Published in: J Biol Chem (2000), vol. 275, iss. 18, pp. 13183-13186
Status: Postprint (Author's version)

Proteolytic Cleavage Confers Nitric Oxide Synthase Inducing Activity upon Prolactin

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Abstract

Prolactin (PRL), originally associated with milk secretion, is now known to possess a wide variety of biological actions and diverse sites of production beyond the pituitary. Proteolytic cleavage is a common post-trans-lational modification that can either activate precursor proteins or confer upon the peptide fragment unique biological actions not exerted by the parent molecule. Recent studies have demonstrated that the 16-kDa N-terminal proteolytic cleavage product of PRL (16K-PRL) acts as a potent inhibitor of angiogenesis. Despite previous demonstrations of 16K-PRL production *in vivo*, biological functions beyond its antiangiogenic actions remain unknown. Here we show that 16K-PRL, but not full-length PRL, acts to promote the expression of the inducible isoform of nitric oxide synthase (iNOS) and nitric oxide (˙NO) production by pulmonary fibroblasts and alveolar type II cells with potency comparable with the proinflammatory cytokines interleukin-lβ, interferon γ, and tumor necrosis factor α. The differential effect of 16K-PRL *versus* PRL occurs through a receptor distinct from known PRL receptors. Additionally, pulmonary fibroblasts express the PRL gene and endog-enously produce 16K-PRL, suggesting that this pathway may serve both autocrine and paracrine roles in the regulation of ˙NO production. These results reveal that proteolytic cleavage of PRL confers upon this classical hormone potent iNOS inducing activity, suggesting its role in inflammatory/immune processes.

Abbreviations used: PRL, prolactin; 16K-PRL, 16 kDa N-terminal proteolytic cleavage product of PRL; iNOS, inducible nitric oxide synthase; ˙NO, nitric oxide; IL-1β, interieukin 1β; IFN-γ, interferon γ; TNF-α, tumor necrosis factor-α; L-NAME, L-N^G-arginine methyl ester; D-NAME, D-N^G-arginine methyl ester; RT-PCR, reverse tran-scription-polymerase chain reaction.

Although PRL was originally identified as a lactotrophic hormone secreted by the pituitary gland, accumulating evidence has implicated PRL in a strikingly diverse array of physiological functions, including osmoregulation, reproduction, and behavioral modifications (1, 2). PRL synthesis has been demonstrated in numerous extra-pituitary tissues, including endothelial (3), neuronal, and immune cells (*i.e.* lymphocytes, mononuclear cells, and thymocytes) (1). Moreover, the emerging role of PRL in immunoregulation has led to the concept of a dual function for PRL as both a circulating hormone and a cytokine (1). The tenet of PRL as a cytokine is further established by studies demonstrating its structural similarity to members of the cytokine/hematopoietin family (4) and that PRL receptors belong to the cytokine/hematopoietin receptor superfamily (5). Perturbation of PRL physiology appears to have significant immunologic effects in humans, where hyperprolactinemia is associated with autoimmune diseases, including systemic lupus erythematosus, rheumatoid arthritis, multiple sclerosis, and uveitis (6). All these conditions are also associated with elevated tissue iNOS expression and ˙NO production (7). Consistent with these observations, hyperprolactinemia has been associated with elevated ˙NO levels in a rat model of acute inflammation (8).

PRL can be post-translationally processed by proteolytic cleavage, giving rise to 16K-PRL, a fragment that has unique antiangiogenic actions not shared with the full-length molecule (9-11). 16K-PRL has been detected in the pituitary and serum of humans, rats, and mice (12, 13) and acts as a potent inhibitor of angiogenesis *in vivo* and *in vitro*, inhibiting endothelial cell proliferation (9-11) and stimulating type 1 plasminogen activator inhibitor expression (11). Finally, 16K-PRL has been shown to increase endothelial cell cAMP levels (14), an occurrence that has been shown to induce iNOS expression (15). 16K-PRL adds to a growing list of proteins that acquire specific functions or become activated as the result of the proteolytic cleavage of large precursors. This is the

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case of other potent antiangiogenic factors, like angiostatin (16) and endostatin (17), some cytokines, like TNFα (18) and IL-lβ (19), and matrix metalloproteinases (20).

Although PRL manifests cytokine-like signaling properties and can be elevated in conditions where iNOS expression is increased, the influence of the proteolytic processing to 16K-PRL on ˙'NO production and iNOS expression remains unknown. Here we report that 16K-PRL, but not full-length PRL, promotes the expression of iNOS and ˙NO production by rat pulmonary fibroblasts and alveolar type II cells. The stimulatory effect of 16K-PRL appears to occur through a receptor distinct from known PRL receptors, since a specific, high affinity, saturable binding site for 16K-PRL was found, while classical PRL receptors were not expressed at detectable levels in resting pulmonary fibroblasts. Additionally, pulmonary fibroblasts express the PRL gene and endogenously produce 16K-PRL, suggesting a local function of this fragment in the regulation of ˙NO production in the lung.

EXPERIMENTAL PROCEDURES

Reagents—Rat pituitary PRL was from the National Hormone and Pituitary Program (NHPP). Rat 16K-PRL was generated after enzymatic proteolysis of PRL by an extract from rat mammary gland (10). Human recombinant PRL and 16K-PRL were generated as described previously (10). Endotoxins were determined by the *Limulus* amebocyte lysate assay (E-Toxate, Sigma). Recombinant murine interleukin-1β (IL-1β), interferon-γ (IFN-γ), and tumor necrosis factor α (TNF-α) were from R&D Systems (Minneapolis, MN). L- and D-N^G-Arginine methyl ester (L- and D-NAME) were from Sigma.

Cell Culture—Rat lung fibroblasts and type II alveolar epithelial cells were isolated from Sprague-Dawley rat fetuses of 19-20 days of gestational age (21). Rat aortic smooth muscle cells were isolated as described previously (22). All cells were cultured in Ham's F-12 medium with 10% heat-inactivated fetal bovine serum. Cells grown to confluence were treated with human 16K-PRL (0.1-10 nM), rat 16K-PRL (1-100 nM), or full-length PRL (100 nM, human or rat). For comparison, additional cells were treated with a combination of IL-1β (5 ng/ml), IFN-γ (100 units/ml), and TNF-α (500 units/ml).

Nitrite and Nitrate Assay—The oxidation products of ˙NO, nitrite (NO_2^-), and nitrate (NO_3^-) were determined in cell culture medium as described previously (23).

Reverse Transcriptase Polymerase Chain Reaction and Southern Blot—For rat iNOS detection, complementary primers (forward: 5'-GTGTTCCACCAGGAGATGTTG-3'; reverse: 5'-CTCCTGCCCACT-GAGTTCGTC-3'), 30 cycles, and an annealing temperature of 60 °C were used. For rat PRL detection, RT-PCR and Southern blot were performed as described previously (24). For the rat PRL receptors a common forward primer (5'-ATCCTGGGACAGATGGAGGAC-3') and a common reverse primer (5'-ATCCACACGGTTGTGTCCTTC-3') were used to detect the short, intermediate, and long isoforms. Reverse primers were used to specifically detect the short (5'-TGGCTGAGGCT-GACAAAAGAG-3') or long (5'-AGACAGTGGGGCTTTTCTCCT-3') isoforms. Forty cycles and an annealing temperature of 56 °C were used. Amplification of β-actin mRNA was used as control for efficiency as described previously (24).

Western Blot—Fibroblasts were homogenized in lysis buffer and subjected to reducing SDS-polyacrylamide gel electrophoresis as described previously (24). Proteins were electroblotted onto nitrocellulose, probed with 2.5 μg/ml anti-iNOS polyclonal IgG (Upstate Biotechnology Inc., Lake Placid, NY) or 1:500 16K-PRL rabbit antiserum (13) and developed as described previously (24).

PRL Cleavage—The activity of the enzymes that cleave PRL to 16K-PRL was assayed in fibroblast homogenates as described previously (24).

PRL Binding Assays—Binding of 16K-PRL to isolated fibroblasts membranes was performed as described previously (25). Binding parameters (K_d and B_{max}) were derived from the competition studies using the LIGAND program (26).

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RESULTS AND DISCUSSION

To evaluate the influence of PRL and 16K-PRL on ˙NO production and iNOS expression, primary cultures of fetal rat lung fibroblasts, fetal rat alveolar type II cells, and rat aortic smooth muscle cells were utilized to model the response to proinflammatory stimuli. Quantitation of ˙NO production was determined by measurement of its metabolites nitrite (NO_2^- and nitrate (NO_3^-) accumulated in culture media. While human (Fig. 1*a*) and rat PRL (not shown) had no stimulatory effect, 16K-PRL potently stimulated ˙NO production by rat fibroblasts and type II cells, but not by aortic smooth muscle cells (Fig. l*a*). Increasing concentrations of 16K-PRL stimulated ˙NO production by fibroblasts in a dose-dependent manner, with highest effects observed with 10 and 100 nM of human and rat 16K-PRL, respectively (Fig. 1*b*). The higher potency of human compared with rat 16K-PRL is consistent with previous observations in regard to their antiangiogenic effect (10). The maximal effect of 16K-PRL was comparable with that exerted by a mixture of proinflammatory cytokines (TNF-*α*, IFN-γ, and IL-l*β*). Stimulation of ˙NO production by 16K-PRL or cytokines was completely suppressed by L-NAME (1 mM), a competitive inhibitor of iNOS, but was not affected by the inactive isomer D-NAME (Fig. l*c*). The possibility of a nonspecific action of 16K-PRL due to endotoxin contamination was excluded, because native PRL had no positive effect on iNOS expression even though the concentration used had higher or equivalent endotoxin content (0.15-0.5 ng of lipopolysaccharide/ml of medium) than 16K-PRL (0.03-0.5 ng of lipopolysaccharide/ml of medium) as determined by the *Limulus* amebocyte lysate assay.

To determine whether ˙NO production induced by 16K-PRL was mediated through iNOS expression, total RNA of fibroblasts treated with 16K-PRL, PRL, or cytokines was subjected to RT-PCR. Inducible NOS cDNA was detected following exposure of fibroblasts to 16K-PRL and cytokines, with no detectable iNOS expression in control or PRL-treated cells (Fig. *2a).* Similar amounts of amplified products for *β*-actin were observed in all cases. Inducible NOS protein synthesis was confirmed by Western blot of fibroblast lysates probed with an anti-iNOS polyclonal antibody (Fig. 26). Human and rat 16K-PRL dramatically increased iNOS protein expression, while iNOS was not detectable in lysates from control or PRL-treated cells. Expression of iNOS was clearly evident as early as 8 h following stimulation with 16K-PRL (not shown).

The expression of PRL receptors by lung fibroblasts and type II cells was studied to further characterize these novel actions of 16K-PRL. The presently identified PRL receptors arise from the same transcript by alternative splicing and differ in the length and composition of the cytoplasmic domain (2). Expression of the long (Fig. 3*a*) and short (not shown) PRL receptors was demonstrated in type II cells, while no PRL receptor variants were observable in fibroblasts. Although type II cells express PRL receptors, the influence of 16K-PRL on ˙NO production seems to be independently mediated, since PRL, the natural ligand, has no stimulatory effect. Moreover, 16K-PRL stimulated fibroblast iNOS expression and ˙NO production in the absence of detectable PRL receptors, motivating investigation of a possible specific binding site for 16K-PRL on these cells. Binding of ^{125}I-labeled rat 16K-PRL by fibroblast membrane preparations was high affinity (K_d = 7.3 ± 3.9 nM), saturable (B_{max} = 7200 ± 1730 receptors/cell) and potently displaced by rat 16K-PRL but not by full-length PRL (Fig. 36). This is consistent with a previously observed specific binding site for 16K-PRL in vascular endothelium (25) and suggests that lung fibroblasts express a specific receptor for 16K-PRL.

Although 16K-PRL has been detected in serum (12), the proportion of the fragment present in patients with elevated PRL levels is unknown, since the methodology used to detect PRL in serum (radioimmunoassay or enzyme-linked immunosorbent assay) does not distinguish between molecular variants. Moreover, local cell production of PRL and its cleavage to 16K-PRL may not be reflected in circulating PRL levels and this could be a source of 16K-PRL in the lung. To reveal the possibility of pulmonary PRL production, total RNA from fibroblasts and type II cells was subjected to RT-PCR using primers derived from rat pituitary PRL cDNA. PRL gene expression was detected in fibroblasts, but not in type II cells (Fig. 4*a*), and both PRL and 16K-PRL protein was demonstrated by Western blot analysis of cell lysates from fibroblasts (Fig. 4*b*). Moreover, incubation of exogenous PRL with fibroblast lysate resulted in the formation of 16K-PRL (Fig. 4*b*), demonstrating that fibroblasts can locally process PRL to the fragment. These results suggest that paracrine and autocrine effects of 16K-PRL can occur in the lung.

This study shows that 16K-PRL acts as a potent proinflammatory cytokine that stimulates iNOS expression and ˙NO production by pulmonary fibroblasts and type II cells, a biological effect not shared by full-length PRL. The differential effect of 16K-PRL *versus* PRL appears to occur through a specific receptor for 16K-PRL, since a specific, high affinity, saturable binding site for 16K-PRL was apparent, and no PRL receptor expression was detectable in pulmonary fibroblasts under the culture conditions. Moreover, pulmonary fibroblasts are competent to both express the PRL gene and produce 16K-PRL, suggesting that this PRL fragment can exert autocrine and paracrine roles in the regulation of pulmonary inflammatory ˙NO production. The absence of a stimulatory effect

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of PRL contradicts recent studies where the full-length molecule stimulated ˙NO production by the rat glioma cell line C6 (27), suggesting that PRL effects on ˙NO production are cell-type specific or that proteolytic processing of PRL to 16K-PRL was ocurring.

The results reported herein reveal that proteolytic cleavage of PRL confers a potent iNOS-inducing activity on prolactin reminiscent of proinflammatory cytokines such as IL-l*β*,

IFN-γ, and TNF-*α* (23, 28, 29). Locally produced 16K-PRL may contribute to the high levels of ˙NO produced by iNOS activity during inflammatory and host defense processes of the respiratory tract (30-32). In view of the unique properties of 16K-PRL *versus* PRL, the protease(s) responsible for processing PRL also would critically influence local regulation of ˙NO production. Thus, the observation that estrogens regulate the enzymatic cleavage of PRL to 16K-PRL in the rat neurohypophyseal system (24) adds additional significance to pathways regulating PRL processing and warrants further investigation.

FIG. 1. The 16-kDa N-terminal proteolytic fragment of PRL, but not full-length PRL, stimulates ˙NO production by rat pulmonary fibroblasts and alveolar type II cells. α, production of ˙NO by rat fibroblasts, alveolar type II epithelial cells, and smooth muscle cells in the absence (Con) or presence of cytokines (Cyt), 10 nM human 16K-PRL (16K) or 100 nM human PRL (23K). b, potency of human (▲) and rat (■) 16K-PRL on ˙NO production by fibroblasts. For comparison, cells were cultured in the absence (O) or presence of cytokines (●). c, 16K-PRL-induced ˙NO production by fibroblasts is inhibited by the NOS inhibitor L-NAME, but not the inactive isomer D-NAME. Cells were cultured in the absence (Con) or presence of cytokines (Cyt), 10 nM human (hl6K) or 100 nM rat 16K-PRL (r16K), in absence (open bars) or presence of 1 mM L-NAME (black bars) or D-NAME (shaded bars). In all cases, cytokine treatment (Cyt or Cytokines) consisted of a mixture of IL-1/3 (5 ng/ml), IFN-γ (100 units/ml), and TNF-α (500 units/ml). ˙NO production was measured by accumulation of NO^-_2 + NO^-_3 in culture medium after 72 h. Values are the mean ± S.E.

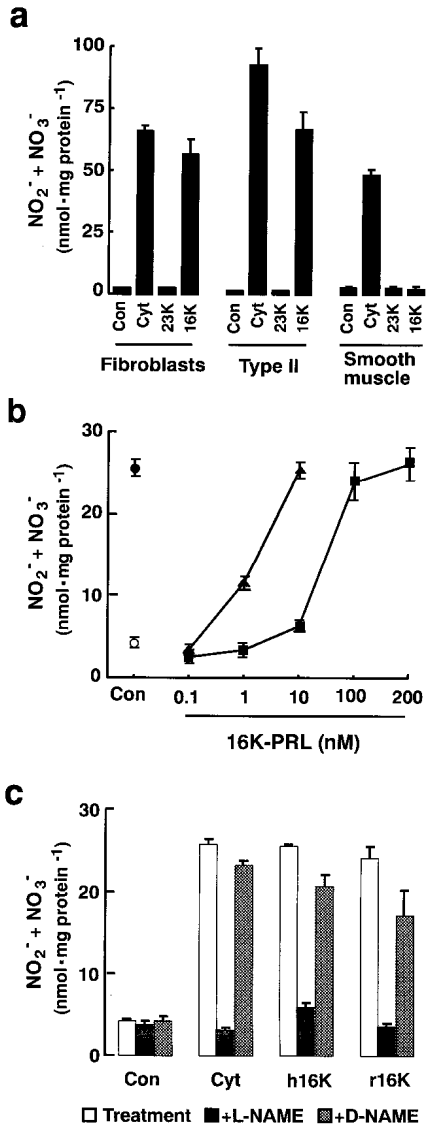

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FIG. 2. 16K-PRL induces nitric oxide synthase (iNOS) mRNA and protein expression in rat lung fibroblasts, a, RT-PCR was performed on total RNA extracted from fibroblasts cultured for 20 h in the absence (Control) or presence of a mixture of cytokines (Cytokines), human (10 nM) or rat (100 nM) 16K-PRL (16K), or human or rat PRL (100 nM) (23K). Amplification of β-actin is shown as a loading control. b, Western blot detection of iNOS in fibroblasts following treatment (72 h) in the absence (Control) or presence of cytokines, human or rat PRL (23K), or 16K-PRL (16K). Doses were the same as in Fig. 2a. A cell lysate of cytokine-activated murine macrophages is shown as a positive control (AM). Cytokine treatment consisted500 of a mixture of IL-1β(5 ng/ml), IFN-γ (100 units/ml), and TNF-α (500 units/ml).

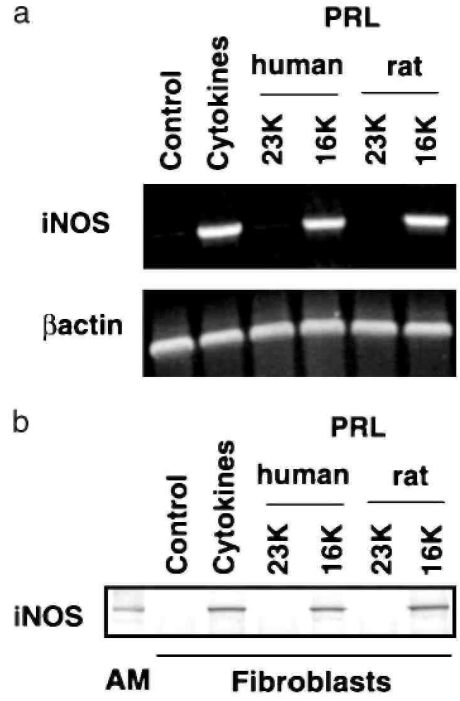

FIG. 3. Detection of a specific 16K-PRL binding site in rat lung fibroblasts, a, Southern blot analysis of the long PRL receptor mRNA expression in rat fibroblasts and alveolar type II epithelial cells. Total RNA was subjected to RT-PCR and Southern blot as described under "Experimental Procedures." PRL receptor cDNA (PRLR cDNA) with or without reverse transcriptase (-RT) are shown as controls. 6, displacement of ^{125}I-labeled rat 16K-PRL binding to fibroblast membranes by increasing concentrations of unlabeled rat 23K-PRL (O) and 16K-PRL (●).

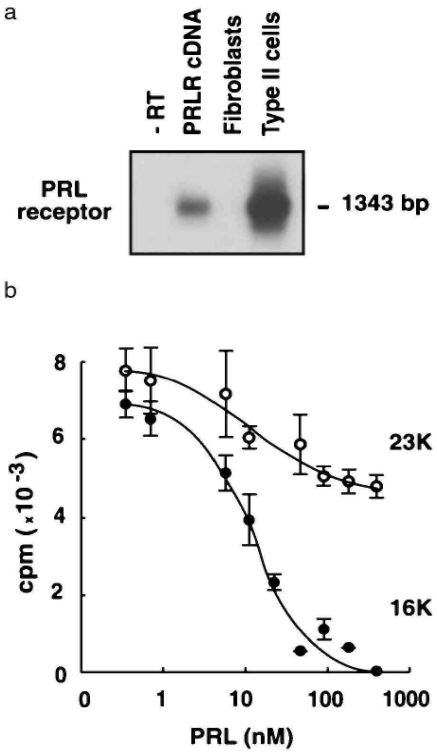

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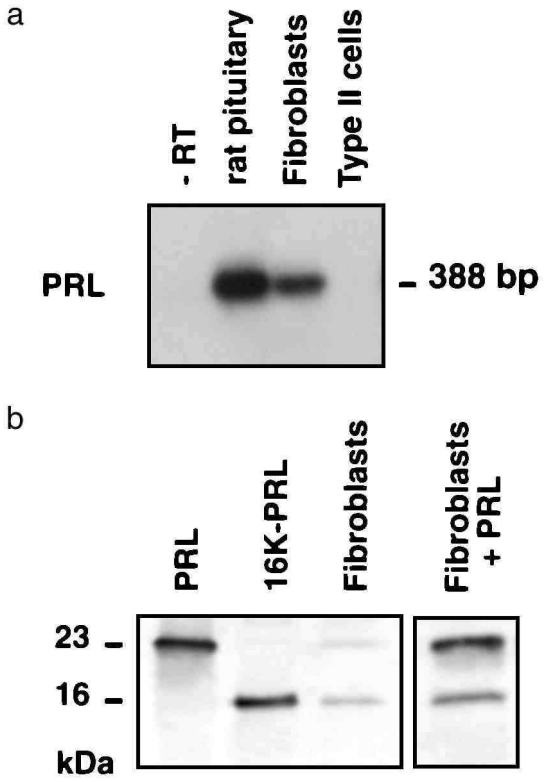
FIG. 4. Prolactin expression and proteolytic cleavage of PRL by rat lung fibroblasts. α, Southern blot analysis of PRL mRNA expression in rat fibroblasts and alveolar type II epithelial cells. RNA from rat anterior pituitary was used as a positive control, and omission of reverse transcriptase (-RT) served as a negative control. Total RNA was subjected to RT-PCR and Southern blot analysis as described under "Experimental Procedures." b, Western blot analysis of endogenous PRL immunoreactive proteins in homogenates from rat lung fibroblasts maintained in serum-free medium for 24 h (Fibroblasts). Exogenous PRL is proteolytically cleaved following incubation with fibroblast homogenates (Fibroblasts + PRL). PRL and 16K-PRL standards are shown.



Acknowledgments

We thank Fernando López Barrera, Andrés Falcon, Thaddeus V. Bamberg, and Phillip H. Chumley for their expert technical assistance.

This work was supported by Howard Hughes Medical Institute Grant 75197-554801, National Council of Science and Technology of México Grant 27950-N, National Autonomous University of México Grant IN226799 (to C. C.), and National Institutes of Health Grants RO1-HL6493, RO1-HL58115, and P6-HL58418 (to B. A. F. and J. P. E.).

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