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Structural and Functional Effects of High Prolactin Levels on Injured Endothelial Cells

Evidence for an Endothelial Prolactin Receptor

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Stress has been linked to health problems such as atherosclerosis and prolonged wound healing, which involve the responses of injured endothelial cells. Though prolactin (PRL) levels become increased during the physiological response to stress, the significance and effects of these increases are largely unknown. Here we examined the effects of elevated, though physiological, concentrations of PRL on the responses of cultured endothelial cells after mechanical injury to cell monolayers. When treated at the time of injury with PRL levels of 62.5–1000 ng/mL, cells at the wound front became abnormal in shape and had reductions in f-actin staining in comparison to controls that were not PRL-treated. High PRL concentrations also inhibited the adhesion of cells to their growth surface in a dose-dependent manner. Using rhodamine-labeled PRL, we observed specific PRL uptake by these cells that suggested the presence of a PRL receptor. Finally, mRNA for the long form of the PRL receptor was detected by RT-PCR. To our knowledge, this is the first report demonstrating that (1) high PRL concentrations alter the actin cytoskeleton and adhesion of injured endothelial cells and (2) endothelial cells express the transcript for the PRL receptor. Thus, we report novel effects of PRL that may be mediated by activation of an endothelial cell PRL receptor.

Key Words: Prolactin; endothelial cell; actin cytoskeleton; prolactin receptor.

Introduction

PRL was originally described as an anterior pituitary hormone involved in breast growth during pregnancy and milk production post partum. Now PRL is considered to be a pleiotropic cytokine (reviewed in refs. *1* and *2*) that is released by many cell types *(3)* including lymphocytes *(4)* and endothelial cells *(5)*. The role of PRL as a cytokine is of interest because PRL has long been known to become elevated during the physiological response to stress exhibited by humans and other animals *(6–9)*. In humans, for example, serum levels are known to increase from 2 to 20 ng/mL to concentrations ranging from 80 to 350 ng/mL for days following surgical and emotional stress *(8)*, and levels over 1000 ng/mL have been reported. Despite documentation of its occurrence, neither the effects nor the significance of stress-induced PRL elevation have been elucidated *(10)*.

We are interested in the effects of stress hormones on injured endothelial cells because stress has been linked to health problems, such as atherosclerosis *(11,12)* and prolonged wound healing *(13,14)*, which involve the responses of endothelial cells to injury. There is limited evidence that PRL has effects on the vascular endothelium. A recent report *(15)* demonstrated that human PRL stimulated new capillary and blood vessel formation in the late stages of the chick chorioallantoic membrane angiogenesis assay, but proliferation of endothelial cells was unaffected. In other studies, PRL has been demonstrated to be an autocrine growth factor for bovine brain capillary endothelial cells, with both basal and bFGF-stimulated growth inhibited by anti-PRL antibodies *(16)*. Though endothelial cells are responsive to PRL in these model systems, identification of an endothelial cell PRL receptor has not yet been reported, and the role of this hormone in endothelial cell function remains unclear.

Several PRL-related proteins have been reported to regulate endothelial cell function and angiogenesis. Proliferin and proliferin-related protein exert opposing effects on angiogenesis *(17)*. Anti-angiogenic activity is also pro-

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duced by 16 kDa PRL, a proteolytic fragment of the 23 kDa PRL hormone *(18,19)*. In addition, other PRL/growth hormone (GH) family proteins and their fragments affect endothelial cells *(15)*. Therefore, endothelial cells are affected by several PRL/GH family proteins. However, the effects of 23 kDa PRL itself on these cells has received little experimental attention.

In preliminary studies on the responses of bovine pulmonary artery endothelial cells (BPAECs) to mechanical injury, we unexpectedly found that additions of PRL to culture medium caused marked changes in endothelial cell shape. In view of the importance of the vascular endothelium and the observations discussed above, we felt that 23 kDa PRL effects on endothelial cell function deserved further investigation. The purpose of the present study was twofold: to elucidate the effects of high PRL concentrations, such as induced by stress, on injured endothelial cells, and to determine if the known PRL receptor was present in endothelial cells, as suggested by the observed PRL effects following injury.

The results of the experiments reported here showed that addition of PRL altered several characteristics of endothelial cells after injury. These included changes in endothelial cell shape and disruption of the actin cytoskeleton. In addition, high PRL levels markedly inhibited cell–substrate adhesion. Finally, a specific PRL binding site was observed on these cells, and mRNA for the long form of the PRL receptor was detected by RT-PCR. Therefore, the effects reported here may have been mediated by the PRL receptor.

Results

Effects of Injury on Endothelial Monolayers

In this study, an in vitro injury model was used. Confluent BPAEC monolayers were injured with a pipet tip or cell scraper to produce cell-free wound spaces. After injury, cells were further incubated up to 16 h in the presence or absence of PRL.

In control Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (FCS), BPAECs grew to confluence with each cell having a peripheral actin band and few stress fibers, as shown in tetramethyl rhodamine isothiocyanate (TRITC)-phalloidin stained cells (Fig. 1A). Injury (Fig. 1B) created cell-free wound spaces (W) that were bordered by ridges of rounded cells (R). The rounded cells remained attached to uninjured portions of the monolayer (U). Four hours after injury (Fig. 1C), the rounded cells in the ridge had begun reattaching to the chamber slide and migrating to fill the wound space. Migrating cells typically appeared fan-shaped and contained numerous stress fibers. (The bright band in Fig. 1C is due to out-of-focus cells that were rounded from injury.) By 16 h after injury, most of the rounded cells had reattached and migrated into the wound space. Cell migration was characterized by a pattern of different cell morphologies (Figs. 1D,E). At the migration front, cells were fan-shaped and contained many stress fibers (Fig 1D, arrows). Though these cells were connected to each other, occasional gaps (G) were seen between them. The fan-shaped cells were connected to well-spread cells that contained prominent peripheral actin bands (arrowheads) and numerous stress fibers. The well-spread cells were attached to BPAECs that were elongated, pointed toward the wound space, and filled with stress fibers (Fig. 1E, arrows), unlike cells rounded from injury (R) or in uninjured portions of the monolayer (U).

PRL Effects on Endothelial Responses to Injury

When BPAECs were treated with PRL upon injury, several changes became apparent after TRITC-phalloidin staining. First, there were less stress fibers and reduced fluorescence intensity in cells at the migration front of samples treated with 62.5 ng/mL and higher PRL, as seen in Fig. 2C showing cells treated with 500 ng/mL PRL. Measured fluorescence intensity was significantly reduced ($p \leq 0.05$), with a 25–40% reduction in fluorescence intensity in samples treated with 62.5 ng/mL and higher as compared to controls (Fig. 2A). Quantitation of these changes is shown graphically in Fig. 3A. Please note that these data (and those in Fig. 6) are presented as box plots in which broken lines represent means, solid lines within boxes represent median values, ends of boxes represent 25th and 75th percentiles, and capped bars represent 10th and 90th percentiles (and not error bars). Secondly, cell shape was altered in the presence of added PRL. In the presence of 15.6 ng/mL, cells at the migration front appeared to be losing the fan shape that was typically seen in controls (compare Fig. 2A to 2B). After treatment with 500 and 1000 ng/mL PRL, cells at the migration front became elongated with thin processes and less prominent lamellipodia (compare Fig. 2A control to Fig. 2C, a 500 ng/mL PRL sample). The shape changes seen in PRL-treated samples were quantified by calculating area to perimeter ratios; it was found that samples treated with 500 and 1000 ng/mL PRL had significantly lower area to perimeter ratios when compared to controls (Fig. 3B). We also observed that there appeared to be more gaps between cells at the migration front when cells were treated with PRL. When gap to area ratios were analyzed, we confirmed that 250 ng/mL and higher concentrations of PRL demonstrated a dose-dependent increase in gap area between cells ($p \leq 0.05$, Fig. 3C). Thus, high concentrations of PRL appeared to cause morphological differences in BPAECs at the migration front 16 h after injury.

PRL Effects on Endothelial Cell Adhesion

Because reattachment of cells to the surface of slides was an early postinjury event likely to be affected by cytoskeletal changes such as those discussed above, we further examined the effects of PRL on BPAEC adhesion (Fig. 4). In these experiments, cells were harvested in the absence of trypsin to prevent loss of adhesion molecules and receptors, then replated into gelatin-coated 96-well

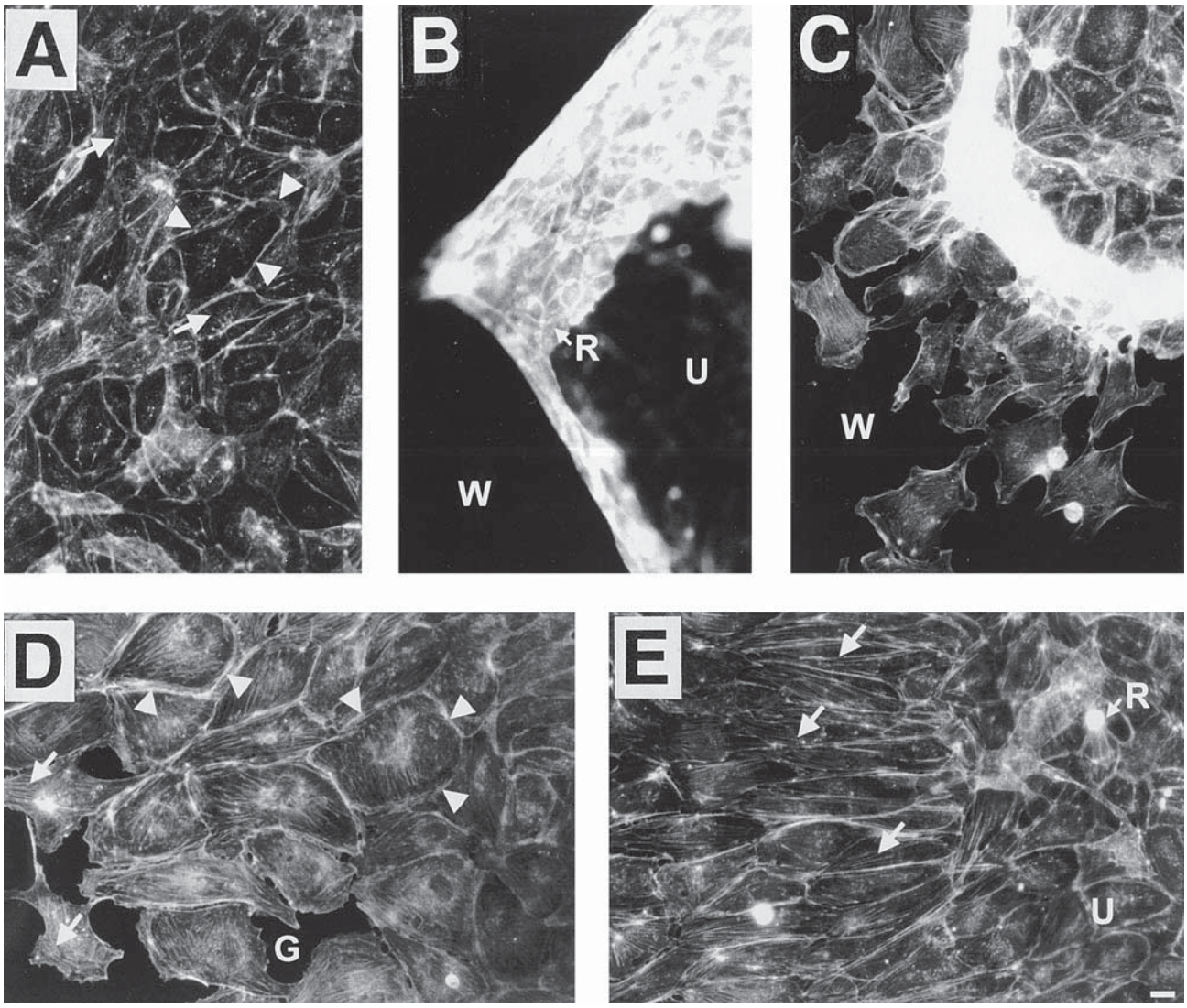

Fig. 1. Photomicrographs of TRITC-phalloidin stained BPAECs before (A) and after injury (B, 2 min; C, 4 h; and D and E, 16 h) in DMEM containing 10% FCS. In A, uninjured monolayers contained closely attached cells that are surrounded by peripheral actin bands (arrowheads) and have occasional stress fibers (arrows). In B, mechanical injury produced cell-free "wounds" (W) and rounded cells (R) that remained attached to uninjured portions of the monolayer (U). In C, by 4 h after injury, some of the rounded cells had reattached, spread, and migrated into the wound space (W). In D and E, at 16 h postinjury, most of the previously rounded cells were migrating into the wound. At the migration front (shown in D), there were rows of fan-shaped cells with stress fibers (arrows) and some gaps (G) between cells. Fan-shaped cells were attached to well-spread cells with prominent peripheral bands of F-actin (arrowheads). The spread cells were connected to linear-shaped cells, shown in E. These elongated cells were arranged in parallel with long-stress fibers (arrows) directed toward the wound space. Some cells still rounded from injury (R) remained attached to uninjured portions of the monolayer (U). In E, bar = 16 μm; A–E have identical magnifications.

plates in the presence and absence of added PRL. At various times thereafter, the plates were washed to remove nonadherent cells, and the remaining adherent cells were quantified using the MTS assay. As expected, attachment was rapid and time-dependent under all treatment conditions. However, PRL concentrations of 62.5, 500, and 1000 ng/mL caused a concentration-dependent decrease in attached cell numbers in comparison to controls at 45 min and 90 min post-plating ($p \leq 0.01$ by ANOVA). At 90 min, these differences were significant at $p \leq 0.05$ for each PRL concentration except 15.6 ng/mL (pair-wise multiple comparison). In this experiment, the maximum effect, 30% inhibition of adhesion was produced by 1000 ng/mL PRL.

Evidence for an Endothelial Cell PRL Receptor

Our observations described above strongly suggested the presence of a PRL receptor on these cells, but an endothelial cell PRL receptor has not been reported. Therefore, it was crucial to address this issue. In our initial experiments, we labeled PRL with the fluorescent dye, tetramethyl rhodamine (TMR), and determined that it had full biological activity in the Nb2 lymphoma PRL bioassay

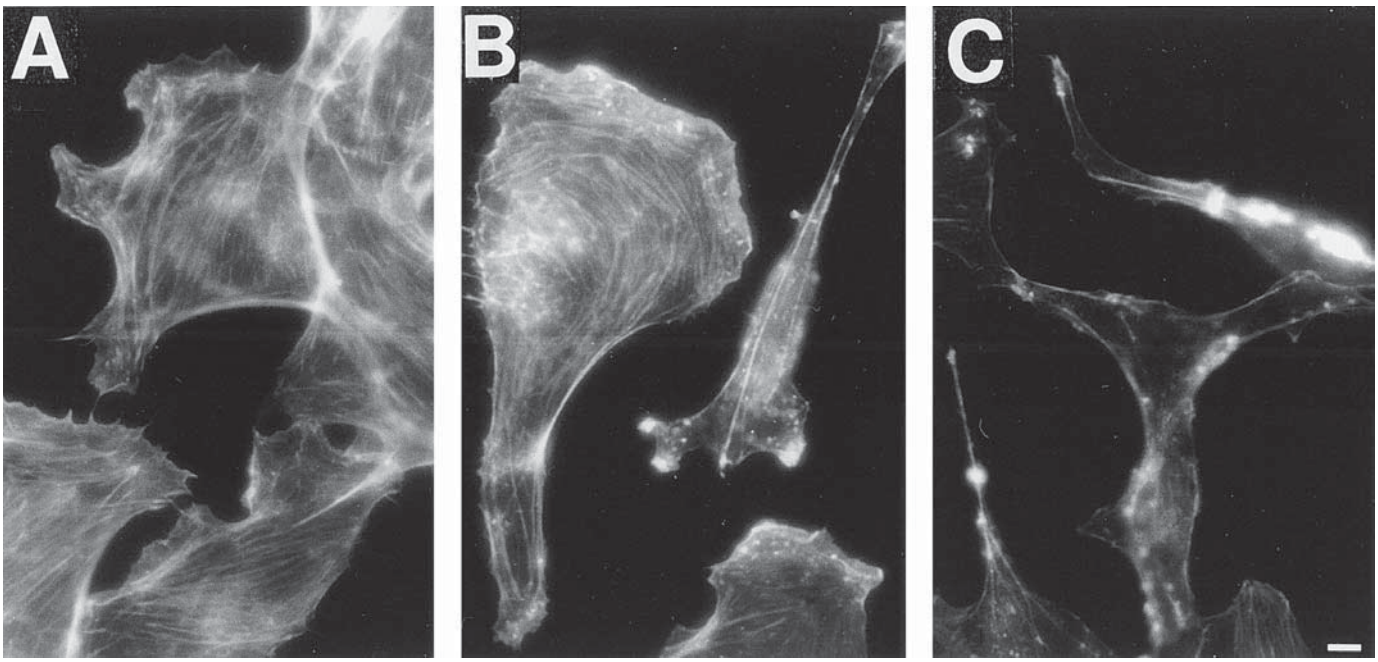

Fig. 2. Photomicrographs of TRITC-phalloidin stained BPAECs at the migration front 16 h after injury in the absence (A) and presence of added PRL (B 15.6 ng/mL; C 500 ng/mL). Figures have identical magnifications. The bar = 5 μm.

(data not shown). Experiments were then performed on BPAEC monolayers grown on gelatinized plastic chamber slides to determine if specific binding of tetramethyl rhodamine (TMR)-PRL occurred. When cells were incubated with 1 μg/mL TMR-PRL at 4°C, no cell surface binding was detectable by epifluorescence microscopy at 6 h. Longer times were not used because cells detached from the slides after 6 h (data not shown). When samples were incubated at 37°C for 16 h with 1 μg/mL TMR-PRL, cells internalized TMR-PRL producing very bright, punctate intracellular fluorescence (Fig. 5A). In adjacent wells that contained BPAECs incubated with 1 μg/mL TMR-PRL plus the addition of either a 25-fold (Fig. 5B) or 50-fold (Fig. 5C) excess of unlabeled PRL, the punctate fluorescence was markedly decreased. These observations were verified by image analysis in which the number of pixels having intensities greater than 50 arbitrary intensity units (AIU) were determined in digital images of BPAECs treated as described above (Fig. 6). In the presence of a molar excess of unlabeled PRL, the area (pixels) occupied by punctate bright spots was reduced by four- to five-fold in comparison to BPAECs incubated with TMR-PRL alone. This was not due to a non-PRL specific effect of added PRL protein, since all experiments were performed in the presence of 3 mg/mL BSA. These differences were statistically significant ($p \leq 0.01$, Kruskal–Wallis ANOVA on ranks). Comparison between individual treatment groups (pairwise multiple comparison method) demonstrated that competition of TMR-PRL with nonlabeled PRL resulted in marked and significant ($p \leq 0.05$) reduction of fluorescence at both 25- and 50-fold excess of unlabeled PRL. These data showed that BPAECS internalized TMR-PRL in a PRL-specific manner, and suggested the presence of a specific binding site for PRL on these cells.

Because our data did not address the identity of this PRL binding site, we sought to determine if the known PRL receptor is expressed in BPAECs using RT-PCR and Southern blot analysis to confirm PCR product identity. Total RNA from both uninjured and injured monolayers of these cells (Fig. 7) contained readily detectable transcripts for the long form of the PRL receptor, while the short form was not detected. Both forms were present in bovine cotyledonary cells (positive control), as previously reported *(20,21)*, and the negative control lane was empty, ruling out contamination as the source of PRL receptor signals. Although not shown, these bands were easily detected by ethidium bromide staining after 30 PCR cycles, indicating that PRL receptor transcripts are not exceptionally rare. Taken together, our data provide strong evidence for the presence of the long-form PRL receptor in BPAECs.

Discussion

We have demonstrated that treatment of injured endothelial cell monolayers with high, but physiologically occurring, concentrations of PRL, results in marked alterations of the cellular cytoskeleton and cell morphology at the migration front. These include a large decrease in f-actin and stress fibers, loss of the lamellipodia characteristic of motile cells, induction of oddly-shaped cells, and increased numbers of gaps between cells at the migration front after injury. PRL also inhibited attachment of cells to their growth surface. Taken together, these data support the

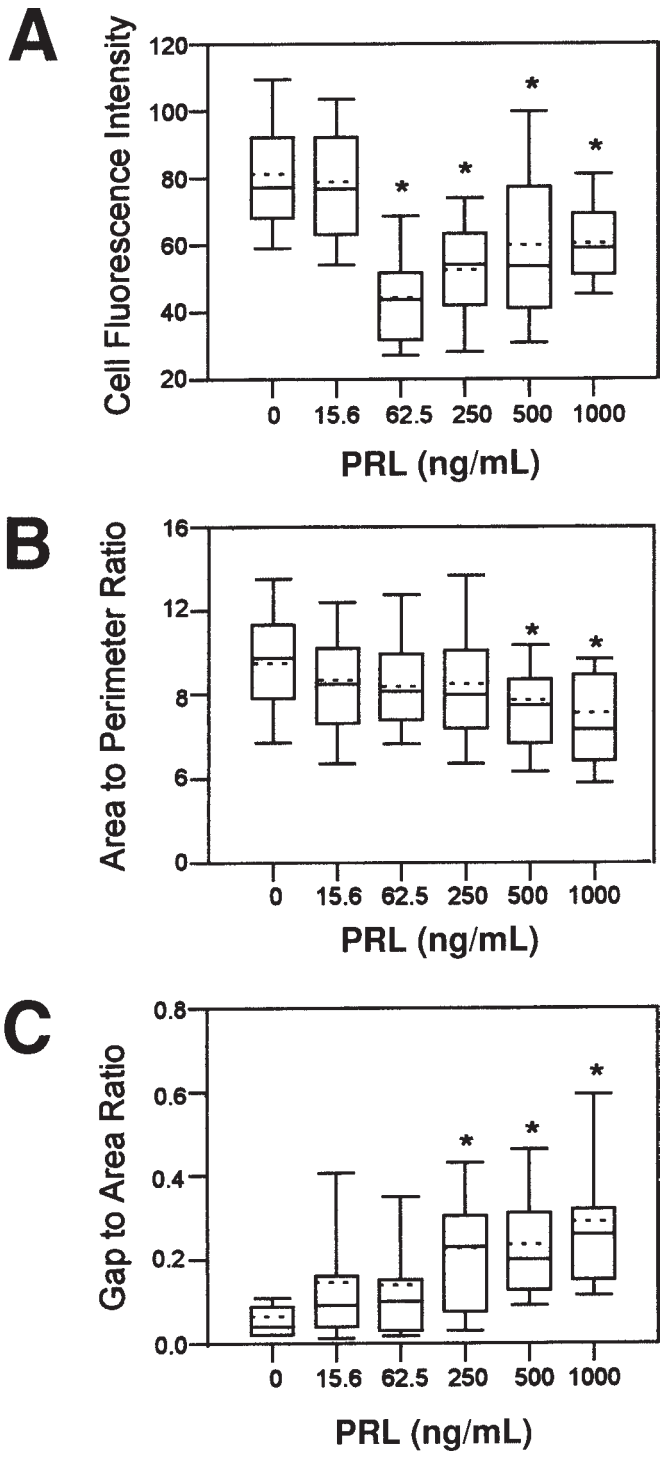

Fig. 3. The effects of PRL on BPAECs in an in vitro injury model. Data are presented as box plots in which broken lines represent means, solid lines within boxes represent median values, ends of boxes represent 25th and 75th percentiles, and capped bars represent 10th and 90th percentiles (and not error bars). Data were analyzed using Kruskal–Wallis ANOVA on ranks and Dunn's test to compare treatments to controls when indicated. Asterisks denote statistical significance ($p < 0.05$) between treatment and control. In A, PRL concentrations of 62.5 ng/mL and higher reduced the mean and median fluorescence intensity associated with TRITC-phalloidin staining in individual cells in the migration front, in comparison to no added PRL controls and 15.6 ng/mL PRL. Number of cells ranged from 57 to 70 per treatment group. In B, PRL reduced the mean and median area to perimeter ratios in cells of the migration front. Number of individual cells measured ranged from 36 to 41 per treatment group. In C, PRL increases mean gap area to total area ratios in digital images cropped to include cells one row back from the foremost cells to the elongated cells in the migration front. Number of images ranged from 10 to 28 per treatment group. Statistical significance was achieved between median values of controls and 250, 500, and 1000 ng/mL PRL samples.

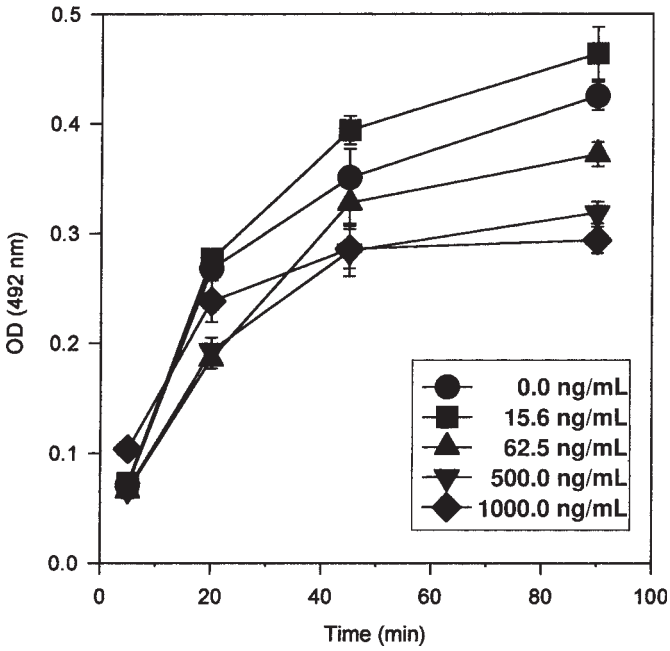

Fig 4. PRL inhibited BPAEC adhesion. Subconfluent cells were harvested without trypsin and plated in gelatinized 96 well culture plates in the presence or absence of PRL (0, 15.6, 62.5, 500, and 1000 ng/mL). At the times shown in the figure, nonadherent cells were removed by washing the wells, and the relative number of remaining adherent cells was determined using the MTS assay. Data are mean optical density (OD 492 nm) ± SE for six replicate wells at each condition.

hypothesis that high PRL levels alter responses to injury in this model. Moreover, we have identified the long form of the PRL receptor as a potential mediator of these PRL effects by demonstrating that a specific PRL binding site is present and that mRNA for the PRL receptor protein is expressed. To our knowledge, this is the first report of these effects of 23 kDa PRL on endothelial cells or of the detection of PRL receptor mRNA in this cell type.

Our data provide three lines of evidence in support of an endothelial cell PRL receptor. First, we report several strong effects of this hormone that are PRL-concentration dependent. However, this is circumstantial evidence for a receptor. Second, we found that TMR-PRL was internalized by BPAECs at 37°C. Addition of a large excess of unlabeled PRL competed with TMR-PRL to markedly decrease TMR-PRL uptake. Therefore, the binding site

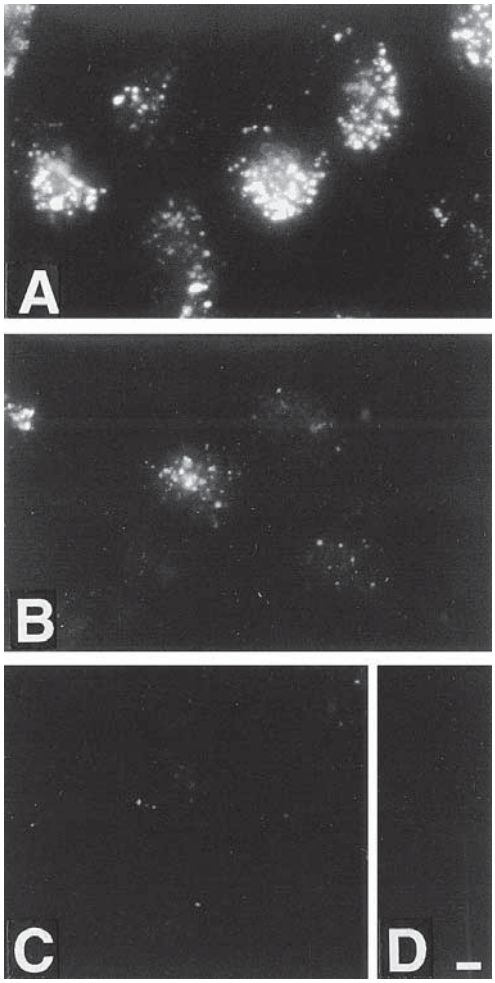

Fig. 5. Photomicrographs of BPAECs incubated in the presence of 1 μg/mL TMR-PRL and no unconjugated (UC)-PRL (A), 1 μg/mL TMR-PRL and 25 μg/mL UC-PRL (B), 1 μg/mL TMR-PRL and 50 μg/mL UC-PRL (C), and no TMR-PRL (D). After addition of PRL, samples were incubated for 18 h at 37°C. Figures A–D have identical magnifications; in D bar = 4.8 μm.

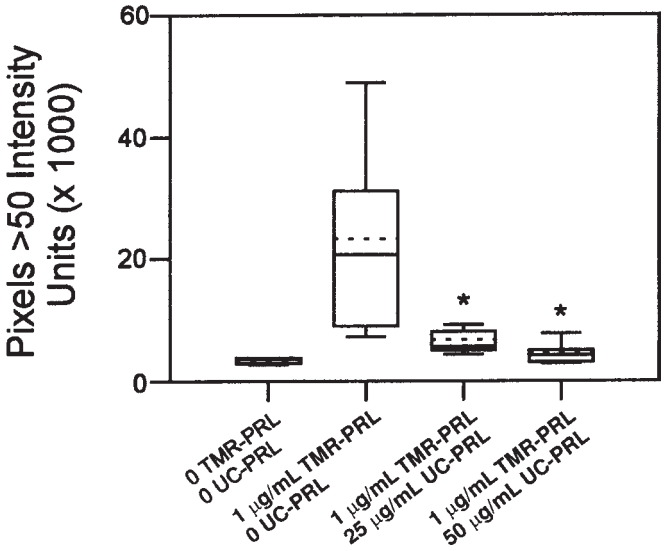

Fig. 6. Uptake of TMR-PRL by BPAECs. Median values for number of pixels greater than 50 AIU are greater in BPAECs incubated in the presence of 1 μg/mL TMR-PRL with no UC-PRL than in cells incubated in the presence of 1 μg/mL TMR–PRL with molar excess of UC-PRL (either 25 or 50 μg/mL). Kruskal–Wallis ANOVA on ranks gave $p < 0.05$. Asterisks represent statistical significance ($p < 0.05$) in comparison to 1 μg/mL TMR-PRL with no UC-PRL sample values using Dunn's test.

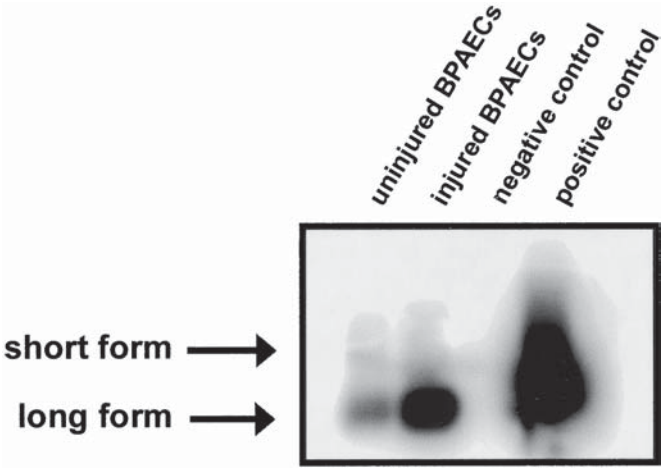

Fig. 7. Identification of PRL receptor mRNA in endothelial cells. Total RNA from injured and uninjured endothelial cell monolayers and bovine placental cotyledonary cells (positive control) were amplified by RT-PCR as described in Methods. Reactions run without the addition of RNA served as negative controls. PRL receptor cDNA products were detected by Southern blotting with the full-length bovine PRL receptor cDNA. The locations of the short (313 bp) and long (274 bp) form cDNA products are shown on the left.

mediating uptake was PRL-specific, a key characteristic of receptors. Internalization is also consistent with previous work showing that PRL bound to its receptor is endocytosed via a mechanism involving clathrin-coated pits *(22–24)*. Internalized TMR-PRL appeared as small, intensely fluorescent spots with a submembrane distribution, suggesting its presence in endocytic vesicles. We were unable to detect cell surface binding of TMR-PRL after incubation at 4°C to prevent internalization. However, this was likely due to a low level of PRL receptor expression on the cell surface, a common observation in PRL target tissues. Third, and most important, we detected mRNA for the long form of the PRL receptor in these cells by RT-PCR, and confirmed its identity by Southern blotting. This procedure is highly specific *(20)*, because each step (reverse transcription, PCR, Southern blotting) uses a different PRL receptor-specific DNA primer or probe to recognize the receptor sequence. Therefore, our conclusion that long form PRL receptor transcripts are present in these cells is strongly supported. It is noteworthy that the PRL receptor band was markedly stronger in samples from injured than from uninjured BPAECs, even though an equal amount of RNA was used in each reaction. Although the RT-PCR method used was not quantitative,

this suggests that investigating the effects of injury on PRL receptor expression by these cells is warranted.

Taken together, our data suggest that the PRL effects reported here may have been due to its interaction with the long form of the PRL receptor. Another explanation is that a novel receptor for PRL is present in these cells, with the PRL receptor transcript reported here being irrelevant. This appears a less likely explanation for our findings, since no such alternate PRL receptor gene or protein has been found in any species or tissue. While a specific endothelial cell binding site for the 16 kDa PRL fragment has been reported, this site does not bind the 23 kDa PRL *(25)*. It is unlikely that our results resulted from in vitro production of the 16 kDa PRL from 23 kDa PRL, since this requires the presence of a specific protease and disulfide bond reduction to free the active fragment *(26,27)*. Moreover, the 16 kDa PRL did not produce cytoskeletal effects in our system (data not shown).

The effects of PRL on the endothelial cytoskeleton and cell adhesion were surprising, in view of the limited data showing functional effects of PRL on these cells, and the absence of published reports showing similar PRL effects in any cell type. Therefore, our observations demonstrate new actions of this hormone. However, other hormones and cytokines have been shown to alter the actin cytoskeleton in various types of cells including endothelial cells *(28–30)*. Moreover, the PRL receptor is a cytokine receptor that activates JAK/STAT signaling pathways *(1,2)* now known to be present in endothelial cells *(31–33)* and to mediate cytokine actions. Tumor necrosis factor alpha, for example, causes endothelial cell cytoskeletal disassembly similar to the changes we have reported here *(34)*. Loss of the endothelial permeability barrier also occurs, likely due to dissolution of tight contacts between these cells *(35)*, causing vascular leak syndrome in vivo. Marked PRL-induced cell shape changes occurred in our studies. Similar, but less profound, effects are also produced by interleukin 1 *(36,37)* and interferon gamma *(36)*. Therefore, alterations in the endothelial cell cytoskeleton are recognized actions of several cytokines. Growth hormone (GH), also a cytokine and a hormone whose receptor closely resembles the PRL receptor *(1,2)*, induces actin depolymerization and loss of stress fibers in Chinese hamster ovary cells that have been transfected by the GH receptor *(28)*. These changes were dependent on phosphatidylinositol 3-kinase activation and calcium influx, pathways also activated by the PRL receptor *(38–40)*. This is a potential mechanism for the PRL-induced cytoskeletal changes reported here.

PRL also caused a concentration-dependent decrease in cell adhesion to their growth surface. The process of cell reattachment, an important early event in our in vitro injury model, is known to be mediated by the integrins in association with the focal adhesion complex and the actin cytoskeleton (reviewed in *refs. 41* and *42*). The focal adhesion complex is an assembly of multiple intracellular proteins that regulate cell attachment to the extracellular matrix, affect cell shape, and control motility. This is a dynamic complex capable of forming and dissociating under the control of extracellular signals. The protein tyrosine kinase, focal adhesion kinase (FAK), is a key regulator of focal adhesion complex turnover that has been reported to be altered by PRL treatment of human breast cancer cells (*43*). Low levels of PRL stimulated increased tyrosyl phosphorylation of FAK, increasing its activity and the phosphorylation of its substrate paxillin, also an important component of the focal adhesion complex. In contrast, high concentrations of PRL (100 ng/mL) such as those used here decreased these events *(43)*, effects known to disrupt the cytoskeleton and reduce the number of focal adhesions mediating cell–substrate attachment *(41,42)*. We have recently found that PRL stimulation of endothelial cell monolayers results in marked inhibition of paxillin tyrosyl phosphorylation and loss of this protein from the focal adhesion complex *(44)*. Therefore, PRL action on the FAK signaling pathway may have caused disruption of the focal adhesion complexes in endothelial cells, producing the decreased cell–substrate adhesion reported here.

The PRL-induced changes in endothelial cells after injury reported here have possible physiological relevance, particularly in view of the high levels of circulating hormone found during stress *(6–9)*. Hyperprolactinemia may alter endothelial barrier function as a result of gap formation between cells. In addition, high PRL levels could adversely affect the ability of these cells to engage in angiogenesis by altering cytoskeletal structures required for motility or by decreasing cell–substrate adhesion. These effects of PRL could potentially contribute to altered health states such as delayed wound closure and progression of atherosclerosis. It is also possible that elevated PRL levels, reported to be involved in autoimmune diseases such as lupus erythematosis *(45)*, may play a role in the vascular dysfunctions that occur in these diseases. Future studies will evaluate some of these possibilities. However, the physiological response to stress is a complex process that involves altered levels of many hormones and cytokines. Hence, the effects of PRL elevation in vivo are likely to be the end result of multiple factors acting in concert. Nevertheless, the findings reported here increase our understanding of PRL as a cytokine and further establish that the vascular endothelial cell is a target for PRL action.

Materials and Methods

Cells and Materials

Bovine pulmonary artery endothelial cells (BPAECs), previously described in detail *(46)*, were used between passages 9 and 20. These cells tested negative for *Mycoplasma* infection using the Mycofluor™ Mycoplasma Detection Kit (Molecular Probes; Eugene, OR). For the experiments, BPAECs were sparsely plated and grown to

confluence in either fibronectin-coated, gelatinized two-well glass chamber slides or gelatinized plastic ware. BPAECs were grown and studied in Dulbecco's modified Eagle's medium (DMEM; Sigma Chemical Co., St. Louis, MO) containing 10% fetal calf serum (FCS), penicillin (100 units/mL), streptomycin (0.1 mg/mL), and gentamicin (50 μg/mL). Two sources of FCS were used. Serum obtained from HyClone™ Laboratories, Inc. (Logan, UT) was used in most of the studies reported here. This FCS was assayed for lactogenic activity by the Nb2 node lymphoma bioassay *(47)* and found to contain between 20 and 50 ng/mL PRL, depending upon the lot. Medium using this serum contained 2–5 ng/mL PRL. Serum obtained from Bio-Whittaker (Walkersville, MD) was lot-specific (lot 8S066F) and contained 0.7 ng/mL PRL by ELISA assay (Holly Brown-Borg, personal communication). Medium prepared using this FCS contained 0.07 ng/mL PRL ("low PRL" medium).

Purified bovine PRL was obtained from the National Hormone and Pituitary Program (NIH NIDDK). Stock solutions were passed through a Detoxi-Gel column (Pierce Chemical Co., Rockford, IL) following the manufacturer's protocol to ensure an endotoxin-free PRL preparation. The absence of endotoxin was confirmed by the Limulus amebocyte assay (E-TOXATE™, Sigma Chemical Co.), following kit instructions. PRL was stored frozen at –20°C and thawed immediately prior to use.

Both tetramethylrhodamine isothiocyanate (TRITC)-phalloidin and tetramethyl rhodamine (TMR) were obtained from Molecular Probes (Eugene, OR). Formaldehyde was obtained from Electron Microscopy Sciences (Fort Washington, PA) and was a grade suitable for electron microscopy. Other chemicals used were reagent grade and were purchased either from Sigma Chemical Co. or J.T. Baker (Phillipsburg, NJ).

Preparation of TMR-PRL

PRL was conjugated to TMR using the FluoReporter™ labeling kit from Molecular Probes following the manufacturer's protocol. First, PRL was reacted with a 20:1 molar excess of TMR for 1.25 h. After the reaction was stopped, free TMR was removed with a desalting column (D-Salt column, Pierce Chemical Co.) that had been preequilibrated with HEPES storage buffer containing 0.025 *M* HEPES, 0.15 *M* NaCl, pH 8.0. Column fractions (1 mL) were collected and the absorbance of each fraction measured spectrophotometrically at 280 nm and 555 nm (TMR absorbance peak) to permit calculation of the protein concentration and the conjugation ratio of TMR:PRL. To confirm that PRL was not degraded and that free TMR was removed, 1 μg of PRL from each fraction was electrophoresed by nonreducing 12.5% SDS-PAGE, and the fluorescent protein observed with a UV transilluminator. Two fractions were selected for use and pooled. The final conjugation ratio of these was 1.30 molecules of TMR/molecule of PRL. The conjugate was aliquoted and stored at –20°C until used. The Nb2 lymphoma PRL assay *(47)* was used to assess the bioactivity of the TMR-PRL conjugate in comparison to the unlabeled hormone from the same shipment.

Injury Model and TRITC-Phalloidin Staining Experiments

For the injury model, BPAECs were grown to confluence in gelatinized glass chamber slides. Four days after reaching confluence, the monolayers were mechanically injured using a sterile pipet tip and returned to the incubator. At various times after injury (2 min, 4 h, and 16 h), injured monolayers were fixed in 4% formaldehyde, permeabilized in 0.15% Triton X-100, and incubated in TRITC-phalloidin (1 unit/mL normal saline). After mounting in Vectashield (Vector Laboratories, Inc., Burlingame, CA) slides were viewed by epifluorescence microscopy (Leica Laborlux S). Photographs were taken at standard settings using a 35 mm camera and Kodak T-Max 400 film. Film was processed and printed at set conditions for each experiment.

To test the effects of added PRL, BPAECs were injured in DMEM with 10% FCS without added PRL as controls, and 15.6, 62.5, 250, 500, and 1000 ng/mL PRL in the treatment groups. Sixteen hours later, samples were fixed, TRITC-phalloidin stained, photographed, and printed as described.

Cell Adhesion Assay

Flasks of sub-confluent BPAECs were harvested in Hank's solution containing EDTA (200 mg/L) in the absence of trysin. After centrifugation, the cell pellet was resuspended in DMEM with 10% low PRL FCS, and the cell concentration adjusted to 1.5×10^5 cells/mL. BPAECs in 100 μL aliquots were added to gelatinized 96-well flat bottom plastic culture plates containing 100 μL/well of DMEM with 10% low PRL FCS with and without the addition of PRL to produce final PRL concentrations of 0, 15.6, 62.5, 250, 500, or 1000 ng/mL (six wells per concentration). BPAECs were incubated and allowed to settle for 5, 20, 45, and 90 min. At the end of the settling times, wells were gently washed using phosphate-buffered saline (PBS) containing 5% horse serum for 5–7 s with a hand-held plate washer (Skatron Instruments, Sterling, VA). Next, wells were aspirated to dryness with the plate washer and 100 μL fresh DMEM with 10% low PRL FCS added. The number of remaining, adherent cells was determined using the Cell Titer™ 96 Aqueous MTS cell proliferation assay (Promega, Madison, WI) following the manufacturers's protocol. Plates were read at 492 nm wavelength using a Tecan SpectraFluor plate reader (Tecan US, Research Triangle Park, NC). These data were analyzed by one-way analysis of variance (ANOVA) and the Student–Neuman–Keuls method for pair-wise multiple comparisons

using SigmaStat™ (Jandel Scientific Software, San Rafael, CA). Statistical significance was set at $p \leq 0.05$.

TMR-PRL Experiments

For these experiments, cells were grown to confluence in chamber slides, then treated without injury. The culture medium was changed to serum-free DMEM containing 0.3% bovine serum albumin (BSA). After a 1 h preincubation, BPAECs were further incubated in either 0.3% BSA in DMEM alone, or 0.3% BSA in DMEM containing one of the following: 1 μg/mL TMR-PRL with no added unconjugated PRL (UC-PRL), 1 μg/mL TMR-PRL with 25 μg/mL UC-PRL, or 1 μg/mL TMR-PRL with 50 μg/mL UC-PRL. Cells were incubated at either 4°C or 37°C for 6–16 h. After washing to remove unbound TMR-PRL, samples were formaldehyde-fixed and mounted for microscopy. Pictures were taken using the 100× objective at 60 s exposures.

Data Collection and Analysis

Photographic prints were laser scanned (Hewlett Packard ScanJet 4c), then digital images were analyzed using SigmaScan/Image™ (Jandel Scientific Software). For cell shape evaluation, images of individual cells were first traced on the video screen using the computer mouse. Then perimeter and area values were determined in pixels and used to calculate perimeter to area ratios. Average intensity values were measured in TRITC-phalloidin stained BPAECs at the migration front by positioning "squares" over individual cells to determine average intensity within the measurement field. The area of gaps between cells and total area were determined and were used to calculate ratios of gap to total area ratios. Data from three experiments were pooled by treatment group. Digital image analysis of pixels having intensities greater than 50 arbitrary intensity units (AIU; scale ranged from 0 for "darkest black" to 255 for "brightest white") was used to quantify uptake of TMR-PRL in the presence and absence of UC-PRL in confluent BPAECs.

The Kruskal–Wallis one-way ANOVA on ranks in SigmaStat™ was used to analyze these data, which departed from normality. When treatment effects were detected, data were further analyzed using Dunn's test as a multiple comparison method. Box plots were used to present data and included means, medians, 25th and 75th percentile values presented as box ends, and 10th and 90th percentiles as capped bars.

Detection of PRL Receptor mRNA

BPAECs were grown to confluence on gelatin-coated 75 cm^2 flasks. Postconfluent (2–4 d) BPAECs were either injured twice making a "V" shape in the monolayer using a 1.8-cm-wide cell scraper or were studied without injury. After an additional 16 h incubation, cells were washed twice with ice-cold PBS and removed with a rubber policeman. After centrifugation, total RNA was extracted from pellets using the guanidinium isothiocyanate method (RNA Isolation Kit, Stratagene, La Jolla, CA). RNA quality was assessed by spectrophotometry (260/280 ratios) and by agarose gel electrophoresis.

RT-PCR was employed to examine BPAECs for PRL receptor transcripts to distinquish those encoding the "short" and "long" isoforms, as previously described *(20)*. In brief, PRL receptor-specific mRNA was reverse-transcribed with a 12-mer primer complementary to both forms of the PRL receptor. A portion of the cytoplasmic domain of both long and short form transcripts was amplified for 30 cycles from reverse transcriptase products derived from 750 ng total RNA. PCR primers flanked the insertion site of the 39 bp sequence containing a premature stop codon that creates the short form of the receptor in ruminant species *(21)*. This procedure yields PCR products of 274 bp from amplification of long form transcripts, and 313 bp from the short form of the receptor. Southern analysis using the full-length PRL receptor cDNA was used to confirm the identity of the amplified products. Southern blots were imaged using a Fluoroimager and ImageQuant™ software (Molecular Dynamics, Sunnyvale, CA).

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