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Prolactin Stimulates Mitogen-Activated Protein Kinase in Human Leiomyoma Cells

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The effects of prolactin (PRL) on proliferation of cultured human uterine leiomyoma-derived smooth muscle cells (SMC) and its mechanism of action were investigated. PRL stimulated DNA synthesis and the expression of PRL receptor was identified by ribonuclease protection assay. Moreover, the regulation of mitogen-activated protein (MAP) kinase by PRL in leiomyoma-derived SMC was investigated. PRL stimulated MAP kinase activity, as detected by ^{32}P incorporation into MAP-2, in a dose-dependent manner. PRL also rapidly stimulated MAP kinase phosphorylation as detected by in vivo phosphorylation using ^{32}P labeling and phosphotyrosine immunoblotting. These results suggest that PRL stimulates the proliferation of human leiomyoma cells via the MAP kinase cascade.

Prolactin (PRL) is a 198-amino acid polypeptide originally identified as a lactogen in extracts of the anterior pituitary gland. In addition to regulating mammary gland growth and lactation, PRL has been reported to activate cellular proliferation in nonreproductive tissues such as the liver (1-3), spleen, and thymus (4). The binding of PRL to its cell-surface receptor regulates diverse physiological processes. However, little is known regarding early biochemical events in signal transduction through the PRL receptor.

Uterine leiomyomas, or fibroids, are the most common pelvic tumors in women, occurring in 20-25% of women of reproductive age. These smooth muscle cell (SMC) tumors rarely become malignant. Although the pathogenesis of leiomyoma is not clearly understood, there is considerable evidence that estrogen and/or progesterone are involved in tumor growth (5-8). However, other investigators have also indicated lack of effects of estradiol and progesterone on both DNA synthesis and cell number in leiomyoma-derived SMC in culture (9). It was reported that leiomyoma-derived SMC in explant or monolayer culture can secrete PRL (10-12). Normal uterine myometrial cells secrete less PRL in vitro than leiomyoma cells (12,13). Moreover, PRL mRNA was detected in human leiomyoma cells (14). However, the physiological role of PRL in uterine tissue remains unclear.

Mitogen-activated protein (MAP) kinase is well known to be activated by growth factors and various agents which have mitogenic effects (15). Taken together, we examined whether PRL has a mitogenic effect in leiomyoma-derived SMC, the expression of PRL receptors in leiomyoma cells, and whether stimulation of leiomyoma-derived SMC by PRL leads to MAP kinase activation to clarify the role of PRL in uterine leiomyomas. Here, we report that PRL stimulated DNA synthesis in leiomyoma-derived SMC, PRL receptor was identified in human uterine leiomyoma cells by ribonuclease protection assay, and stimulation by PRL of cultured human uterine leiomyoma-derived SMC resulted in rapid MAP kinase activation and phosphorylation.

MATERIALS AND METHODS

Materials

[γ-^{32}P]ATP (3000Ci/mmol) was purchased from New England Nuclear Corp. (Bannockburn, IL). [^{32}P]orthophosphate (285Ci/mg) was obtained from ICN (Irvine, CA). Anti-MAP kinase and anti-phosphotyrosine antisera were obtained from Upstate Biotechology (Lake Placid, NY). ECL Western blotting detection reagents were obtained from Amersham (Buckinghamshire, UK). TRI REAGENT was obtained from Molecular Research Center INC (Cincinnati, OH).

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Methods

Preparation of human uterine leiomyoma-derived SMC. The tissues of uterine leiomyoma were obtained from women who underwent hysterectomy for uterine leiomyoma, and decidual tissue at term was obtained after dissection away from amniotic and chorionic membranes after informed consent was received from the women for their use. The tissues were cut into 1-2 mm^3 fragments and digested with 0.1% trypsin for 1 h at 37°C in calcium-magnesium free Hanks' solution. The tissues were digested with 0.1% collagenase, 0.1% hyaluronidase, 0.1% deoxyribonucrease, and 0.1% protease for 1 h at 37°C in Ca-Mg-free Hanks' solution. Cell aggregates were isolated by gentle pipetting. Nondispersed fragments were separated by filtration through gauze cloth. The cells were maintained at 37°C under an atmosphere of 95% air and 5% CO_2 in RPMI1640 medium containing 10% fetal bovine serum supplemented with penicillin (200 U/ml) and streptomycin (200 mg/ml).

Determination of thymidine incorporation. Following starvation for 24 h, leiomyoma-derived SMC were incubated with PRL at the indicated concentrations for 12 h. Then, the medium was replaced with 1 mCi/ml [^{3}H]thymidine in RPMI1640 medium containing 0.1% BSA. After further incubation for 4 h, cells were washed twice with PBS, incubated with 5% trichloroacetic acid for 6 h at 4°C, washed twice with 5% trichloroacetic acid, and lysed with 1 N NaOH. Thymidine incorporation was then determined by scintillation counting.

Ribonuclease protection assay. PRL receptor mRNA were quantified in hybridization solution supplied with a ribonuclease protection assay kit from Ambion Inc., Austin, TX. Antisense transcripts for use as probes were synthesized from linearized plasmid DNA using ^{32}P-UTP (16). The residual DNA template was removed by incubating for 30 min at 37°C with 1U RNase-free DNase I (Promega). Radiolabeled transcripts were purified by polyacrylamide gel electrophoresis. Total RNA from uterine leiomyoma tissue was isolated by the guanidium isothiocyanate-phenol-chloroform extraction method of Chomczynski and Sacci (17) using Tri-reagent. Aliquots of 5 μg of total RNA were hybridized with 1×10^5 cpm of labeled probes in buffer containing 80% formamide at 45°C for 16 h. Residual transcripts were digested with RNase A and RNase T1 at 37°C for 30 min. The protected transcripts were ethanol-precipitated and resolved by electrophoresis through 5% polyacrylamide/8M urea DNA sequencing gels. Dried gels were exposed to Phosphorimager screens and the bands were analyzed using ImageQuant 3.2 Software (Molecular Dynamics). The sizes of the protected fragments were determined by reference to an RNA marker transcribed with ^{32}P-UTP (Ambion Inc.). The intensity of the PRL receptor transcripts was normalized to the intensity of protected GAPDH (316nt) fragments in the same sample, and results are expressed as corrected Phosphorimager units. All ribonuclease protection assays shown were performed in duplicate.

Assay of MAP kinase activity. The activity of MAP kinase was assayed as previously described (18). Briefly, 10 μl aliquots of cell lysates were incubated with bovine brain microtubule-associated protein-2 (MAP-2) and 40 μM [γ-^{32}P]ATP (1 μCi). The reaction was stopped by addition of Laemmli sodium dodecyl sulfate (SDS) sample buffer (19), and phosphorylated MAP-2 was resolved by 8% SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

^{32}P labeling of MAP kinase. Human uterine leiomyoma-derived SMC were grown in 60-mm dishes. The cells were washed once with phosphate-free DMEM and incubated for 2 h in phosphate-free DMEM with 1 mCi [^{32}P]orthophosphate. After hormone treatment, the cells were washed once with ice-cold PBS, followed by addition of 100 μl 1% SDS. The lysates were heated for 5 min at 100°C and diluted 1:10 with ice-cold HNTG lysis buffer (20). The lysates were cooled to 0°C for 5 min, followed by the addition of 40 μl of rabbit immunoglobulin G-agarose. After incubation at 4°C for 15 min, samples were incubated at 4°C for 1 h with anti-MAP kinase antiserum. Immunocomplexes were precipitated with protein-G-protein-A agarose and washed three times with HNTG buffer. Isolated proteins were analyzed by 8% SDS-PAGE (21).

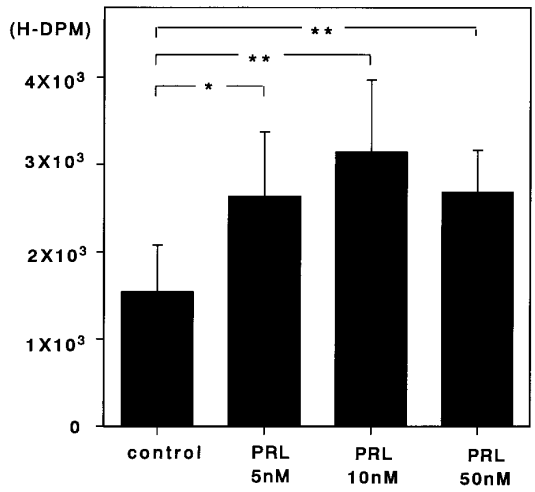

FIG. 1. Mitogenic effect of PRL on leiomyoma-derived SMC. Leiomyoma-derived SMC (0.5×10^6/ml) were incubated without (control) or with the indicated concentration of PRL. At the end of 12 h incubation, [^{3}H]thymidine uptake was measured with a scintillation counter. Each bar shows the mean value, and figures in parentheses indicate the S.E. for five independent experiments. Significant differences (versus the control) are shown by asterisks. **, $p<0.01$; *, $p<0.05$.

Immunoblots. For analysis of protein tyrosine phosphorylation in whole cell lysates, cells were grown in 60-mm dishes. After hormone treatment, the cells were washed once with ice-cold PBS, followed by the addition of 100 μl of Laemmli SDS sample buffer (19). Samples were heated at 100°C for 5 min, and 30-μg aliquots of protein were separated by 8% SDS-PAGE. Proteins were transferred to nitrocellulose paper and immunoblotted with anti-phosphotyrosine antiserum, as described previously (22).

Statistics. Statistical analysis was performed by Student's *t* test. $p<0.05$ was considered significant. Data are expressed as the means +/− S.E.

RESULTS

Mitogenic Effect of PRL on Leiomyoma-Derived SMC

To examine whether PRL has a mitogenic effect on leiomyoma-derived SMC, cells were treated with the indicated concentrations of PRL for 12 h, followed by 4h incubation with [^{3}H]thymidine for pulse labeling. PRL stimulated thymidine uptake in leiomyoma-derived SMC dose-dependently (Fig.1). The lowest stimulatory dose was 5 nM, and maximal stimulation was achieved at 10 nM. The dose-response relationship of thymidine uptake in leiomyoma-derived SMC by PRL was similar to that in Nb2 cells, a PRL-dependent rat pre-T lymphoma cell line, evoked by PRL (23).

Detection of PRL Receptor mRNA in Leiomyoma Cells

The initial step in the biological action of PRL involves its interaction with a specific transmembrane

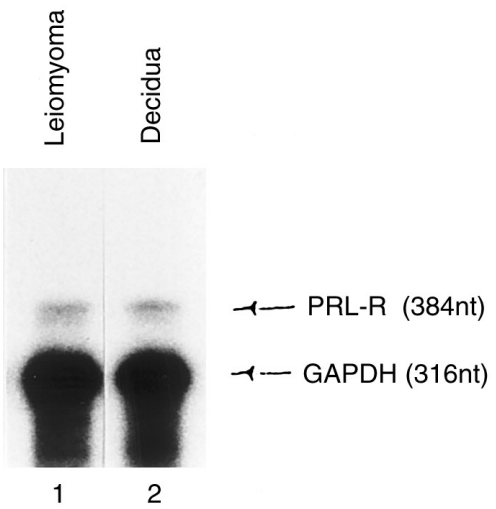

FIG. 2. Detection of PRL receptor mRNA in leiomyoma cells. Ribonuclease protection assays were performed using an RNA probe complementary to the 5′ region of the PRL receptor. Aliquots of 5 μg of total RNA were analyzed from human leiomyoma tissue (lane 1) or human term decidual tissue (lane 2). The simultaneous detection of GAPDH transcripts was used to normalize the intensity of the signal for PRL receptor for the same sample upon analysis of the data by Phosphorimage screens.

receptor. Although it was reported that leiomyoma-derived SMC in explant or monolayer culture can secrete PRL (10-12), PRL receptors have not been identified on these cells. Therefore, we examined whether PRL receptor mRNA was expressed in human leiomyoma by ribonuclease protection assay using an RNA probe complementary to the 5′ region of the PRL receptor at approx. 384 nucleo tides (nt) (16). PRL receptor mRNA was detected previously in term decidua (16). PRL receptor mRNA levels in leiomyoma cells were comparable to those in term decidua (Fig.2).

PRL Stimulates MAP Kinase Activity

The mechanism of the mitogenic effect of PRL was investigated. Since various cytokines and growth factors stimulate MAP kinase activation and cell proliferation, we examined whether PRL also stimulates MAP kinase activation. Cultured human leiomyoma-derived SMC were treated with the indicated concentration of PRL for 5 min. Cell lysates were assayed for MAP kinase by examining the incorporation of ^{32}P into MAP-2, followed by SDS-PAGE and autoradiography (Fig.3). PRL produced a marked dose-dependent increase in this kinase activity compared with the control cells.

PRL Stimulates Phosphorylation of MAP Kinase

Mitogenic stimuli activate MAP kinase by increasing tyrosine and serine or threonine phosphorylation of the protein, due to the activity of the dual specificity of MAP kinase kinase (24). To further characterize the effect of PRL, we evaluated the hormone-dependent phosphorylation of the predominant form of MAP kinase detected in cultured human leiomyoma-derived SMC. Following preincubation with [^{32}P]orthophosphate, cells were treated for the indicated times with PRL. After hormone treatment, lysates were prepared and immunoprecipitated with anti-MAP kinase antiserum, followed by SDS-PAGE and autoradiography (Fig.4A). PRL caused the rapid phosphorylation of the 42-kDa MAP kinase protein in cultured human leiomyoma-derived SMC. The maximal activation of MAP kinase by PRL was detected as early as at 5 min and declined thereafter.

The effects of PRL on protein tyrosine phosphorylation were also evaluated in cultured human leiomyoma-derived SMC by immunoblotting with antiphosphotyrosine antiserum (Fig.4B). PRL and EGF caused tyrosine phosphorylation of a protein with a molecular mass of 42 kDa, which has been well documented as a tyrosine kinase substrate after stimulation of quiescent cells by mitogens acting through receptor tyrosine kinases (25), endothelin (26,27), thyrotropin-releasing hormone (28), or oxytocin (29).

DISCUSSION

The results presented in this study demonstrated that, in the non-immune system such as leiomyoma-derived SMC cultures, PRL has a mitogenic effect and PRL receptor ligation is coupled to the activation and

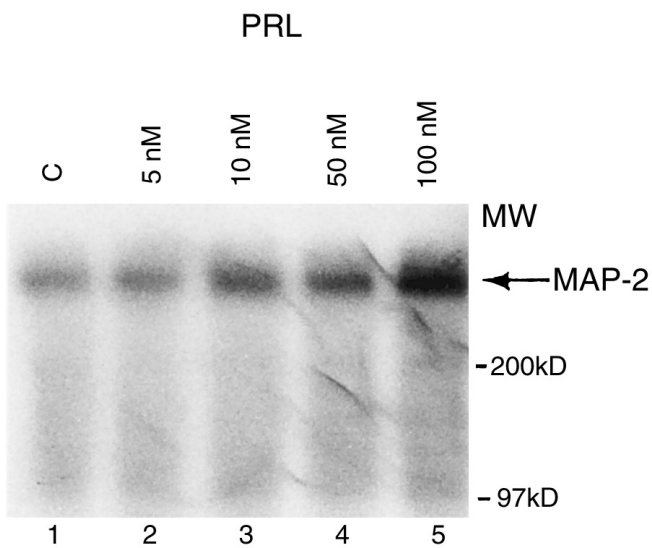

FIG. 3. PRL stimulates MAP kinase activity. Cells were grown in 60-mm dishes and treated with the indicated concentration of PRL for 5 min. Lysates were incubated with [γ-^{32}P]ATP in the presence of MAP-2, as described in Materials and Methods. Reactions were stopped with Laemmli sample buffer, followed by SDS-PAGE and autoradiography. Experiments were repeated 3 times with essentially identical results.

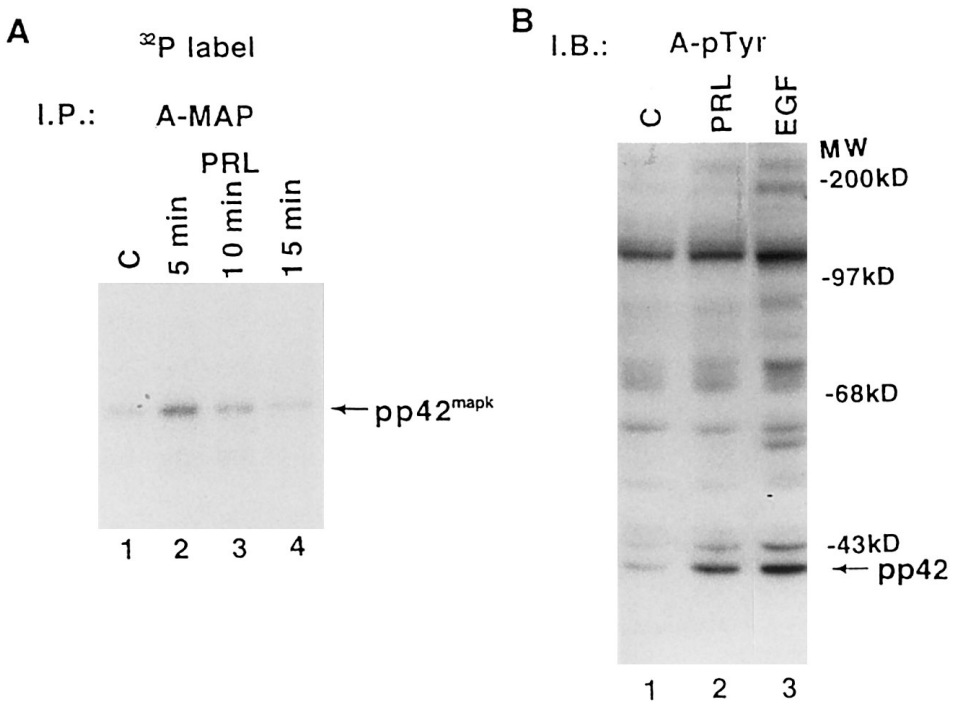

FIG. 4. PRL stimulates the phosphorylation of MAP kinase. Cells were grown in 60-mm dishes. (A) Cells were labeled with 1 mCi [^{32}P]orthophosphate for 2 h, followed by incubation with 100 nM of PRL for the indicated times. Cell lysates were subsequently immunoprecipitated with an anti-MAP kinase antiserum, and the immunoprecipitates were subjected to SDS-PAGE followed by autoradiography. (B) Cells were treated with 100 nM PRL (lane 2) or with 1 nM EGF (lane 3) for 5 min. After treatment, 30 μg aliquots of protein from each whole-cell lysate were analyzed by SDS-PAGE, followed by immunoblotting with anti-phosphotyrosine antiserum, as described in Materials and Methods. Experiments were repeated 3 times with essentially identical results.

phosphorylation of MAP kinase. The rapidity with which MAP kinase was activated following PRL addition suggested that it plays a central role in PRL signal transduction in leiomyoma-derived SMC cultures.

The question remains as to how PRL stimulates the activation of MAP kinase. The initial step in the biological action of PRL involves its interaction with a specific transmembrane receptor which belongs to the cytokine receptor family, members of which share similar extracellular structural domains (30,31). Three forms of the PRL receptor have been identified which differ primarily in the length of their cytoplasmic domains (30). Of the two predominant forms of the PRL receptor (long-form of 82 kDa, and short-form of 42 kDa), only the long form confers PRL-dependent signaling when transfected into CHO cells along with a PRL-responsive reporter gene construct (32). Recently, an intermediate-length form of the PRL receptor (60-62 kDa) was identified in Nb2 cells, a PRL-dependent rat pre-T lymphoma cell line (33). Although this receptor lacks 198 amino acids present in the cytoplasmic domain of the long-form PRL receptor, it is capable of conferring PRL-dependent signaling when expressed in CHO cells (34). Since PRL receptors are members of the cytokine receptor family, JAK-family tyrosine kinases have been identified as signal transducers (35). Many cytokines stimulate a molecular cascade coupled with Ras activation (36). In addition, Raf-1 kinase which regulates MAP kinase activity upstream was activated by PRL in Nb2 cells (37). Thus, similarly to T lymphocyte systems, Raf-1 may activate the multifunctional tyrosine and serine/threonine kinase MAP kinase kinase in leiomyoma SMC, which in turn likely phosphorylates MAP kinase on threonine and tyrosine residues resulting in its activation.

What is the role that MAP kinase might play in eliciting physiological responses to PRL? There is considerable evidence that MAP kinases play an important role in the control of cellular growth (15). These enzymes are activated in a variety of cell types and in response to numerous growth factors whose receptors are structurally unrelated, suggesting the existence of distinct pathways that converge at this site of regulation. However, the physiological response of PRL in the uterus is not clear. Endometrial stromal cells, which give rise to decidual cells, myometrium and uterine leiomyoma all have the capacity to synthesize and secrete PRL (38). Leiomyomas have been shown to secrete larger amounts of PRL in vitro than normal myometrial tissue (12). In addition, the secretion of PRL by leiomyoma explants obtained from women treated with the GnRH agonist leuprolide acetate was reduced compared to

that in placebo-treated women. In this study, we showed that PRL has a mitogenic effect in leiomyoma-derived SMC and PRL receptor was identified in uterine leiomyoma. Taken together, these observations suggest that MAP kinase plays a role in PRL-induced cellular growth and PRL may have important autocrine or paracrine regulatory functions in uterine leiomyoma SMC. Further studies will be required to fully delineate the precise relationship between MAP kinase and the physiological role of PRL in uterine leiomyoma SMC.

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