16-kDa Prolactin Down-Regulates Inducible Nitric Oxide Synthase Expression through Inhibition of the Signal Transducer and Activator of Transcription 1/IFN Regulatory Factor-1 Pathway

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Abstract

Angiogenesis plays a key role in promoting tumorigenesis and metastasis. Several antiangiogenic factors have been shown to inhibit tumor growth in animal models. Understanding their mechanism of action would allow for better therapeutic application. 16-kDa prolactin (PRL), a NH2-terminal natural breakdown fragment of the intact 23-kDa PRL, exerts potent antiangiogenic and antitumor activities. The signaling mechanism involved in 16-kDa PRL action in endothelial cells remains unclear. One of the actions of 16-kDa PRL is to attenuate the production of nitric oxide (NO) through the inhibition of inducible NO synthase (iNOS) expression in endothelial cells. To delineate the signaling mechanism from 16-kDa PRL, we examined the effect of 16-kDa PRL on interleukin IL-1β–inducible iNOS expression, which is regulated by two parallel pathways, one involving IFN regulatory factor 1 (IRF-1) and the other nuclear factor-κB (NF-κB). Our studies showed that 16-kDa PRL specifically blocked IRF-1 but not NF-κB signaling to the iNOS promoter. We found that IL-1β regulated IRF-1 gene expression through stimulation of p38 mitogen-activated protein kinase (MAPK), which mediated signal transducer and activator of transcription 1 (Stat1) serine phosphorylation and Stat1 nuclear translocation to activate the IRF-1 promoter. 16-kDa PRL effectively inhibited IL-1β–inducible p38 MAPK phosphorylation, resulting in blocking Stat1 serine phosphorylation, its subsequent nuclear translocation and activation of the Stat1 target gene IRF-1. Thus, 16-kDa PRL inhibits the p38 MAPK/Stat1/IRF-1 pathway to attenuate iNOS/NO production in endothelial cells. (Cancer Res 2005; 65(17): 7984-92)

Introduction

Prolactin (PRL) is originally identified as a lactotrophic hormone secreted by the pituitary gland. PRL exists in several forms as a result of post-translational modifications, such as glycosylation (1), phosphorylation (2), and proteolysis (3–5). Intact PRL (also called 23-kDa PRL) is further proteolyzed into fragments of various sizes (6). One predominant proteolytic PRL fragment has an apparent molecular weight of 16 kDa and is produced by removal of about a quarter of the PRL molecule from the COOH terminus (7, 8). 16-kDa PRL was found to be present in the hypothalamus, pituitary gland, and mammary gland and in the circulation in humans (3–5, 8–10).

Rather than being an inactive breakdown product of PRL, 16-kDa PRL has potent antiangiogenic effects. 16-kDa PRL inhibited the basal, basic fibroblast growth factor (bFGF)–stimulated and vascular endothelial growth factor (VEGF)–stimulated proliferation of endothelial cells (11, 12). Consistent with its antiangiogenic activities in vitro, 16-kDa PRL inhibited the growth of new capillaries in a chick embryo chorioallantoic membrane assay (11, 13) and blocked bFGF-induced capillary growth in a corneal angiogenesis assay (14).

Angiogenesis is important not only for normal physiology but also for pathophysiology, such as tumorigenesis and metastasis (15). Angiostatin (16) and endostatin (17), which are fragments of plasminogen and collagen XVIII, respectively, inhibit angiogenesis and tumor growth (16–19). Similarly, 16-kDa PRL was shown to have antitumor activities in vivo. Bentzien et al. (13) showed that expression of 16-kDa PRL in HCT116 human colon cancer cells inhibited the tumorigenicity of HCT116 cells in a Rag1 mouse model. Using a recombinant adenovalov vector containing the 16-kDa PRL gene to infect DU145 and PC-3 human prostate cancer cells, Kim et al. (20) showed that overexpression of 16-kDa PRL reduced the tumorigenicity of the prostate cancer cells in a xenograft nude mouse model. These studies suggest that 16-kDa PRL, through its antiangiogenic activity, inhibits tumor growth in vivo.

The mechanism by which 16-kDa PRL mediates its antiangiogenic effects is not clear. 16-kDa PRL may block tumor angiogenesis by antagonizing the effects of angiogenic factors. The cytokine interleukin (IL-1β) is one of the factors that stimulate endothelial cell proliferation in tumorigenesis (21–23). A high level of vascularization and enhanced tumor growth was observed in tumors derived from lung carcinoma cells overexpressing IL-1β (21). A fibrosarcoma cell line transfected with IL-1β exhibited rapid tumor growth, invasiveness, and metastasis to the lung, which resulted in increased mortality in tumor-bearing animals (22). It is likely that 16-kDa PRL may antagonize the effect of IL-1β in tumor angiogenesis.

IL-1β elicits its angiogenic effects in part by inducing the production of nitric oxide (NO), which is an endothelial cell survival factor that inhibits apoptosis and promotes endothelial cell proliferation and migration (24, 25). NO production is regulated...
by NO synthases (NOS). In endothelial cells, the constitutively expressed endothelial NOS (eNOS) is responsible for the low (nanomolar) concentrations of NO that regulates endothelial cell homeostasis (26). In contrast, proinflammatory cytokines induce the transcription of inducible NOS (iNOS), which generates high (micromolar) concentrations of NO that contributes to endothelial cell pathology (27). Proinflammatory cytokines, including IL-1β, tumor necrosis factor-alpha, IFN, and IL-6, regulate iNOS gene transcription through the activation of several transcription factors, including signal transducer and activator of transcription 1 (Stat1), IFN regulatory factor 1 (IRF-1), CAAT/enhancer binding protein (C/EBP), and nuclear factor-κB (NF-κB; refs. 27–29).

In this study, we investigated the effects of 16-kDa PRL on IL-1β stimulation of iNOS and NO production in endothelial cells. Our studies showed that 16-kDa PRL attenuated IL-1β–inducible iNOS and NO expression through inhibition of a signaling cascade involving the p38 mitogen-activated protein kinase (MAPK)/Stat1/IRF-1 pathway but not the NF-κB pathway.

Materials and Methods

Reagents, antibodies, and purified recombinant 16-kDa PRL. Human 16-kDa PRL was expressed and purified from baculovirus-infected insect cells as described previously (30). IL-1β (R&D Systems, Minneapolis, MN); anti-p65 NF-κB and anti-IκB-α (Santa Cruz Biotechnology, Santa Cruz, CA); anti-human iNOS (Research and Diagnostic Antibodies); anti-phospho-Stat1 (Tyr701), anti-phospho-Stat1 (Ser727), anti-Stat1, anti-phospho-p38 MAPK, and anti-p38 MAPK (Cell Signaling Technology, Beverly, MA); and anti-β-tubulin (Sigma-Aldrich, St. Louis, MO) antibodies were purchased from commercial suppliers. Anti-rat IRF-1 antibody was generated as described previously (31).

Cell culture. Rat aortic endothelial cells (RAEC; refs. 32, 33)8 were maintained in DMEM with 10% fetal bovine serum (Invitrogen, Carlsbad, CA) and 1% gentamicin (Sigma-Aldrich). Cells were grown to confluence, placed in serum-free medium for 24 hours, and treated with 20 nmol/L 16-kDa PRL for 1 hour before the addition of 10 ng/mL IL-1β in the continued presence of 16-kDa PRL. Lipopolysaccharide (LPS; Sigma-Aldrich; 5 μg/mL)–treated or IFN-γ (R&D Systems; 100 ng/mL)–treated RAEC cells were used as positive controls for the activation of NF-κB and IRF-1, respectively.

Nitric oxide and citrulline conversion assay. NOS activity in RAEC was determined by measuring the accumulation of nitrite in the culture medium. The culture medium (100 μL) was mixed with 100 μL Griess reagents (Sigma-Aldrich) for 10 minutes at room temperature, and the absorbance at 543 nm was measured. Serial dilutions of sodium nitrite were used as standards (34). NOS activity in cell lysates was analyzed by the 1-arginine to 1-citrulline conversion assay (35) using 1 μCi 3H-1-arginine (53.4 Ci/mmol, Sigma-Aldrich) in the NOS detection kit (Stratagene, La Jolla, CA). The protein extract (30 μg) was used for each assay and the conversion rate was expressed as counts per minute per microgram protein.

Western blot analysis. RAECs were lysed in buffer containing 20 mmol/L Tris (pH 7.4), 100 mmol/L NaCl, 5 mmol/L EDTA, 0.5% Triton X-100, 1 mmol/L phenylmethylsulfonyl fluoride (Sigma), and a protease-inhibitor cocktail (Sigma-Aldrich). Total protein (10–20 μg) was resolved by NuPAGE 4% to 12% Bis-Tris gradient gels (Invitrogen) and transferred to a 0.4% to 12% Bis-Tris gradient gel (Invitrogen) and transferred to 4% to 12% Bis-Tris gradient gels (Invitrogen) and transferred to 4% to 12% Bis-Tris gradient gels (Invitrogen) and transferred to "C2" nitrocellulose membranes (Bio-Rad, Hercules, CA), and the filters were blocked with 5% nonfat milk in TBST [10 mmol/L Tris (pH 7.5), 150 mmol/L NaCl, 0.1% Tween 20]. The filters were blocked with 5% bovine serum albumin (BSA) in TBST when blotting with anti-phospho-antibodies. The blots were incubated with antibodies against iNOS (1:700), IκB-α (1:500), phospho-Stat1 (Ser727; 1:1,000), phospho-Stat1 (Tyr701; 1:1,000), Stat1 (1:1,000), phospho-p38 MAPK (1:1,000), p38 MAPK (1:1,000), or β-tubulin (1:1,000) followed by goat anti-mouse or rabbit horseradish peroxidase secondary antibodies (1:2,000; Santa Cruz Biotechnology) and developed by enhanced chemiluminescence (Pierce, Rockford, IL).

Reverse transcription-PCR. Total RNA (2 μg; RNA-Biz, Tel-Test, Inc., Friendswood, TX) was used for cDNA synthesis. The PCR primers (Sigma-Genosys, The Woodlands, TX) used were as follows: Rat iNOS sense 5′-GGAGGACGTGTTGGAATTTGTA′-3′ and antisense 5′-CTTCGGGCTTCAGGT-TATGTT-5′ (405 bp PCR product); rat IRF-1 sense 5′-GACCTACACAGGCGCATACA-3′ and antisense 5′-AGAGAGACTGTGCTGACGAC-3′ (406 bp PCR product); and histone H3 sense 5′-GACTGCCGGCAATTCCAC-3′ and antisense 5′-GCACAGACGGCTGAAGG-3′ (200 bp PCR product). For iNOS, denaturation was at 95°C for 60 seconds, annealing at 55°C for 60 seconds, and extension at 72°C for 60 seconds followed by a final extension for 5 minutes at 72°C; for IRF-1 and H3, the same conditions were used, except that annealing was at 56°C for 60 seconds. The PCR reaction was carried out for 35 cycles. Fold induction was normalized against H3 IRF-1 controls.

Electrophoretic mobility shift assay. Rat iNOS promoter sequences (27) were used to generate the NF-κB and IRF-1 gel shift oligonucleotides. iNOS NF-κB oligonucleotides: forward 5′-ATAATGGGAAATTACC-TATGCC-3′ and reverse 5′-ACATGGCATTGGTTTTCCATAT-3′; IRF-1 oligonucleotides: forward 5′-CAAAATTTACACTTATTTAAT-3′ and reverse 5′-TTCATTATGTAATGTAATTT-5′, which contain a composite IRF-1 (underlined) and C/EBP (italicized) binding site. The gel shift oligonucleotide pair sequences (Genosys) were labeled with 30 μCi [32P]dGTP and [32P]dATP (3,000 Ci/mmol, ICN, Costa Mesa, CA) by a 30-minute reaction using Klenow polymerase (Promega Corporation, Madison, WI). For the NF-κB gel shift, nuclear extracts (10 μg; Nuclear Extract kit, Active Motif, Carlsbad, CA) were incubated with anti-p65 NF-κB antibody for 30 minutes at room temperature before the addition of labeled probes. For the IRF-1 gel shift, whole-cell extracts (10 μg) were incubated with labeled probes first before the addition of anti-IRF-1 antibody (36, 37). Reactions were resolved on a 5% nondenaturing polyacrylamide gel in 0.5× Tris/boric acid/EDTA. Gels were dried, analyzed by autoradiography, and quantified using a Storm960 PhosphorImager (Molecular Dynamics, Inc., Sunnyvale, CA).

Transient transfection and promoter analysis. The 1.7-kb rat IRF-1 promoter, 1.7-kb mutant IRF-1 promoter containing site-directed mutations in the IFN-γ–activated sequence (GAS) element (mut-IRF-1), three-copy GAS-thymidine kinase (TK) promoter, and three-copy mutant GAS-TK promoter (mut-GAS) were described previously (36) and subcloned from pBL-CAT into pGL3-Basic luciferase reporter (Promega). The three-copy Sp1-TK promoter luciferase reporter was generated by outnumbering the IRF-1 Sp1 element as described previously (37) and subcloning into pGL3-Basic. The pGL3 NF-κB-TK promoter luciferase reporter containing two copies of the NF-κB element was provided by Dr. Natarajan Sivasubramanian (Baylor College of Medicine, Houston, TX). RAEC cells (1 × 106) at 80% to 90% cell confluence were cultured in antibiotic-free DMEM in six-well plates (Becton Dickinson, Franklin Lakes, NJ) and transfected with 4 μg reporter construct in 10 μL LipofectAMINE 2000 (Invitrogen). After 24 hours, 16-kDa PRL (20 mmol/L) was added for 1 hour before the addition of 10 ng/mL IL-1β in the continued presence of 16-kDa PRL. After 4 hours, luciferase activity in 20 μg lystate per sample was assayed in triplicates using the Luciferase Assay System (Promega) in a TD20/20 luminometer (Turner Design, Sunnyvale, CA). Background luminescence from transfection with pGL3-Basic vector alone was used to normalize relative luciferase units (RLU) in each experiment.

Immunofluorescence imaging. RAEC cells were cultured on glass coverslips coated with poly-γ-lysine (Sigma). Cells were maintained in serum-free DMEM for 24 hours before treatment with 50 nmol/L 16-kDa PRL for 1 hour followed by the addition of either 200 ng/mL IL-1β or 500 ng/mL TNF-γ for 30 minutes. Cells were fixed with 4% paraformaldehyde (Polysciences, Inc., Warrington, PA) in PEM buffer (80 mmol/L PIPES [pH 7.0], 1 mmol/L EGTA, 1 mmol/L MgCl2) for 30 minutes and permeabilized with 0.5% Triton X-100 in PEM buffer for 20 minutes. The coverslips were blocked in 2% BSA and 2% goat serum in TBST containing 0.2% sodium azide for 1 hour and incubated with anti-Stat1 antibody (1:500) overnight at 4°C followed by goat anti-mouse IgG conjugated Alexa Fluor 488 (1:1,000; 8 http://cellapplications.com/RAOECA.htm
Molecular Probes, Inc., Eugene, OR) for 1 hour and counterstained with 4,6-diamidino-2-phenylindole (DAPI) using SlowFade Light Antifade kit with DAPI (Molecular Probes). Images were obtained using an Olympus IX71 Microscope (Olympus America, Inc., Melville, NY) and figures were compiled using Adobe Photoshop 7.0 (Adobe Systems, Inc., San Jose, CA).

Statistical analysis. All results were confirmed in multiple independent experiments, with each time point or condition assayed in duplicates or triplicates within each experiment. Densitometry data were analyzed by using the Student’s t test and expressed as mean ± SE. *P < 0.05 was considered statistically significant.

Results

Interleukin-1β–inducible inducible nitric oxide synthase expression and nitric oxide production in aortic endothelial cells. To examine whether IL-1β stimulates NO production, RAECs were stimulated with 10 ng/mL IL-1β and the time course of NO accumulation in the culture medium was determined (Fig. 1A). IL-1β stimulated a robust increase in NO production. Preincubation of RAEC with 20 nmol/L recombinant human 16-kDa PRL for 1 hour resulted in a reproducible and significant reduction in basal as well as IL-1β–induced increase in NO production. The reduction in NO production was accompanied by a reduction in IL-1β–inducible iNOS protein expression in response to 16-kDa PRL (Fig. 1B) and IL-1β–inducible iNOS enzyme activity (Fig. 1C). Further analysis showed that 16-kDa PRL attenuated iNOS gene expression as determined by reverse transcription-PCR (RT-PCR; Fig. 1D). IL-1β stimulated an increase in iNOS RNA expression between 1 and 4 hours, reaching a maximum of 20-fold induction at 24 hours (Fig. 1D, lanes 1-5). Preincubation of RAEC for 1 hour with 16-kDa PRL resulted in a pronounced inhibition of basal as well as the IL-1β–inducible increase in iNOS gene expression (Fig. 1D, lanes 6-10). Control histone H3.3 expression remained unchanged. 16-kDa PRL inhibition of IL-1β–inducible iNOS mRNA levels was further confirmed by RNase protection analysis (data not shown). Thus, in RAEC, 16-kDa PRL attenuated IL-1β–inducible NO production by reducing iNOS gene expression.

Nuclear factor-κB signaling to the inducible nitric oxide synthase gene. Multiple cytokines and signaling pathways regulate the transcription of the iNOS gene (29). IL-1β is known to activate both the NF-κB and the IRF-1 signaling pathways to regulate iNOS gene transcription (27–29). To determine which of the IL-1β–inducible signaling pathways might be a target of 16-kDa PRL inhibition, we analyzed NF-κB or IRF-1 binding to the rat iNOS promoter (27) in electrophoretic mobility shift assays (EMSA). Using the NF-κB oligonucleotide, a complex of three bands was transiently induced at 30 minutes of IL-1β stimulation in RAEC (Fig. 2A, lane 2). This complex was specific as it was competed by increasing molar excess of unlabeled probe (Fig. 2A, lanes 11 and 12). This complex contained p65 NF-κB as it was supershifted by anti-p65 NF-κB antibody (Fig. 2A, lane 13) and by anti-p50 NF-κB antibody (data not shown). The two faster migrating bands represent p50/p65 NF-κB heterodimers and p50/p50 NF-κB homodimers, respectively (data not shown). Interestingly, this NF-κB-containing complex was not altered by 16-kDa PRL treatment (Fig. 2A, lanes 6-10). These results show that 16-kDa PRL did not affect IL-1β–inducible NF-κB binding to the iNOS promoter in RAEC. Consistent with a lack of 16-kDa PRL effect on NF-κB DNA binding at the iNOS promoter, 16-kDa PRL treatment also did not affect IL-1β–inducible transient down-regulation of the NF-κB inhibitor IκBα (Fig. 2B). These results show that 16-kDa PRL did not affect IL-1β–inducible IκBα degradation or NF-κB signaling to the iNOS promoter in RAEC.

16-kDa Prolactin inhibits IFN regulatory factor 1 signaling to the inducible nitric oxide synthase gene. We next analyzed IRF-1 binding to the composite IRF-1/C/EBP binding site in the rat iNOS promoter by incubation with 10 ng/mL IL-1β in the continued presence of 16-kDa PRL. A, NO (nitrite) levels that have accumulated in the culture medium were determined by the Griess reaction (34). Columns, mean of two independent experiments; bars, ± SE; *, P < 0.05; **, P < 0.01. B, Western blot analysis of iNOS (131-kDa) protein expression. p-β-tubulin (55-kDa) was used as a protein loading control. Lane C, positive control from LPS-treated RAW264.7 cells. Columns, mean of four independent experiments; bars, ± SE; *, P < 0.05; **, P < 0.005. C, iNOS enzyme levels were determined by the [3H]-arginine to [3H]-citrulline conversion assay (35). D, RT-PCR analysis of iNOS RNA expression. Histone H3.3 was used as a RT-PCR control. Points, mean of four independent experiments; bars, ± SE; *, P < 0.005; **, P < 0.001.
iNOS promoter (27). Incubation of RAEC with IL-1 \( \beta \) for 1 hour induced the formation of a complex (Fig. 3A, lane 2), which was specific as it was competed by increasing molar excess of unlabeled probe (Fig. 3A, lanes 3 and 4). This IL-1\( \beta \)-inducible complex contains IRF-1 as it was supershifted by anti-IRF-1 antibody (Fig. 3A, lane 5) and exhibited similar mobility with the IFN-\( \gamma \)-inducible IRF-1-positive control (Fig. 3A, lane 6). The darker, slower migrating complex represented C/EBP binding to the IRF-1/C/EBP composite site (data not shown; ref. 27). IL-1\( \beta \) stimulated a transient increase in IRF-1 binding at 1 hour to the rat iNOS promoter (Fig. 3B, lanes 1-4), with binding returning to basal levels by 8 hours. Interestingly, IL-1\( \beta \)-inducible IRF-1 binding was inhibited by 16-kDa PRL treatment (Fig. 3B, lanes 5-8). Thus, 16-kDa PRL selectively blocked only the IL-1\( \beta \)-inducible IRF-1 but not NF-\( \kappa \)B binding to the iNOS promoter in RAEC.

16-kDa Prolactin inhibits signal transducer and activator of transcription 1 signaling to the IFN regulatory factor 1 gene. We further examined whether 16-kDa PRL inhibited IL-1\( \beta \)-inducible IRF-1 gene expression. RAECs were pretreated with or without 20 nmol/L 16-kDa PRL for 1 hour before stimulation with 10 ng/mL IL-1\( \beta \), and IRF-1 gene expression was analyzed by RT-PCR (Fig. 3C). IL-1\( \beta \) stimulated a rapid but transient increase in IRF-1 gene expression, which was maximal at 1 hour and declined without 16-kDa PRL.
to basal levels by 24 hours (Fig. 3C, lanes 1-5). 16-kDa PRL inhibited the IL-1β–inducible increase in IRF-1 gene expression (Fig. 3C, lanes 6-10). Histone H3.3 control remained essentially unchanged. 16-kDa PRL inhibition of IL-1β–inducible IRF-1 mRNA levels was further confirmed by RNase protection analysis (data not shown).

Little is known about how IL-1β stimulates IRF-1 expression (38). We have shown that Stat1 and Sp1 mediate transcriptional activation of the IRF-1 gene through a GAS element at -120 bp and an Sp1 element at -200 bp, respectively (36, 37, 39, 40). To determine which elements are involved in IL-1β stimulation of the IRF-1 gene, we analyzed IRF-1 promoter activity by transient transfection of luciferase reporter constructs into RAEC. IL-1β stimulated both Stat1 Tyr701 and Ser727 phosphorylation in RAEC (Fig. 4A). (91-kDa) and Stat1(84-kDa) remained essentially unchanged. As positive controls, IFN-γ stimulated both Stat1 Tyr701 and Ser727 phosphorylation in RAEC (Fig. 4B, lane +). Together, these results suggest that 16-kDa PRL inhibited IL-1β–inducible Stat1 serine phosphorylation as one mechanism to inhibit IRF-1 gene transcription in RAEC.

16-kDa Prolactin inhibits interleukin-1β–inducible p38 mitogen-activated protein kinase phosphorylation of signal transducer and activator of transcription 1 and its nuclear translocation. It has been shown that Stat1 phosphorylation on Ser727 is mediated by the MAPK family of kinases (41). To identify which MAPK may be involved in IL-1β–inducible Stat1 serine phosphorylation, we pretreated RAEC for 1 hour with specific MAPK inhibitors before IL-1β stimulation (Fig. 5A). IL-1β–inducible Stat1 Ser727 phosphorylation was significantly blocked by the p38 MAPK inhibitor SB203580 (Fig. 5A, lane 2 versus lane 3, and B) but not by the extracellular signal-regulated kinase 1/2 (ERK1/2) inhibitor PD98059 (Fig. 5A, lane 4) or the c-Jun NH2-terminal kinase (JNK) inhibitor SP600125 (Fig. 5A, lane 5). The levels of Stat1 remained essentially unchanged. These data suggest that p38 MAPK signaling is required for IL-1β–inducible Stat1 Ser727 phosphorylation in RAEC. We further examined if 16-kDa PRL inhibited p38 MAPK phosphorylation in response to IL-1β stimulation in RAEC. IL-1β stimulated a rapid induction in p38 MAPK phosphorylation, which was maximal at 5 minutes and declined by 15 minutes (Fig. 5B, lanes 1-3).

We have shown previously that Stat1 activates IRF-1 gene transcription through the GAS element (36, 39). We therefore examined Stat1 phosphorylation in response to IL-1β stimulation in RAEC. IL-1β stimulated a transient induction between 15 and 30 minutes in Stat1 Ser727 phosphorylation but not Stat1 Tyr701 phosphorylation (Fig. 4B, lanes 1-5). 16-kDa PRL treatment abolished IL-1β–inducible Stat1 serine phosphorylation (Fig. 4B, lanes 6-10). The levels of Stat1α (91-kDa) and Stat1β (84-kDa) remained essentially unchanged. As positive controls, IFN-γ stimulated both Stat1 Tyr701 and Ser727 phosphorylation in RAEC (Fig. 4B, lane +). Together, these results suggest that 16-kDa PRL inhibited IL-1β–inducible Stat1 serine phosphorylation as one mechanism to inhibit IRF-1 gene transcription in RAEC.
PRL inhibited IL-1β–inducible p38 MAPK phosphorylation in RAEC (Fig. 5B, lanes 4–6). The levels of p38 MAPK remained essentially unchanged. These data show that 16-kDa PRL inhibited IL-1β–inducible p38 MAPK phosphorylation in RAEC.

To examine the functional consequence of p38 MAPK inhibition on IL-1β–inducible Stat1 signaling, we analyzed IRF-1 promoter activity by transient transfection assays (Fig. 5C). Addition of 16-kDa PRL, the p38 MAPK inhibitor SB203580, or both all significantly reduced IL-1β signaling to the IRF-1 promoter. The extent of inhibition was similar for 16-kDa PRL and/or p38 MAPK inhibitor, suggesting that 16-kDa PRL and the p38 MAPK inhibitor acted in the same pathway to inhibit IL-1β–inducible IRF-1 promoter activity. On the other hand, neither 16-kDa PRL nor the p38 MAPK inhibitor reduced IL-1β–inducible NF-κB-dependent TK promoter activity, in agreement with our observation that 16-kDa PRL does not affect the NF-κB signaling pathway in RAEC (Figs. 2 and 4A). Together, the data suggest that 16-kDa PRL inhibited IL-1β–inducible p38 MAPK phosphorylation of Stat1 and blocked Stat1-mediated activation of the IRF-1 gene in RAEC.

As a further step in understanding how 16-kDa PRL inhibits Stat1 signaling, we examined Stat1 nuclear translocation by immunofluorescence microscopy (Fig. 5D). For these experiments, higher levels of cytokines were used to maximize Stat1 nuclear translocation.
entry (42). In control RAEC, Stat1 (green) was localized primarily in the cytoplasm as shown by the lack of Stat1 staining in the nucleus (DAPI in blue) in the merged image. Stimulation of RAEC with IL-1β for 30 minutes induced the nuclear translocation of Stat1. Interestingly, 16-kDa PRL effectively blocked IL-1β–inducible Stat1 nuclear translocation. Neither 16-kDa PRL alone nor p38 MAPK inhibitor SB203580 (data not shown) had any effect on Stat1 cytoplasmic localization. IFN-γ, which induced Stat1 phosphorylation on both Tyr701 and Ser727 (Fig. 4B), stimulated Stat1 nuclear translocation (Fig. 5D). These results reveal for the first time that 16-kDa PRL, via a mechanism that involved p38 MAPK inhibition, blocked Stat1 serine phosphorylation and nuclear translocation. The inhibition of Stat1 activation blocked IL-1β–inducible IRF-1 gene expression and attenuated iNOS/NO expression in RAEC.

Taken together, our data indicate that 16-kDa PRL modulates endothelial cell activity through the inhibition of a specific intracellular pathway (Fig. 6). Although IL-1β regulates iNOS gene expression and NO production in aortic endothelial cells by activating both IRF-1 and NF-κB signaling pathways, 16-kDa PRL attenuates IL-1β effects by antagonizing one of these signaling pathways. 16-kDa PRL effectively inhibits IL-1β–inducible p38 MAPK phosphorylation, resulting in blocking Stat1 Ser727 phosphorylation, its subsequent nuclear translocation and activation of Stat1 target genes, such as IRF-1. In contrast, 16-kDa PRL does not affect IL-1β–inducible NF-κB signaling to the iNOS gene. This pathway results in 16-kDa PRL attenuation of IL-1β–induced iNOS expression and NO production in aortic endothelial cells.

Discussion

We have shown that 16-kDa PRL attenuates IL-1β–inducible iNOS gene expression in RAECs, and this effect is mediated in part through the inhibition of the expression of IRF-1, a factor necessary for iNOS expression. IRF-1 is a transcription factor that regulates the expression of genes involved in immune and inflammatory responses (43, 44). However, the role and regulation of IRF-1 in endothelial cells are not known. Results from our study suggest that IRF-1 may play a role in the regulation of angiogenesis through modulating iNOS expression in endothelial cells. Although 16-kDa PRL has been shown to inhibit angiogenesis and tumorigenesis in both in vitro and in vivo studies (11–14, 20), the mechanism leading to the antiangiogenic activity of 16-kDa PRL remains largely unknown. Our study elucidates one of the molecular mechanisms that may be involved in the antiangiogenic and antitumor effect of 16-kDa PRL.

IL-1β–inducible Stat1 Ser727 phosphorylation plays an important role in activating IRF-1 expression (Figs. 3 and 4). Although both Tyr701 and Ser727 phosphorylation of Stat1 occurs in response to cytokine and growth factor stimulation (41, 45), IL-1β did not induce Stat1 Tyr701 phosphorylation but stimulated Stat1 Ser727 phosphorylation through activation of p38 MAPK in RAEC (Figs. 4 and 5; refs. 41, 46). The phosphorylated Ser727 residue on Stat1 has been shown to mediate the association of Stat1 with coactivators to regulate chromatin remodeling at target genes (47). We have shown previously that extensive histone acetylation and chromatin remodeling at the IRF-1 promoter is required for IRF-1 gene transcription (48). Because mutations in the GAS element abolished IRF-1 promoter activity (Fig. 4A), it is likely that serine-phosphorylated Stat1 regulates IL-1β–inducible IRF-1 expression through the GAS element in the IRF-1 promoter. We thus suggest that 16-kDa PRL inhibits IRF-1 gene expression through down-regulation of the Stat1 signaling pathway via inhibition of Stat1 Ser727 phosphorylation and Stat1 nuclear translocation. Consistent with our observation that Stat1 is involved in antiangiogenic signaling mechanisms, recent studies also show that down-regulation of Stat1 levels contributes to the antiangiogenic effects of endostatin (49), but it is not known whether Tyr701 and/or Ser727 phosphorylation of Stat1 is involved.

Our studies indicate that the inhibition of iNOS expression by 16-kDa PRL in aortic endothelial cells is not mediated through the NF-κB pathway (Fig. 2). This is in contrast to the studies by Macotela et al. (50) and Tabruyn et al. (51), which showed that 16-kDa PRL activates NF-κB activity in primary mouse embryonic lung fibroblasts and bovine brain capillary endothelial cells, respectively. Because the IL-1β–inducible NF-κB pathway is essentially unperturbed by 16-kDa PRL in RAEC, these results may explain why 16-kDa PRL only inhibited ~40% iNOS gene transcription, which is likely contributed by the IL-1β–inducible IRF-1 pathway. Interestingly, 16-kDa PRL also inhibited basal IRF-1 (Fig. 3C) and basal iNOS (Fig. 1D) gene expression in the absence of IL-1β stimulation, suggesting that other pathways might be involved. Thus, 16-kDa PRL regulates multiple signaling pathways, including Stat1 (Figs. 4 and 5), IRF-1 (Fig. 3), Ras, Raf, ERK1/2 MAPK (12, 52), p38 MAPK (Fig. 5), and NF-κB (50, 51), in a cell type– and tissue-specific manner. Several antiangiogenic proteins (i.e., angiostatin and endostatin) are fragments of larger protein counterparts (16, 17). Both
16-kDa PRL, likely functions through a cell surface receptor that is distinct from the 23-kDa PRL receptor. Clapp and Weiner (57) showed the presence of specific, high-affinity (κD = 9.9 mmol/L), and saturable (Dmax = 48 pmol/mg protein) binding sites for 16-kDa PRL on bovine brain capillary endothelial cells. However, the receptor(s) for 16-kDa PRL has not been identified. Our observation that 16-kDa PRL regulates signaling in RAEC suggests that the RAEC express the 16-kDa PRL receptors and will provide an excellent source for further receptor studies.

That 16-kDa PRL has antiangiogenic and antitumor properties has been observed for many years (8, 11–14, 20, 52, 58, 59). However, the lack of understanding of 16-kDa PRL signaling mechanisms has hampered the possibility of applying 16-kDa PRL in therapy. Understanding the signaling mechanism of 16-kDa PRL in endothelial cells will allow the design of better therapeutic strategies targeting the angiogenic process. Our studies raise the possibility that 16-kDa PRL can be used for the treatment of diseases characterized by abnormal angiogenesis, such as tumor-metastasis, and proliferative retinopathies, and/or by chronic angiogenesis, such as rheumatoid arthritis and inflammatory bowel disease (15, 60, 61).

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