Cytokine induction of prolactin receptors mediates prolactin inhibition of nitric oxide synthesis in pulmonary fibroblasts

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Abstract Prolactin (PRL) has been implicated as a modulator of immune function, and some of its actions may be linked to NO synthesis. Because NO acts as a mediator of inflammation, we speculated that an inflammatory milieu could unmask pathways by which PRL could affect NO synthesis. Here, we show that pro-inflammatory cytokines induce the expression of PRL receptors in pulmonary fibroblasts, allowing PRL to inhibit cytokine-induced NO production and the expression of the inducible nitric oxide synthase (iNOS). Inhibition of iNOS expression by PRL correlates with the phosphorylation of STAT-5b (signal transducer and activator of transcription 5b) and the suppression of expression of IRF-1 (interferon regulatory factor 1), a transcription factor for iNOS. These results reveal previously unrecognized mechanisms by which PRL and PRL receptors may play significant modulatory roles during immune–inflammatory processes.

Key words: Prolactin; Prolactin receptor; Nitric oxide; iNOS; Pro-inflammatory cytokine; STAT-5b; IRF-1

1. Introduction

NO is a gaseous biological mediator with effects on smooth muscle relaxation, neurotransmission, host defense, immunity and inflammation [1–3]. NO produced by inducible nitric oxide synthase (iNOS) plays critical roles in modulating inflammation and responses to infectious and degenerative diseases [3]. iNOS expression is induced by a variety of immunologic and inflammatory stimuli, including pro-inflammatory cytokines (e.g. interleukin 1β [IL-1β], tumor necrosis factor α [TNFα], interferon γ [IFNγ]) and lipopolysaccharides.

Prolactin (PRL) acts as a circulating hormone and as a cytokine, eliciting a wide variety of actions [4]. Moreover, PRL can be proteolytically cleaved to generate 16K PRL, a PRL fragment with potent anti-angiogenic properties [5,6]. We have recently shown that 16K PRL stimulates iNOS expression and NO production by rat pulmonary fibroblasts [7], resembling the action of pro-inflammatory cytokines. Unlike 16K PRL and consistent with undetectable PRL receptors, PRL showed no effect on either iNOS expression or NO synthesis in these pulmonary cells [7]. Since NO acts as a mediator of inflammation, we speculated that an inflammatory milieu could uncover PRL actions on iNOS expression in pulmonary fibroblasts, which are important cells in inflammatory reactions of the lung. Here, we investigate the effects of PRL on NO production and iNOS expression in pulmonary fibroblasts treated with a combination of pro-inflammatory cytokines (IL-1β, TNFα and IFNγ), and we analyze the receptors and signal transduction pathways involved. Our findings reveal previously unrecognized anti-inflammatory properties of PRL.

2. Materials and methods

2.1. Reagents

Rat pituitary PRL was from the National Hormone and Pituitary Program (NHPP). Recombinant murine IL-1β, IFNγ, TNFα, transforming growth factor β (TGFβ), angiotensin II (Ang II) and platelet-derived growth factor (PDGF) were from R&D Systems (Minneapolis, MN, USA). IRF-1 (interferon regulatory factor 1) cDNA and anti-STAT-5b (signal transducer and activator of transcription 5b) rabbit polyclonal antibody were kindly provided by L.-Y. Yu-Lee (Baylor College of Medicine, Houston, TX, USA) [8], and anti-phosphotyrosine (anti-pY) monoclonal antibody was a gift of C. Rosales (UNAM, Mexico City, Mexico) [9].

2.2. Cell culture

Rat pulmonary fibroblasts were isolated from Sprague–Dawley rat fetuses as previously described [10]. Cells were grown in Ham's F-12 medium with 10% heat-inactivated fetal bovine serum (HI FBS), and experiments were performed in Ham's F12 containing 0.5% HI FBS. Unless otherwise stated, for all experiments fibroblasts were pretreated for 6 h with 2.5 ng/ml IL-1β, 50 U/ml IFNγ and 12.5 ng/ml TNFα and further incubated with 10 nM rat PRL for 24 h.

2.3. Reverse-transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted using Trizol (Invitrogen, Carlsbad, CA, USA) and treated with RNase-free DNase for 15 min at 37°C followed by phenol–chloroform extraction. Reverse transcription was performed using 5 μg of total RNA, 5 μM oligo(dT)$_{16}$ primers, and MMLV retrotranscriptase (Invitrogen) at 37°C for 1 h. For PRL receptors, a common forward primer (5′-ATCCTGGGCAGATGGAGGAC-3′) and a common reverse primer (5′-ATCCACACGGTT-

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Abbreviations: PRL, prolactin; iNOS, inducible nitric oxide synthase; IL-1β, interleukin 1β; IFNγ, interferon γ; TNFα, tumor necrosis factor α; STAT-5b, signal transducer and activator of transcription 5b; IRF-1, interferon regulatory factor 1

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GTGTCCTTC-3′) were used to detect the short, intermediate and long isoforms. Reverse primers that specifically detect the short (5′-TGGCTGAGGCTGACAAAAGAG-3′) or long (5′-AGACAGTGGGGCTTTTCTCCT-3′) isoform were also used. For iNOS detection, the forward primer 5′-GTGTTCCACCAGGAGATGTTG-3′ and the reverse primer 5′-CTCCTGCCCACTGAGTT CGTC-3′ were employed. Messenger RNAs for ribosomal protein L19 (RPL19) and glucose-6-phosphate dehydrogenase (G6PDH) were amplified as internal controls. For RPL19, forward and reverse primers were 5′-CGAAATCGCCAATGCCAACTC-3′ and 5′-TGCTCCATGAGAATCCGCTTG-3′, respectively. For G6PDH, forward primer 5′-GACCTGCAGAGCTCCAATCAAC-3′ and reverse primer 5′-CACGACCCTCAGTACCAAAGGG-3′ were used. Conventional PCR was performed as previously described [11], using 40 cycles for iNOS and PRL receptors, and 21 cycles for RPL19 and G6PDH. Real-time PCR was carried out using RPL19 or G6PDH as internal standards, in reactions that contained 1 μl aliquots of the reverse transcription reaction described above, 7.5 μl of quantitect SYBR Green PCR (Qiagen, Hilden, Germany) and 400 nM forward and reverse primers in 15 μl total volume. Detection and data analyses were carried out on a LightCycler Instrument according to the manufacturer's instructions (Roche Molecular Biochemicals, Mannheim, Germany).

2.4. Nitrite and nitrate assay

The oxidation products of •NO, nitrite (NO_2^-) and nitrate (NO_3^-) were determined in cell culture media by the Griess reaction as previously described [12].

2.5. Western blot and immunoprecipitation

To determine the tyrosyl-phosphorylation of STAT-5b, fibroblasts were pre-treated with cytokines for 6 h and incubated in the absence or presence of 10 nM PRL for 30 min. A combination of immunoprecipitation and Western blot analyses was performed as previously described [13]. Briefly, cells were lysed in RIPA buffer (50 mM Tris pH 7.4, 0.5% NP-40, 0.2% sodium deoxycholate, 100 mM NaCl, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 mM sodium orthovanadate, and 1 mM NaF). Lysates were centrifuged and pre-cleared with protein-A beads for 1 h, then incubated with anti-STAT-5b antibody (2 μg/ml) overnight. 60 μl of protein-A beads was added, and the immunoprecipitated proteins were resolved on 7.5% SDS–PAGE. Membranes were probed with anti-pY antibodies (5 μg/ml) and re-probed with anti-STAT-5b (2 μg/ml) antibodies.

2.6. Northern blot

To detect IRF-1 mRNA, 10 μg of RNA extracted from fibroblasts was denatured and electrophoresed in a denaturing formaldehyde/1% agarose gel. Northern blotting was performed using a 1.5 kb fragment of a specific cDNA probe for rat IRF-1 labeled with [α-^{32}P]dCTP (Amersham Biosciences, Piscataway, NJ, USA). The blots were re-probed with ^{32}P-RPL19 cDNA as an internal standard for RNA loading.

2.7. Statistical analysis

All results were replicated in three or more independent experiments. Data are expressed as mean ± S.E.M. Statistical analysis was performed by the unpaired Student's *t*-test. $P \ll 0.05$ was considered statistically significant.

3. Results and discussion

Based on the hypothesis that an inflammatory milieu would unmask PRL actions on NO synthesis, we analyzed PRL effects on NO production by resting versus cytokine-stimulated pulmonary fibroblasts (Fig. 1A). Consistent with previous observations [7], PRL did not modify basal NO levels in the untreated cells (lower curve). However, in cells pre-treated with a mixture of IL-1β, TNFα and IFNγ, known to act synergistically to stimulate NO production [12], PRL produced a significant ($P \ll 0.05$) inhibition of NO synthesis in a dose-dependent manner (upper curve, Fig. 1A). Inhibition by PRL was similar to effective doses of TGFβ (0.4–10 nM), Ang II (10 nM) and PDGF (0.4–10 nM), three known inhibitors of cytokine-induced NO [14–16] (Fig. 1B). Finally, PRL inhibition was not restricted to rat pulmonary cells, as it also occurred in the A549 human lung epithelial cell line; however, rat aortic smooth muscle cells were not affected (Fig. 1C).

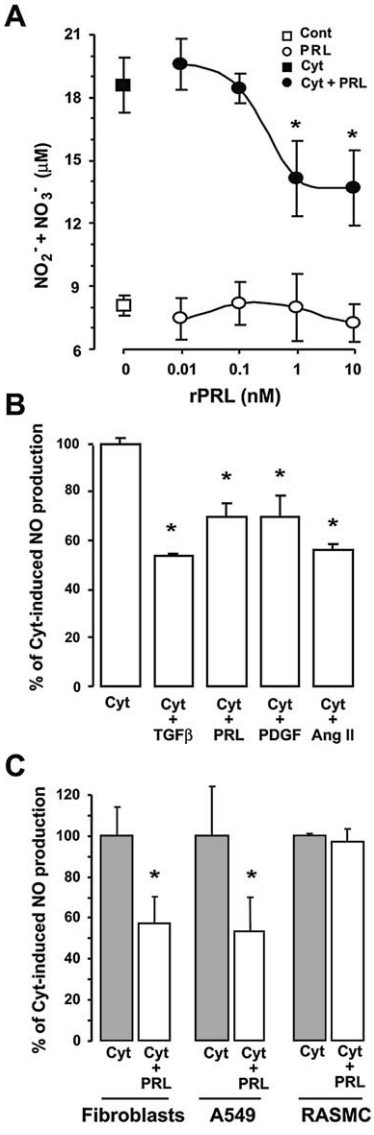

Fig. 1. A: PRL inhibits cytokine-induced NO production in a dose-dependent manner in rat pulmonary fibroblasts. B: Comparative effect of PRL (10 nM), TGFβ (0.4 nM), Ang II (10 nM) and PDGF (0.4 nM) on cytokine-induced NO production by rat pulmonary fibroblasts. C: PRL effect on cytokine-induced NO production by rat pulmonary fibroblasts, A549 human lung epithelial cells and rat aortic smooth muscle cells (RASMC). For all experiments, cells were pre-treated for 6 h with or without cytokines (2.5 ng/ml IL-1β, 50 U/ml IFNγ and 12.5 ng/ml TNFα) and further incubated with PRL (10 nM), TGFβ, Ang II or PDGF for 24 h. NO production was measured by accumulation of $NO_2^- + NO_3^-$ in the culture medium. Values are the mean ± S.E.M. *$P \ll 0.05$ versus cytokines alone (Cyt).

Consistent with the inhibition of NO production, conventional and real-time RT-PCR showed that PRL significantly decreased ($P \ll 0.05$) cytokine-induced iNOS expression, but not basal expression (Fig. 2). While cytokines induced a 16-fold increase in iNOS mRNA, PRL decreased the cytokine effect four-fold (Fig. 2B). However, the magnitude of the reduction of iNOS mRNA did not correlate well with that of

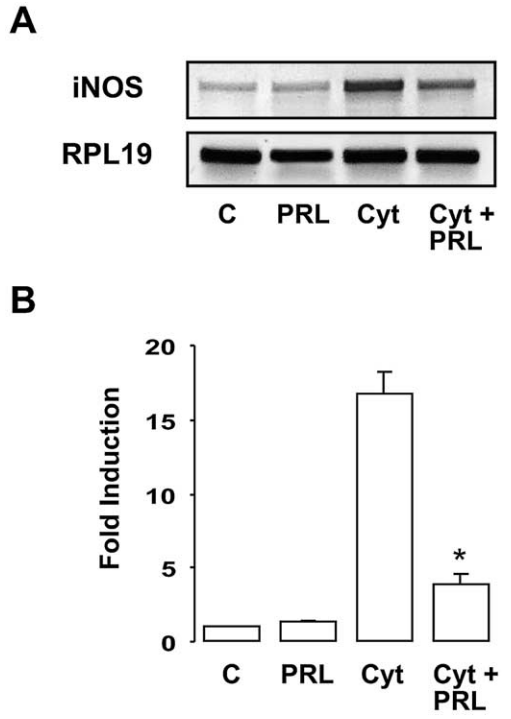

Fig. 2. PRL inhibits cytokine-induced iNOS expression in rat pulmonary fibroblasts. A: iNOS mRNA was analyzed by RT-PCR performed on total RNA extracted from cells. Amplification of RPL19 is shown as control. B: Quantitation of iNOS mRNA expression using real-time PCR. Cells were pre-treated for 6 h with or without cytokines (2.5 ng/ml IL-1β, 50 U/ml IFNγ and 12.5 ng/ml TNFα), and then incubated in the absence or presence of 10 nM PRL for 24 h. *$P \ll 0.05$ versus cytokines alone (Cyt).

NO (Fig. 1). The reason for this difference is unclear but may reflect the fact that the NO measured represents the accumulation of stable NO metabolites over time, whereas iNOS mRNA can have a short, 1 h half-life [17], and its values reflect a point in time after PRL treatment.

We previously showed that the expression of PRL receptors in resting fibroblasts is undetectable [7]; however, here we show that PRL is able to inhibit cytokine-induced iNOS expression and NO production in the same cell type. Hence, we hypothesized that the cytokines used to induce iNOS also induce the expression of PRL receptors that in turn could mediate PRL action. To address this possibility, we investigated the expression of PRL receptors in cytokine-treated cells. While PRL receptors were not detected in control fibroblasts, either in the absence or presence of PRL, the expression of the long PRL receptor isoform was markedly induced by cytokine treatment (Fig. 3). The mRNA for the short PRL receptor was not detectable. In support of these findings, analysis by real-time PCR showed an approximately six-fold induction of the long PRL receptor by cytokine treatment, both in the absence and presence of PRL (Fig. 3B). Therefore, the combination of IL-1β, TNFα and IFNγ acts to promote the expression of PRL receptors in pulmonary fibroblasts, enabling PRL to inhibit cytokine-induced iNOS expression.

To characterize the mechanisms underlying PRL effects on iNOS expression, the activation of the classical signal transduction pathway associated with the PRL receptor was investigated. The interaction of PRL with its receptor leads to the downstream phosphorylation of STAT factors, primarily STAT-5 or STAT-1 [4]. Phosphorylated STAT-5 or STAT-1 homo-dimerize and translocate to the nucleus, where they regulate the transcription of specific genes, including IRF-1 [18]. While STAT-1 can activate IRF-1 [13,19], STAT-5 can inhibit IRF-1 transcription [8,20,21]. In fact, IRF-1 is one of the transcription factors that activates the iNOS promoter [22,23]. Here, we investigated the extent of phosphorylation of the STAT-5b protein and the levels of IRF-1 mRNA in fibroblasts treated with PRL. Consistent with the absence of functional PRL receptors, treatment with PRL alone did not increase STAT-5b phosphorylation (Fig. 4A); however, after cytokine treatment, PRL stimulated STAT-5b phosphorylation, when compared with control cells or cells treated with cytokines alone (Fig. 4A). These results suggest that after cytokine treatment, pulmonary fibroblasts express functional PRL receptors and become responsive to PRL signaling as determined by STAT-5b tyrosine phosphorylation. Furthermore, PRL inhibited cytokine-induced IRF-1 expression (Fig. 4B). While similar basal levels of IRF-1 mRNA were observed in the absence of cytokines, a clear induction of IRF-1 was observed after cytokine treatment (Fig. 4B). This result was expected since IFNγ is known to induce IRF-1 [22]. Remarkably, PRL was able to counteract the effect of the cytokine treatment by reducing IRF-1 mRNA levels (Fig.

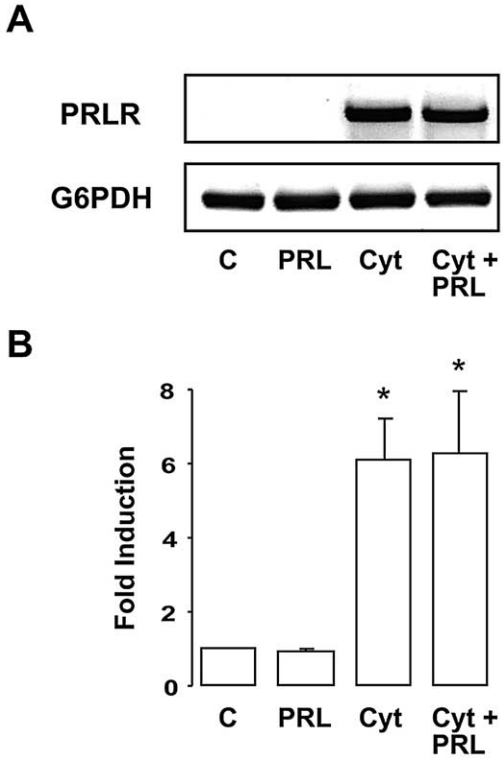

Fig. 3. Pro-inflammatory cytokines induce the expression of the long PRL receptor in rat pulmonary fibroblasts. A: The mRNA for the long PRL receptor was analyzed by RT-PCR performed on total RNA. Amplification of G6PDH is shown as control. B: Quantitation of mRNA for the long PRL receptor using real-time PCR. Cells were pre-treated for 6 h with or without cytokines (2.5 ng/ml IL-1β, 50 U/ml IFNγ and 12.5 ng/ml TNFα) and then incubated for 24 h in the absence or presence of 10 nM PRL. For both figures: untreated control cells (C), PRL alone (PRL), cytokines alone (Cyt) and cells pre-treated with cytokines and further incubated with PRL (Cyt+PRL). *$P \ll 0.05$ versus basal and PRL-treated controls.

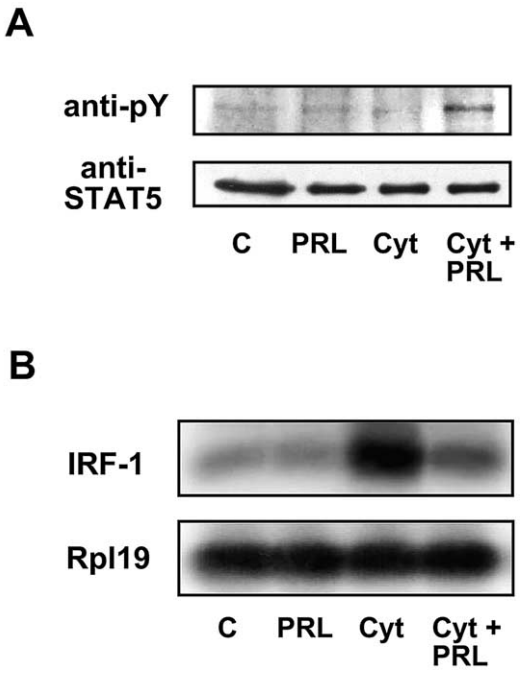

Fig. 4. PRL induces STAT-5b phosphorylation and inhibits IRF-1 expression in cytokine-treated fibroblasts. A: STAT-5b phosphorylation was induced by PRL in cytokine-treated cells. Cells were pretreated with cytokines for 6 h and further incubated for 30 min with or without 10 nM PRL. Proteins were immunoprecipitated with anti-STAT-5b antibody and immunoblotted with anti-pY antibody (upper panel) and anti-STAT-5b antibody (lower panel). B: Northern blot analysis for IRF-1. Cells were pre-treated with cytokines for 6 h and further incubated with 10 nM PRL for 24 h. For both figures, cytokines were 2.5 ng/ml IL-1β, 50 U/ml IFNγ and 12.5 ng/ml TNFα. Groups were as follows: untreated control cells (C), PRL alone (PRL), cytokines alone (Cyt) and cells pretreated with cytokines and further incubated with PRL (Cyt+PRL).

4B). These results show for the first time in a non-genetically modified primary culture cell type that PRL acts to induce STAT-5b activation and to inhibit the expression of IRF-1. Because IRF-1 is one of the transcription factors required for iNOS expression [22,23], PRL inhibition of IRF-1 could explain the downstream PRL inhibitory effect on iNOS mRNA observed in fibroblasts. These findings agree with a previously proposed mechanism by which phosphorylated STAT-5b can inhibit the induction of the IRF-1 via protein–protein interactions and competition for limiting transcriptional co-activators [8,21].

In conclusion, our results demonstrate that PRL acts to inhibit cytokine-induced iNOS expression and NO production by rat pulmonary fibroblasts. The PRL effect appears to be mediated by cytokine-induced PRL receptors, which upon activation lead to the phosphorylation of STAT-5b and the inhibition of IRF-1 expression. To our knowledge, the present findings represent the first evidence in which up-regulation of the PRL receptor occurs in response to pro-inflammatory cytokines and is a requirement for any PRL action.

The PRL inhibitory effect on cytokine-induced NO production and iNOS expression occurs in pulmonary cells (fibroblasts and epithelial) from different species (rat and human) but not in other cell types (rat smooth muscle cells). These findings suggest that the lung is a site for the anti-inflammatory effect of PRL. However, in other cells PRL can activate NO synthesis. Previous investigations have shown that PRL elevates NO production in mouse macrophages [24] and induces iNOS expression in human leukocytes [25] and in rat C6 glioma cells [26]. Opposite effects are not unexpected since the regulation of iNOS expression is complex and known to be cell- and species-specific [27]. For example, regulatory factors, such as retinoic acid and TGFβ, can either inhibit [28–30] or potentiate [31–33] iNOS expression, depending on the cell type investigated.

This study, together with our previous work [7], reveals that PRL and 16K PRL have dichotomous effects on NO production by rat pulmonary fibroblasts. While 16K PRL acts as a pro-inflammatory cytokine inducing the expression of iNOS in resting conditions, PRL acts as an anti-inflammatory factor, attenuating the cytokine-induced iNOS expression. The fact that pro-inflammatory cytokines induced the expression of PRL receptors further supports the idea that the inhibitory effect of PRL is secondary to the action of pro-inflammatory cytokines, and it suggests that relevant PRL functions may become apparent during the inflammatory process. Because NO acts as a mediator of inflammation [3], the outcome of the hormonal effect could promote or inhibit the inflammatory response, depending on the activity of the proteases responsible for processing PRL into 16K PRL and on the selective expression of the receptors for each PRL isoform.

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