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Prolactin triggers pro-inflammatory immune responses in peripheral immune cells

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ABSTRACT. The peptide hormone prolactin (PRL) is produced by specialized cells in the anterior pituitary gland and in a number of sites outside the pituitary. Its biological actions consist of various roles in reproduction, lactation, and of a number of homeostatic biological activities that also include immune functions. Elevated serum PRL concentrations often correlate with abnormalities in immune responses. To determine the influence of PRL on human immune cells, human whole blood cultures were stimulated with lipopolysaccharide (LPS), supplemented with various concentrations of human recombinant PRL. We found that PRL, at concentrations achievable during pregnancy, anesthesia and medication, significantly amplified interleukin (IL)-12 and tumor necrosis factor-α (TNF-α) synthesis in LPS-stimulated cultures, in a dose-dependent manner. Conversely, synthesis of the anti-inflammatory cytokine IL-10 only increased significantly at very high concentrations of supplemented PRL. PRL alone was not able to induce any measurable secretion of TNF-α, IL-10, or IL-12 in non-stimulated, whole blood cultures. However, we demonstrated that PRL, by itself or in combination with LPS, causes an increase in the binding activity of the transcription factors nuclear factor-κB (NFκB) and interferon regulatory factor-1 (IRF-1), which are known to promote TNF-α and IL-12 secretion. These data suggest that PRL promotes pro-inflammatory immune responses via NFκB and IRF-1, which may affect pathophysiological processes in physiological hyperprolactinemic states.

Keywords: prolactin, NFκB, IRF-1, IL-12, TNF-α, IL-10

INTRODUCTION

Prolactin is established as a pituitary hormone that induces growth and differentiation of the mammary gland and exerts effects on other targets important for reproduction. It is now recognized that PRL synthesis and secretion are not restricted to the anterior pituitary gland alone, since they also occur in other organs and tissues. Extrapituitary PRL synthesis has been described in the brain, placenta, decidua, uterus, epithelial cells and cells of the immune system [1]. PRL-receptors are present in a wide range of peripheral cells including T and B lymphocytes, natural killer cells, and intestinal epithelial cells [2, 3]. PRL acts as an immunomodulator hormone which may interfere with lymphocyte activation and cytokine production [4]. An increase in serum PRL concentration is observed with stress, pregnancy, breast-feeding, epilepsy, and medication [5-7]. During pregnancy, a continuous rise from low serum PRL concentrations (10-20 ng/ml) to levels greater than 200 ng/ml have been measured [5]. Routine administration of opioids during general anesthesia may rapidly increase serum PRL in patients, from normal levels up to 150-250 ng/ml [8, 9]. Similar elevations of serum PRL concentrations have been demonstrated in patients taking neuroleptic drugs [10]. Excessive levels of serum PRL greater than 1,000 ng/ml have been found in patients with pituitary prolactinomas [11].

A number of autoimmune diseases exist involving disturbances in the balance of pro-inflammatory and anti-inflammatory processes, and where increased circulating PRL is suspected of being involved in their pathogenesis [12]. The immunomodulatory activities of PRL may arise from increasing nuclear transcription factors such as IRF-1 and NFκB, which play a pivotal role in many immune functions. To examine the impact of human recombinant PRL on the human immune response, we investigated the *in vitro* production of IL-10, IL-12, and TNF-α, in response to the ubiquitous bacterial product lipopolysaccharide (LPS), in whole blood cultures. We further analysed the IRF-1 and NFκB binding activity in nuclear extracts of peripheral blood mononuclear cells (PBMC) in response to LPS and PRL.

DONORS AND METHODS

Immune response triggering: we used a modified whole blood assay, as established by Kirchner *et al.* [13]. Blood samples were obtained from 10 healthy blood donors in lithium-heparin tubes (15 IU Li-heparin/ml blood)

(Sarstedt Monovetten, Nümbrecht, Germany). Using 5 ml test tubes (PNN, Greiner, Nürtingen, Germany), 100 µl blood were pipetted into 800 µl RPMI 1640 medium (Biochrome KG, Berlin, Germany), supplemented with 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (GIBCO GmbH, Karlsruhe, Germany), containing concentrations of either 0 ng/ml, 100 ng/ml, 200 ng/ml, or 300 ng/ml human recombinant prolactin (R&D Systems GmbH, Wiesbaden-Nordenstadt, Germany). The medium-blood suspensions were incubated for one hour before LPS (Sigma, Deisenhofen, Germany, consisting of purified *Escherichia coli* OIII:B4 endotoxin) was added in aliquots of 100 µl and at a concentration of 100 ng/ml. As a negative control we added 100 µl of culture medium in place of the mitogen solution. Blood cell cultures were incubated at 37 °C in 95% air/5% CO_2, under 95% humidity. Supernatants were harvested and stored at – 70 °C after 12 h for measurement of IL-10, IL-12, and TNF-α.

Human peripheral blood mononuclear cells were isolated using Ficoll-Hypaque (Biochrome) for gradient centrifugation at 800 g, at room temperature for 20 min. The isolated PBMC were washed three times with phospate-buffered saline (Sigma) and suspended in supplemented RPMI 1640 medium with 10% fetal bovine serum. PBMC (3×10^6 cells) were incubated at 37 °C in 95% air/5% CO_2, under 95% humidity with or without 300 ng/ml PRL for 90 min in 6-well plates (Greiner). After 60 min of incubation, LPS was added to some specimens at 100 ng/ml final concentration for another 30 min before nuclear extracts were prepared.

Cytokine assay: the concentrations of the cytokines were determined using a quantitative enzyme-linked immunosorbent assay (ELISA). The TNF-α ELISA was obtained from R&D Systems GmbH; IL-10 and the IL-12 ELISAs were purchased from Bender (Bender GmbH, Vienna, Austria). The manufacturer's protocol was closely adhered to. The color intensity of the enzymatic indicator reaction was measured photometrically in an ELISA plate reader (Anthos Labtec, Salzburg, Austria). The detection limit of the ELISA kits was approximately 3 pg/ml for each of the cytokines.

Gel shift assay: nuclear extracts of purified PBMC were prepared according to Trede *et al.* [14] with minor modifications. As probes for the gel shift assay, oligonucleotides of the NFκB consensus binding sequence of the TNF-α promoter (GAT CCT CAG AGG GGA CTT TCC GAT G published in [15] and the – 44 to – 66 IRF-1 binding site of the human IL-12 p40 promoter (AGT TTC TAG TTT AAG TTT CCA published in [16] and [17] were synthesized. They were ^{32}P-labeled with 5'γ-P-ATP (3,000 Ci/mmol, Amersham, Braunschweig, Germany) using a polynucleotide kinase kit (Pharmacia, Freiburg, Germany). Labeled probes (20,000 cpm), 5-10 µg of nuclear extracts, and binding buffer (10 mM Tris-HCL pH 7.5-8.0, 100 mM NaCl, w/o Ca^{2+}, 1 mM EDTA, 10% glycerol, 2 µg of poly (dIdC), 0.5 mM dithiothreitol, 0.05% NP-40) were incubated for 30 min at room temperature in a final volume of 20 µl, following the manufacturer's recomendations, using a BandShift Kit (Pharmacia). The reaction mixture was analysed by electrophoresis for two hours at 20 mA on a 4-8% polyacrylamide gel. Gels were dried, and conventionally and digitally evaluated using a phosphoimager (Digitaler Auto-Radiograph, EG G Berthold, Bad Wildbad, Germany). In order to compare the strength of shifted bands, the cpm of the respective band was divided by the cpm of the whole lane so that the labeled free probe could be considered properly.

Statistical analysis: all data were normally distributed, as ascertained by the Kolmogorov-Smirnov test, and were analyzed using Student's t-test for dependent samples to test for significant differences between means. A computer statistics package was used for all statistical analyses (SPSS, Chicago, Illinois, USA). *P* values < 0.05 were considered to be significant.

RESULTS

We first examined the cytokine production in the whole blood cultures. There were no measurable concentrations of any of the determined cytokines in the supernatants of non-stimulated (medium control) or PRL-supplemented cultures (data not shown). In response to LPS, a dose-dependent increase in IL-12 *(Figure 1)* and TNF-α *(Figure 2)* synthesis within the range of PRL concentrations tested was demonstrated. All concentrations of PRL up to 300 ng/ml were able to raise the IL-12 and TNF-α output significantly when compared to the production after LPS stimulation without PRL ($p < 0.01$). Conversely, synthesis of IL-10 was not considerably affected up to 200 ng/ml of added PRL *(Figure 3)*. There was a tendency to lower IL-10 production at 100 ng/ml PRL. At 300 ng/ml of PRL, a significant increase in IL-10 synthesis in response to LPS could be demonstrated compared to values obtained without PRL ($p < 0.05$).

To determine the modifying effect of PRL on NFκB and IRF-1 binding activity in the nucleus of immune cells, we performed three experiments in which PBMC were incubated with medium (control), LPS (100 ng/ml), PRL (300 ng/ml), and LPS plus PRL. *Figures 4 and 5* indicate a representative analysis of NFκB and IRF-1 binding by gel shift assays. We found that the presence of LPS or PRL increased NFκB and IRF-1 binding activity to an equal

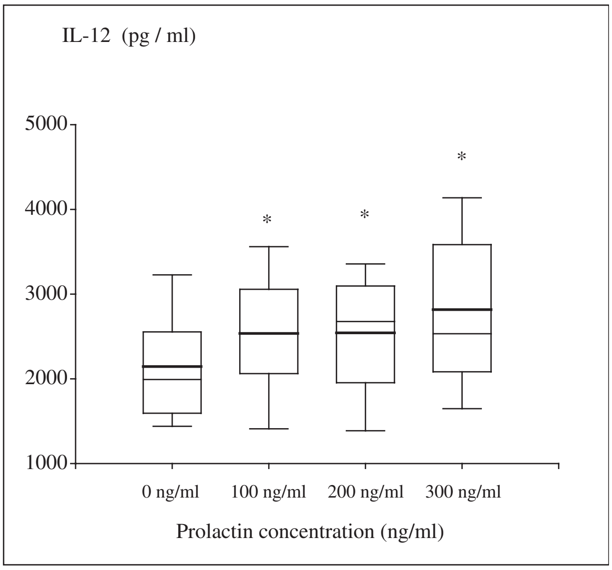

Figure 1
Influence of PRL on IL-12 synthesis in the supernatant of LPS-stimulated whole blood cultures.
Box plots with mean (thick bar) and median (thin bar) are shown. *n* = 10. * = significant *versus* 0 ng/ml PRL: $P < 0.01$.

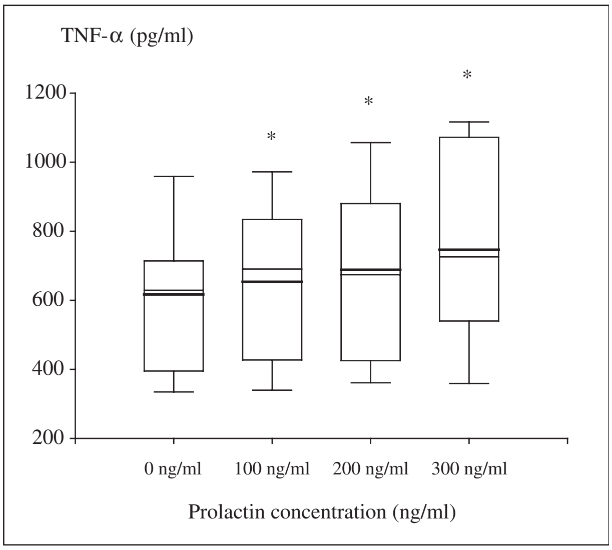

Figure 2
Modulation of TNF-α release by PRL in the supernatant of LPS-stimulated whole blood cultures.
$n = 10$. * = significant *versus* 0 ng/ml PRL: $P < 0.01$.

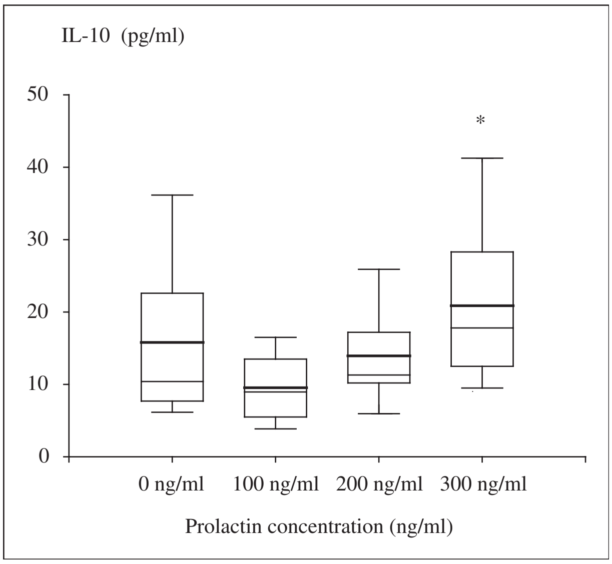

Figure 3
IL-10 synthesis in the supernatant of LPS-stimulated whole blood cultures, influenced by PRL.
$n = 10$. * = significant *versus* 0 ng/ml PRL: $P < 0.05$.

extent in the nuclear extracts of PBMC compared to the medium control. Administration of LPS plus PRL further amplified NFκB and IRF-1 binding activity, resulting in higher binding signals compared to LPS or PRL alone. The phosphoimager-based quantification revealed that LPS and PRL induced a significant 1.5 to 2.0-fold elevation of the NFκB and IRF-1 binding signal from the base line (medium control) ($P < 0.05$). The application of LPS to PRL-supplemented cultures resulted in the highest, (more than three-fold) increase in NFκB and IRF-1 binding activity ($P < 0.01$) *(Figures 6 and 7)*. The enhancement of NFκB binding activity by PRL was even more evident compared to LPS alone (but statistically not significant).

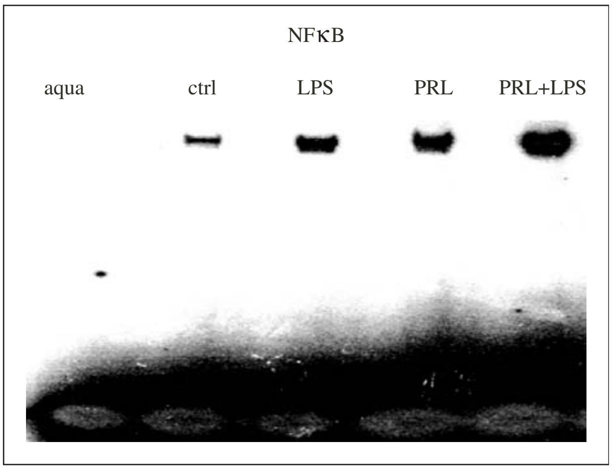

Figure 4
Representative gel shift assay of NFκB binding to the TNF-α promoter in LPS- and PRL-treated PBMC.
ctrl = medium control, aqua = water control.

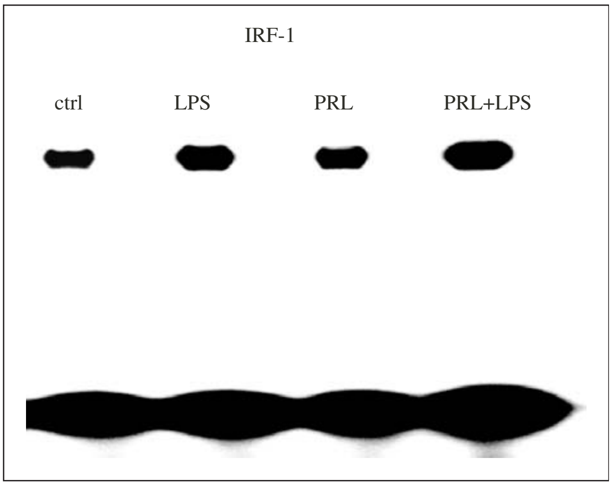

Figure 5
Representative gel shift assay of IRF-1 binding activity to the human IL-12 p40 promoter in LPS- and PRL-treated PBMC.
ctrl = medium control.

DISCUSSION

We used LPS for stimulation since it represents a widespread agent in inflammation, infection and post-infectious sequelae [18]. The doses of supplemented PRL (100, 200, and 300 ng/ml) were chosen to represent upper physiological concentrations often observed during pregnancy or medication with opioids or neuroleptic drugs [5, 6, 9, 10]. We have demonstrated that PRL within physiologically achievable concentrations may selectively increase TNF-α and IL-12 release upon LPS-stimulation. Conversely, IL-10 synthesis, which inhibits pro-inflammatory reactions by downregulating the production of IL-12 and TNF-α [19, 20], was virtually unaffected within elevated levels of PRL up to 200 ng/ml. TNF-α and IL-12 can be considered essential mediators of inflammation, septic complications and autoimmunity [19, 21-23]. High PRL-concentrations (300 ng/ml) may activate a negative feedback system through IL-10, thus limiting the pro-inflammatory reactivity.

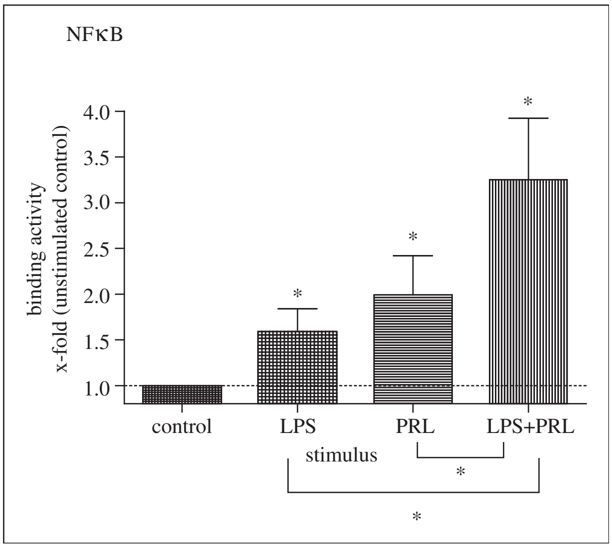

Figure 6
Analysis of 3 gel shift assays of NFκB binding via phosphoimager-quantification.
X-fold amplificated signal compared to medium control. Mean and standard error of the mean is shown. * = significant *versus* medium control or respective column: $P < 0.05$.

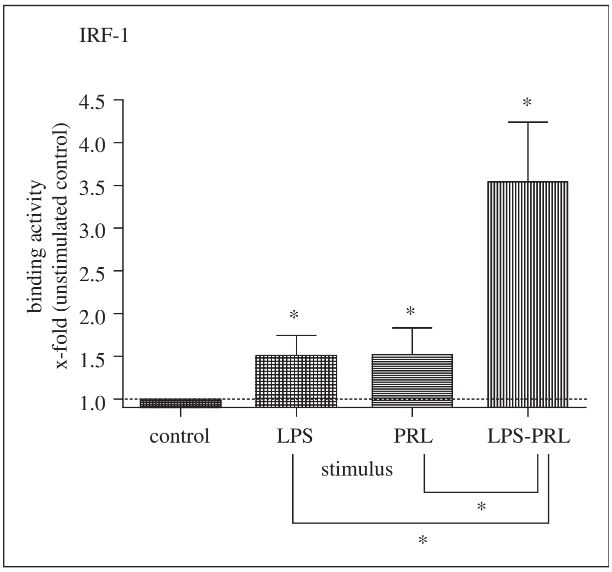

Figure 7
Phosphoimager-quantification of three gel shift assays of IRF-1 binding activity.
X-fold amplification compared to medium control. Results are presented as ratios to the medium control. * = significant *versus* medium control or respective column: $P < 0.05$.

Previous reports have demonstrated that PRL may increase TNF-α release in cultured astrocytes [24], and that PRL-treated rats show a marked increase in TNF-α production by their peripheral neutrophils upon experimentally-induced inflammation [25]. Furthermore, it has also been observed that PRL participates in T lymphocyte and NK cell activation [4, 26]. This effect may be mediated by IL-12, which has been shown to be upregulated in PRL-treated and activated macrophages [27]. The cytokine IL-12 is produced very early during immune responses and may induce differentiation of Th1 cells while inhibiting the generation of Th2 cells [28]. Th1 cells, which produce IL-2 and IFN-γ, are involved in inflammatory reactions and autoimmunity, whereas Th2 cytokines, such as IL-4 and IL-10, encourage antibody production and are found in association with allergic responses [29].

The signaling events that regulate LPS-stimulated induction of cytokines involves NFκB and IRF-1. NFκB mediates the transcription of a broad spectrum of pro-inflammatory genes, including not only those for TNF-α and IL-12, but also for IRF-1. Conversely, IRF-1 is able to enhance NFκB activation via a positive feedback mechanism [30]. The effects of PRL are mediated by the PRL receptor, a member of the hematopoietin cytokine receptor superfamily. It has been demonstrated that PRL interferes with IRF-1 gene expression and NFκB signaling via the JAK/Stat pathway [31]. Obviously, NFκB and IRF-1 activation is not sufficient and other co-signals are involved in IL-12 and TNF-α synthesis as we did not measure any significant amount of cytokine released by PRL alone. Antagonizing factors such as PRL-inducible Stat5 may inhibit NFκB and IRF-1-mediated signaling and interfere with cytokine synthesis [32]. However, here we report that LPS together with PRL markedly increases NFκB and IRF-1 binding, which may participate in the enhancement of IL-12 and TNF-α secretion. Additionally, an amplified IRF-1 binding activity may be responsible for the observed increase of IL-10 secretion because IL-10 gene activation is dependent upon IRF-1 binding to the IL-10 promoter [33].

These effects of PRL may also be relevant for drug-induced autoimmunity, which has been reported to occur with neuroleptic drugs [34, 35]. By modification of cytokine synthesis in the cells participating in the immune response, through elevation of serum PRL which is a typical side effect of these agents, lymphocytes may be permitted to respond strongly to signals which normally are insufficient to initiate a response. Furthermore, antigen-presenting cells may be allowed to abnormally stimulate an immune response, which consequently might lead to autoimmune reactivity. Opioids have been demonstrated to transiently generate a dramatic increase in the plasma concentration of PRL while simultaneously reducing plasma cortisol [36]. For patients undergoing surgery, where translocation of endotoxin regularly occurs through the mucosal barrier of the gut [37], an opioid-based general anesthesia may provoke a higher intra- and post-operative pro-inflammatory cytokine release, which might be involved in the pathophysiology of septic shock and multiple organ failure.

It has been demonstrated that small levels of circulating PRL are necessary for maintaining normal immunocompetence [38, 39]. However, our results emphasize that high levels of PRL may increase pro-inflammatory immune responses, suggesting an involvement in human immune dysfunctions. The role of PRL in the development of autoimmune diseases, such as systemic lupus erythematosus or rheumatoid arthritis, is currently a matter of great discussion [12, 40]. The fact that PRL-antagonizing drugs are able to improve the clinical course could well be the result of their inhibition of pro-inflammatory cytokine release [41, 42]. Pregnancy and the postpartum period, characterized by several hormonal changes (including alterations in PRL), obviously have an influence on the development and activity of autoimmune diseases [43, 44]. Further work should be directed towards the different physiological hyperprolactinemic states and their association with pathophysiological, pro-inflammatory immune responses.

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