MaxiSorp 96-well flat-bottom plates were purchased from Nunc International (Denmark).

Whole Blood Culture

Blood was drawn into sterilized sodium heparin tubes (Vacutainer, Becton Dickinson) and processed within 45 min. Whole blood cytokine production was performed as described below. The blood was diluted 1:9 with RPMI 1640, supplemented with 2 m*M* glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin, without exogenous serum. To each well of the culture plates, 1.8 ml of the diluted blood was added. Sterilized phosphate buffer (0.2 ml) alone or containing either hydrocortisone (10, 50, 100, 500, 1,000 nmol/l), prolactin (5, 15, 30, 100, 300 ng/ml), estradiol (0.2, 1, 3, 19, 100 nmol/l) or progesterone (5, 50, 200, 1,000, 2,000 nmol/l) was added to each well giving a 1:10 final dilution of the blood. These concentrations are based on previous reports of stress induction of cortisol and prolactin [9, 11], and on the hormonal concentrations during preovulatory and pregnancy periods [6–8]. In addition, diluting whole blood 1:10 significantly lowers concentrations of hormones in the samples and therefore makes the effect of the endogenous hormones negligible. The plates were then incubated in 5% CO_2 at 37°C for 24 h. After the first incubation, PHA + LPS or only LPS in 40-μl volumes was added to give final concentrations of 5 and 1 μg/ml for PHA and LPS, respectively, and incubated in 5% CO_2 at 37°C for another 24 h [30, 31]. Using mixtures of PHA + LPS were found to stimulate both monocytes and T cells by producing higher levels of TNF-α and IL-10, but not IL-12, than LPS alone, and in such a system LPS alone did not induce IFN-γ more than the baseline levels [30, 32]. In LPS-stimulated whole blood, monocytes/macrophages are the main inducers of IL-12, TNF-α and IL-10 [33]. The results of Maes et al. [31], Matalka [32] and Elenkov et al. [33] (and others) indicated that LPS is a T-independent antigen and using PHA + LPS mixtures induces cytokines from both monocytes and T cells. After the second incubation, the blood was collected from wells into sterilized tubes and each well was washed with 0.5 ml of RPMI to ensure removal of the entire well content. Tubes were centrifuged and the supernatant was separated, aliquoted and stored in sterilized tubes at –30°C until assayed.

Cell Viability

To ensure that the ethanol (v/v) that was used to prepare the stock solutions of the steroid hormones did not induce cell killing following the proper dilutions, cell viability using the trypan blue exclusion method was performed following cell culture (as described in the section Whole Blood Culture). All percentages of ethanol used (0.036% and lower) revealed no cytotoxic effect on leukocytes (i.e. the viability was similar to baseline levels).

Cytokine Assay

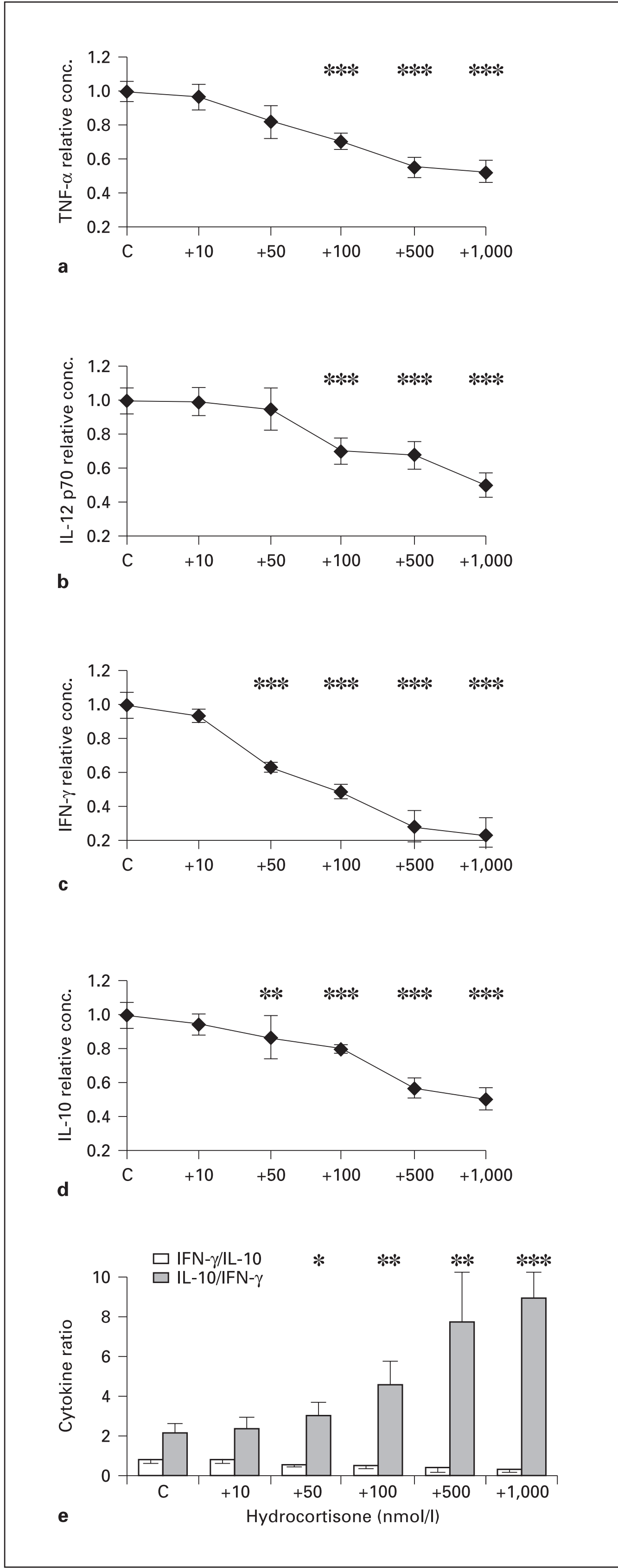
IL-12 p70, IL-10, IFN-γ and TNF-α were assayed by ELISAs (DuoSet R&D Systems, UK). Briefly, the captured antibodies for all cytokines were coated at 4 μg/ml in PBS (pH 7.2–7.4), and anti-cytokine-biotinylated detector antibodies for IL-12 p70, IFN-γ and TNF-α were used at 175 μg/ml and 600 ng/ml for IL-10. Standards (human recombinant) for all assays were used in the range of 15.6–1,000 pg/ml, except for IL-12 p70 (7.8–500 pg/ml) with 7 points of the standard curve with the zero standard. All standards and samples were run in duplicates. Streptavidin-horseradish peroxidase conjugate with H_2O_2-tetramethylbenzidine (R&D, UK) substrate was used. Plates were read by Wellscan Denley ELISA plate reader and absorbance was transformed to cytokine concentrations (pg/ml) using a standard curve computed on Excel. The sensitivities of IL-12 p70, IL-10, IFN-γ and TNF-α assays were 4, 4, 8 and 5 pg/ml, respectively.

Statistical Analysis

All data in the figures are presented as relative concentration of each cytokine (±SE). The relative concentration is a better indicator of changes upon stimulation of a hormone at any given concentration (sample) compared to the control condition (own control). The mean of actual concentration (±SE) for each cytokine measured with PHA + LPS or LPS alone is also given in the figure legends. Comparisons between different conditions of hormone concentrations/conditions were analyzed by one-way ANOVA. The paired t test was used to compare between two conditions (specific concentration with its control) when $n > 10$, whereas the Wilcoxon-rank test was performed when $n < 10$.

Fig. 1. The effect of stress-induced and pregnancy levels of hydrocortisone on cytokine production in PHA + LPS-stimulated whole blood from 16 healthy volunteers (4 males and 12 females with a mean age of 24.9 ± 4.8 years). Blood samples from males or females reacted similarly upon exposure to hydrocortisone. Data are expressed as relative mean ± SE. **a** Mean PHA + LPS-induced TNF-α production was 802.2 ± 69.5 pg/ml. **b** Mean PHA + LPS-induced IL-12 p70 production was 15.9 ± 3.2 pg/ml. **c** Mean PHA+LPS-induced IFN-γ production was 383 ± 68.5 pg/ml. **d** Mean PHA + LPS-induced IL-10 production was 829.9 ± 144.0 pg/ml. **e** Cytokine ratio following incubation of PHA + LPS-stimulated whole blood with different concentrations of hydrocortisone. Each statistically significant point is referred to: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ (see text for more details).

Fig. 2. The effect of stress-induced and pregnancy levels of prolactin on cytokine production in PHA + LPS-stimulated whole blood from 15 normal volunteers (4 males and 11 females with a mean age of 24.4 ± 4.7 years). Blood samples from males or females reacted similarly upon exposure to prolactin. Data are expressed as relative mean ± SE. **a** Mean PHA + LPS-induced TNF-α production was 823.7 ± 162.3 pg/ml. **b** Mean PHA + LPS-induced IL-12 p70 production was 18.8 ± 2.9 pg/ml. **c** Mean PHA + LPS-induced IFN-γ production was 345.7 ± 74.6 pg/ml. **d** Mean PHA + LPS-induced IL-10 production was 776.4 ± 141.6 pg/ml. **e** Cytokine ratio following incubation of PHA + LPS-stimulated whole blood with different concentrations of prolactin. Each statistically significant point is referred to: * $p < 0.05$; ** $p < 0.01$ (see text for more details).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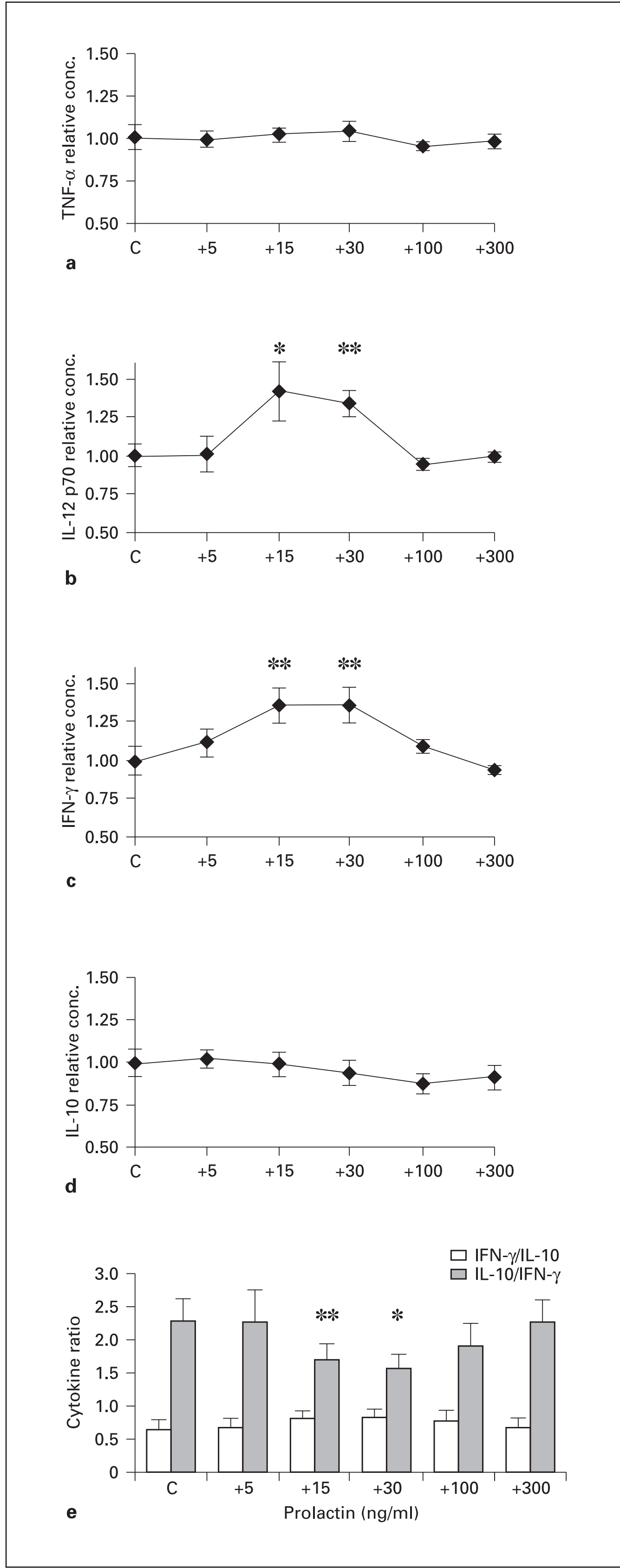
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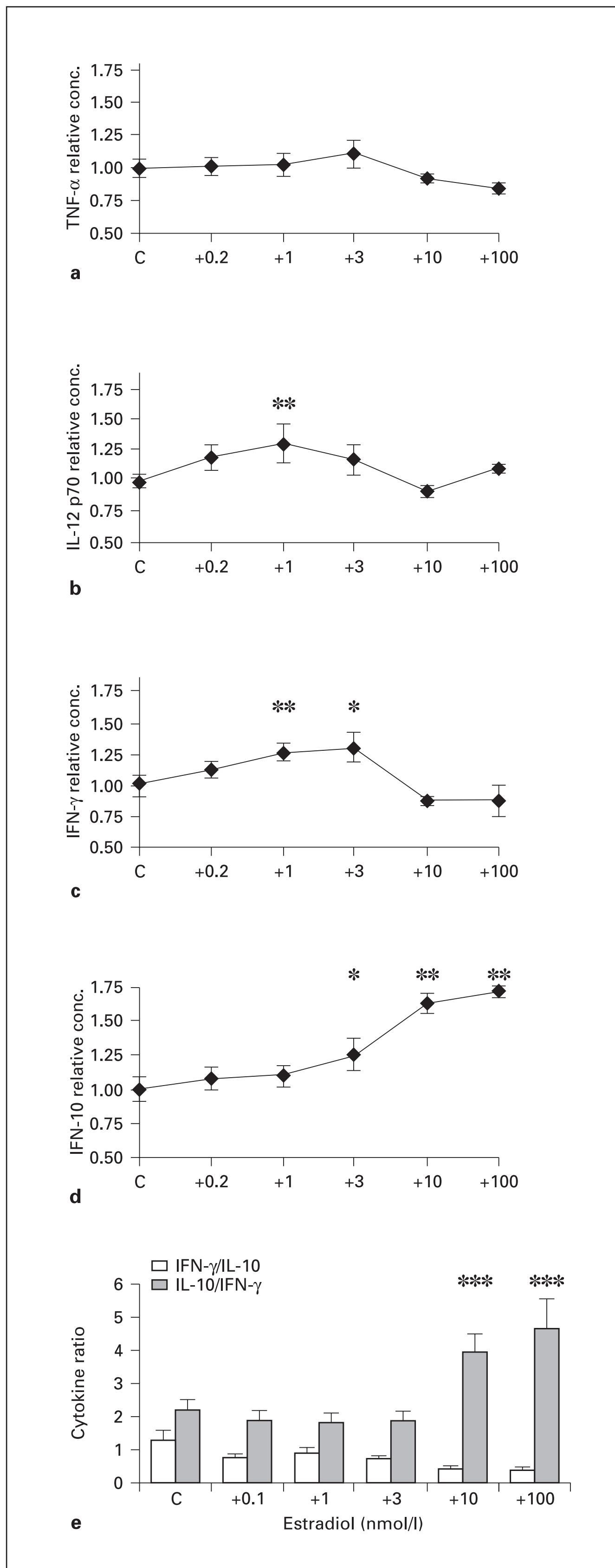

Fig. 3. The effect of preovulatory and pregnancy levels of estradiol on cytokine production in PHA + LPS-stimulated whole blood from 19 normal volunteers (8 males and 11 females with a mean age of 26.5 ± 4.2 years). Blood samples from males or females reacted similarly upon exposure to estradiol. Data are expressed as relative mean ± SE. **a** Mean PHA + LPS-induced TNF-α production was 717 ± 144 pg/ml. **b** Mean PHA + LPS-induced IL-12 p70 production was 16.0 ± 1.8 pg/ml. **c** Mean PHA+LPS-induced IFN-γ production was 313 ± 68 pg/ml. **d** Mean PHA + LPS-induced IL-10 production was 642 ± 127 pg/ml. **e** Cytokine ratio following incubation of PHA + LPS-stimulated whole blood with different concentrations of estradiol. Each statistically significant point is referred to: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ (see text for more details).

Results

Stress-Induced versus Pregnancy Levels of Cortisol and Prolactin in Modulating Cytokine Production

The effects of stress-induced and pregnancy levels of cortisol and prolactin on IL-12, IFN-γ, IL-10 and TNF-α in PHA + LPS-stimulated whole blood are presented in figures 1 and 2. Increasing concentrations of hydrocortisone suppressed significantly ($p < 0.001$) IL-12 [$F(5,90) = 9.98$], IFN-γ [$F(5,90) = 35.2$], IL-10 [$F(5,90) = 13.3$] and TNF-α [$F(5,90) = 21.7$]. At stress-induced (>100 nmol/l) and pregnancy (>500 nmol/l) levels of hydrocortisone, production levels of cytokines were all significantly decreased [$t(16) = 3.23–10.18$, $p < 0.01–0.001$], but the percent of IFN-γ suppression was more evident than of the other cytokines especially at higher cortisol levels (pregnancy levels). This IFN-γ suppression significantly decreased the IFN-γ/IL-10 ratio [$F(5,90) = 2.97$, $p < 0.02$] (fig. 1). On the other hand, stress-induced levels of prolactin (15–30 ng/ml) significantly enhanced IL-12 [$t(15) = 2.2$ and 3.7, $p < 0.05$ and <0.01, respectively] and IFN-γ [$t(15) = 2.93, 3.26$, $p < 0.01$] production levels but not IL-10 and TNF-α. This significantly increased the IFN-γ/IL-10 ratio [$t(15) = 2.3–3.6$ and $p < 0.05–0.01$]. Higher levels of prolactin (>100 ng/ml), as in pregnancy, did not change the production level of any of the tested cytokines in PHA + LPS-stimulated whole blood (fig. 2).

Preovulatory versus Pregnancy Levels of Estradiol and Progesterone in Modulating Cytokine Production

The effects of preovulatory and pregnancy levels of estradiol on IL-12, IFN-γ, IL-10 and TNF-α in PHA + LPS-stimulated whole blood are presented in figure 3. At a preovulatory level of 1 nmol/l of estradiol, IL-12 production levels increased significantly [$t(19) = 2.38$, $p < 0.01$], at 1 and 3 nmol/l of estradiol, IFN-γ levels in-

Table 1. Suppressive effects of hydrocortisone are inhibited by stress-induced prolactin and preovulatory levels of estradiol

	Relative cytokine concentration			
	IL-12	IFN-γ	TNF-α	IL-10
Baseline	1.00 ± 0.04	1.00 ± 0.07	1.00 ± 0.12	1.00 ± 0.10
Cortisol[a]	0.59 ± 0.05	0.49 ± 0.05	0.62 ± 0.03	0.71 ± 0.08
Cortisol + prolactin[b]	1.18 ± 0.14	1.05 ± 0.14	0.66 ± 0.05	0.82 ± 0.09
Cortisol + E_2[c]	1.46 ± 0.15	0.94 ± 0.26	0.63 ± 0.05	1.10 ± 0.11
Cortisol + E_2[d]	1.14 ± 0.12	0.89 ± 0.28	0.63 ± 0.07	1.04 ± 0.11

[a] Wilcoxon-rank test was used to test for significance. p values for the reduction of cytokines were $p < 0.03–0.05$.
[b] Prolactin concentration of 15 ng/ml reversed the levels of all cytokines except TNF-α: $p < 0.05$.
[c] E_2 concentration of 1 nmol/l reversed the levels of all cytokines except TNF-α: $p < 0.05$.
[d] E_2 concentration of 3 nmol/l reversed the levels of all cytokines except TNF-α: $p < 0.05$.

creased significantly [$t(19) = 3.73$ and 2.82, $p < 0.01$ and 0.05, respectively], and at 3 nmol/l of estradiol, IL-10 production levels also increased significantly [$t(19) = 2.25$, $p < 0.05$]. However, this increase in IFN-γ and IL-10 did not reveal any change in IFN-γ/IL-10 ratio. Pregnancy estradiol concentrations (10–100 nmol/l) kept inducing IL-10 levels, but did not have any effect on IFN-γ and IL-12, and TNF-α production levels, which caused a significant decrease in the IFN-γ/IL-10 ratio [$F(5,108) = 3.38$, $p < 0.01$] (fig. 3). No effect of estradiol on the production of TNF-α was observed [$F(5,108) = 0.532$, $p > 0.5$]. Progesterone levels between 5 and 2,000 nmol/l did not change any of the cytokines following PHA + LPS stimulation of whole blood (data not shown).

Stress-Induced Prolactin and Preovulatory Levels of Estradiol Inhibited Cortisol Suppressive Effect on Cytokine Production

The stress-induced levels of prolactin (15 ng/ml) or the preovulatory levels (1–3 nmol/l) of estradiol were mixed with 100 nmol/l of hydorcortisone for 24 h followed by PHA + LPS stimulation. The results of these studies that involved mixing are summarized in table 1. It was observed that 15 ng/ml of prolactin and 1 and 3 nmol/l of estradiol inhibited the suppressive effect of hydrocortisone on the production of IL-12, IFN-γ, and IL-10, but not TNF-α.

Prolactin Increased IL-10 Levels in LPS-Stimulated Whole Blood

Regardless of the prolactin concentration (5–300 ng/ml) used, prolactin significantly increased IL-10 levels [$F(5,30) = 2.99$, $p < 0.03$] in LPS-stimulated whole blood (fig. 4). No effect of prolactin on IFN-γ, IL-12 or TNF-α was observed. Actually, LPS-stimulated whole blood did not increase IFN-γ baseline levels (i.e. did not differ from unstimulated whole blood) [32]. This means that LPS in this human system is a T-independent antigen. In addition, IL-10 and TNF-α production levels were significantly higher in PHA + LPS-stimulated than in LPS-stimulated whole blood, which indicates that in the former stimulation IL-10 and TNF-α are produced from both T lymphocytes and monocytes [32].

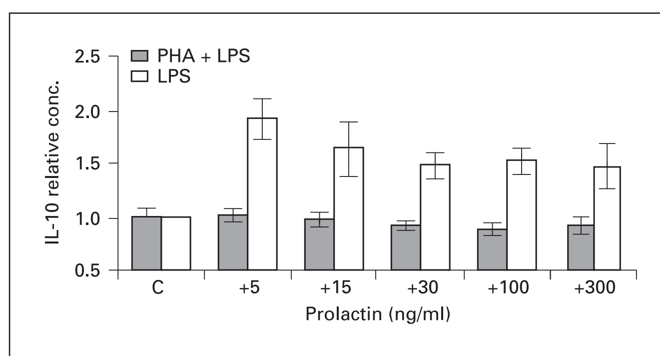

Fig. 4. Prolactin increased IL-10 production in LPS-stimulated whole blood from 6 healthy volunteers (3 males and 3 females with a mean age of 24.0 ± 4.1 years) [$F(5,30) = 2.99$, $p < 0.03$]. Data are expressed as relative mean ± SE. Mean LPS-induced IL-10 production was 149.4 ± 43.4 pg/ml.

Prolactin and Estradiol Alone Can Increase Basal Levels of IL-10 and to a Lesser Extent IL-12 Cytokines

The possibility that prolactin and estradiol (without PHA and LPS) could induce IFN-γ, IL-12 p70, IL-10 and TNF-α was tested in whole blood of 5 healthy volunteers. Increasing concentrations of prolactin did not affect the baseline (without mitogens) concentrations of IFN-γ (~40 pg/ml) and TNF-α (~30 pg/ml). The baseline level of IL-12 p70 was within the range of undetectable (<4 ng/ml) to 5 ng/ml and went up to an average of 11.5, 10.1 and 8.8 pg/ml at prolactin concentrations of 5, 15 and 30 ng/ml, respectively. The relative concentration of IL-10 (baseline levels were ~60 pg/ml) was 1.35, 1.26 and 1.07 at 5, 15 and 30 ng/ml of prolactin, respectively. Regarding estradiol at 1 and 3 nmol/l, IFN-γ went up ~1.2 times the baseline values but no change was observed for the TNF-α level. The baseline level of IL-12 p70 was within the range of undetectable (<4 pg/ml) to 6 pg/ml and went up to an average of 10 pg/ml at estradiol concentrations of 1 or 3 nmol/l, respectively. However, these IL-12 values were not enough to increase IFN-γ levels more than baseline values (data not shown), indicating that T cells at these IL-12 concentrations need another stimulus to secrete IFN-γ. Furthermore, the IL-10 relative concentration was 1.52, 1.87 and 1.78 at 0.2, 1 and 3 nmol/l of estradiol, respectively.

Discussion

The key findings of this study were: (1) 24-hour exposure of prolactin and estradiol to whole blood modulated the production of IFN-γ, IL-12, and IL-10 in a concentration- and stimulus-dependent manner; (2) stress-induced levels of prolactin and preovulatory estradiol concentrations inhibited the suppressive effect of hydrocortisone on IFN-γ, IL-12 and IL-10 productions, and (3) even though the cytokine pattern was different, pregnancy estradiol and cortisol levels in PHA + LPS stimulated the decreased IFN-γ/IL-10 ratio in whole blood, thereby keeping IL-10 levels favored during pregnancy.

The physiological and stress-induced prolactin concentrations (15–30 ng/ml) significantly increased IFN-γ and IL-12 levels in PHA + LPS-stimulated whole blood. Recently, Dimitrov et al. [34] have also shown that 20 ng/ml of prolactin versus antiprolactin antibody increased the percentages of IL-2+ and IFN-γ+CD4+ and CD8+ T cells but not IL-4+CD4+ T cells following stimulation with phorbol myristate acetate, a T cell-dependent mitogen. In the present study, however, prolactin enhanced the production of IL-10, but not IFN-γ, IL-12 or TNF-α, in a non-concentration-dependent manner when LPS was the only immune cell stimulant. This led us to suggest that cytokine modulation by prolactin is not only gene-specific but also stimulation dependent and may explain the dual action of prolactin in proinflammatory (Th1)-mediated autoimmune diseases such as in active rheumatoid arthritis [25, 26] and in antibody (Th2)-mediated autoimmune diseases such as systemic lupus erythematosus [24]. Our data suggest that reducing prolactin levels to minimal levels (<5 ng/ml) in Th1 or Th2-mediated autoimmune diseases might help in the prognosis of such diseases [22, 23, 35].

Prolactin mediates its effects through interacting with prolactin receptors, which are expressed on hemopoietic cell types and belong to the cytokine-hemopoietin receptor family, by activating Janus kinase (JAK)-STAT, mitogen-activated protein kinase (MAPK), and Src kinase pathways [36]. At physiological concentrations of prolactin, activation of prolactin receptor involves prolactin-induced sequential receptor dimerization by a single hormone molecule, whereas in high concentrations (as in pregnancy, breast-feeding or prolactinoma) the receptor becomes engaged and cross-linking cannot occur [36]. This may explain why at high prolactin concentrations, IFN-γ and IL-12 production levels in PHA + LPS stimulation were not increased. However, in LPS stimulation, prolactin increased IL-10 concentrations which remained elevated even at high concentrations of prolactin. Recently, prolactin was also found to regulate another family of proteins called the suppressors of cytokine signaling (SCOS) [37]. These SOCS proteins (SOCS 1–7 and cytokine-inducible SH2-domain-containing protein CIS) behave like a negative regulator of cytokine signal transduction pathways mainly by inhibiting the JAK-STAT kinase pathway. It has been found that prolactin increases the constitutive expression of SOCS-3 in PBMC, and CIS and SOCS-2 in granulocytes [38]. SOCS-2, however, was found to restore prolactin signaling by interfering with the inhibitory activity of SOCS-1 [37]. Since prolactin receptors are expressed on monocytes as well as other leukocytes, prolactin with LPS might enhance the production of IL-10 from monocytes not only through the JAK-STAT pathway but also from MAPK and/or NF-κB, in which expression of SOC-3 does not inhibit the latter two pathways [39]. Furthermore, since IL-10 and prolactin induce SOCS3 as well as SOCS-2 expression and SOCS-2 restores prolactin signaling [38, 40], prolactin enhances LPS in inducing IL-10 from whole blood cells in a non-concen-

tration-dependent manner. Further studies are needed to explain the action of prolactin with LPS.

In the present study, the preovulatory estradiol concentrations significantly increased IFN-γ, IL-12 and IL-10 levels but the IFN-γ/IL-10 ratio did not change. At pregnancy, estradiol concentrations continued to increase IL-10 and thus decreased IFN-γ/IL-10 ratio. These estradiol-induced effects were not observed in LPS-stimulated whole blood [8] but using proteolipid protein-specific T cell clones, estradiol significantly increased IL-10 and IFN-γ in a dose-dependent manner [16]. These observations suggest that estradiol-increased IL-10 production was mainly from stimulated lymphocytes but not monocytes. Since estradiol (at preovulatory concentrations) enhances the production of IFN-γ from stimulated T cells, which could be through estrogen response enhancer at the promoter region [41], it stimulates IL-12 production from monocytes/macrophages [42]. It has been shown that IL-12 can prime T cells for a high IL-10 production [43], which then inhibits IL-12-induced T cell responses. The IL-10 produced from LPS-stimulated monocytes or LPS-stimulated whole blood might contribute to a more inhibitory production of IFN-γ as seen in the present study as well as IFN-γ signaling in monocytes [42, 43]. However, what made IL-10 continue to increase in high estrogen-stimulated blood cells still has to be identified.

The present work also showed that 24-hour exposure of hydrocortisone to immune cells at stress-induced and pregnancy levels suppressed the production of all cytokines, but the suppression of IFN-γ production was greater than that of IL-12, IL-10 and TNF-α in PHA + LPS-stimulated whole blood cultures. This was clearly seen by the significant decrease in the IFN-γ/IL-10 ratio. This decrease in IFN-γ/IL-10 ratio was reversed by glucocorticoid receptor antagonists [13]. Glucocorticoids inhibit NF-κB and AP-1 families of transcription and thus suppress the production of multiple cytokines [44]. Glucocorticoids and also estrogen interact with the JAK-STAT signaling pathway, which could have a positive or a negative impact on cytokine signaling [12, 13, 45]. For instance, Stocklin et al. [45] showed that glucocorticoids enhance JAK/STAT activation by prolactin and that involved glucocorticoid receptors associated with and acted as a coactivator of STAT-5. The data in the present study supports the above observation by showing that prolactin + cortisol and estradiol + cortisol restored cortisol-induced cytokine suppression. These studies also show that the balance between hormones regulates cytokine production from stimulated leukocytes [27] and might prevent stress-induced reactivation of latent Epstein-Barr virus by inducing higher prolactin levels [9, 27].

The present data support the finding that prolactin and estradiol modulate cytokine production and this seemed not only gene-specific but also stimulation dependent. Further studies in the latter direction are mandatory to be able to better explain the dual action of prolactin in autoimmune diseases. Furthermore, the present study shows that pregnancy estradiol concentrations (and higher ones) enhance the production of IL-10 from stimulated whole blood cells. These high IL-10 levels keep anti-inflammatory cytokines favored during pregnancy. Although progesterone did not affect IL-10 levels, recently it has been shown that it promotes the production of IL-4 and abrogates the abortion in mice by reducing inflammatory cytokines through a CD8-dependent pathway [46]. Therefore, estradiol and progesterone keep anti-inflammatory cytokines more dominant during pregnancy and might be useful in regulating inflammatory-mediated autoimmune diseases.

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