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16K-Prolactin Inhibits Activation of Endothelial Nitric Oxide Synthase, Intracellular Calcium Mobilization, and Endothelium-Dependent Vasorelaxation

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Activation of endothelial nitric oxide synthase (eNOS) and subsequent nitric oxide production (NO) are events that mediate the effect of important angiogenic, vasopermeability, and vasorelaxation factors, including vascular endothelial growth factor (VEGF), bradykinin (BK), and acetylcholine (ACh). The N-terminal 16-kDa fragment of prolactin (16K-PRL) acts on endothelial cells to inhibit angiogenesis both *in vivo* and *in vitro*. Here, we show that 16K-PRL inhibits VEGF-induced eNOS activation in endothelial cells. Inhibition of eNOS activation may mediate the antiangiogenic properties of 16K-PRL, because the NO donor (Z)-1-[2-(2-aminoethyl)-*N*-(2-ammonio-ethyl)amino]diazen-1-ium-1,2-diolate (DETANONOate) prevented 16K-PRL from blocking the VEGF-induced proliferation of endothelial cells. In addition, 16K-PRL inhibited eNOS activation by BK and blocked the BK-evoked transient increase in intracellular Ca^{2+} in endothelial cells. This finding suggests that 16K-PRL interferes with the mobilization of intracellular Ca^{2+}, thereby inhibiting the Ca^{2+}-dependent activation of eNOS. Blockage of eNOS activation can lead to inhibition of vasodilation. Consistently, 16K-PRL inhibited BK-induced relaxation of coronary vessels in isolated perfused guinea pig hearts. Moreover, 16K-PRL inhibited eNOS activation induced by ACh, and this action resulted in the inhibition of both ACh-evoked relaxation of coronary vessels in isolated perfused rat hearts and ACh-induced, endothelium-dependent relaxation of rat aortic segments. In conclusion, 16K-PRL can block the Ca^{2+}-mediated activation of eNOS by three different vasoactive substances, and this action results in the inhibition of both angiogenesis and vasorelaxation. (*Endocrinology* 145: 5714–5722, 2004)

THE OUTGROWTH OF new blood vessels is associated with tissue repair after injury and inflammation and is an important mechanism underlying human diseases such as cancer, diabetic retinopathy, rheumatoid arthritis, and heart disease. Several angiogenesis inhibitors have been described, some of which are fragments of larger proteins that themselves lack inhibitory activity. Among these protein fragments are angiostatin, an internal fragment of plasminogen (1); endostatin, a fragment of collagen XVIII (2); a fragment of antithrombin (3); and the N-terminal 16-kDa fragment of prolactin (16K-PRL) (4).

The role of 16K-PRL as an antiangiogenic agent has recently attracted much attention (5–7). This proteolytic product of PRL acts as a potent inhibitor of angiogenesis *in vivo* (4, 8) and *in vitro*, inhibiting the proliferation of endothelial cells (4) and stimulating the expression of type-1 plasminogen activator inhibitor (9) and the apoptosis (10) of endothelial cells. Some of the molecular mechanisms underlying these actions have been investigated and include the inhibition of the MAPK pathway (11, 12) and the activation of the caspase pathway via nuclear factor-κB (13). However, as for many other antiangiogenic proteolytic fragments (14), the receptors for 16K-PRL have not been identified (15), and the signaling molecules that mediate the antiangiogenic activity of 16K-PRL remain poorly characterized (5).

It was recently proposed that inhibition of nitric oxide (NO) production plays an important role in mediating the antiangiogenic activity of endostatin (16) and results in the inhibition of vasodilation by angiostatin (17). NO is a gaseous free radical with multiple biological functions throughout the body. In blood vessels, NO is produced mostly by the endothelial isoform of NO synthase (eNOS) (18), a constitutive, Ca^{2+}-dependent enzyme. eNOS-derived NO is released in response to various chemical and physical stimuli and acts as a principal endothelium-derived relaxing factor and as a mediator of vasopermeability and angiogenesis (19, 20). Strong evidence for the role of endothelial NO in angiogenesis comes from the observation that eNOS mediates

Abbreviations: ACh, Acetylcholine; BK, bradykinin; BUVEC, bovine umbilical vein endothelial cell; eNOS, endothelial nitric oxide synthase; FCS, fetal calf serum; HUVEC, human umbilical vein endothelial cell; 16K-PRL, N-terminal 16-kDa fragment of prolactin; RRCEC, rat retinal capillary endothelial cell; VEGF, vascular endothelial growth factor.

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the proangiogenic activity of VEGF (21–26) and from studies showing attenuated angiogenesis in eNOS knockout mice (27) or after treatment with pharmacological and natural inhibitors of eNOS (16, 28).

We hypothesized that 16K-PRL may inhibit eNOS activation, which would account for some of its effects on angiogenesis. This hypothesis is based on observations that 16K-PRL blocks the mitogenic effect of VEGF on endothelial cells (4), a VEGF effect requiring NO (21–23), and that the action of other antiangiogenic factors, such as endostatin (16) and angiostatin (17), are mediated by the inhibition of eNOS activation. Here, we investigate whether 16K-PRL can inhibit eNOS activity triggered by three different angiogenic and vasodilatory factors. Our findings reveal that 16K-PRL inhibits eNOS activity by blocking intracellular Ca^{2+} mobilization, the primary mechanism controlling eNOS activation. Furthermore, we show that inhibition of eNOS by 16K-PRL contributes to its antiangiogenic properties and results in novel actions of this peptide on vascular tone.

Materials and Methods

Materials

Rat pituitary PRL was from the National Hormone and Pituitary Program, and human recombinant PRL was donated by Genzyme Corp. (Framingham, MA). 16K-PRL was generated by enzymatic proteolysis of rat PRL with a particulate fraction from rat mammary glands, gel filtration, and carbamidomethylation, as described (29). Human 16K-PRL was generated by site-directed mutagenesis using a baculovirus expression system (30). Vascular endothelial growth factor (VEGF) was a gift from Genentech (South San Francisco, CA), and basic fibroblast growth factor was from Scios Inc. (Mountain View, CA). Bradykinin (BK), acetylcholine (ACh), sodium nitroprusside, and polymyxin-B were from Sigma Chemical Co. (St. Louis, MO).

Cell cultures

Human and bovine umbilical vein endothelial cells (HUVECs and BUVECs) were obtained as previously described (31). HUVECs were cultured in M199 with 20% fetal calf serum (FCS), 100 μg/ml porcine heparin, 50 U/ml penicillin/streptomycin, and 25 μg/ml endothelial cell growth supplement (Sigma); BUVECs were cultured in F12K medium with 10% FCS and 50 U/ml penicillin/streptomycin. Rat retinal capillary endothelial cells (RRCECs) were obtained and cultured in DMEM with 10% FCS, 100 μg/ml porcine heparin, 2 ng/ml basic fibroblast growth factor, and 50 U/ml penicillin/streptomycin, as previously described (32).

L-Citrulline assay

eNOS activity was measured by conversion of [^{3}H]L-arginine to [^{3}H]L-citrulline using the method previously described (33). Briefly, cells at approximately 80% confluence were incubated in Hanks' medium with additives [20 mM HEPES (pH 7.4), 0.6 mM $CaCl_2$, trasylol (0.2 IU/ml), and 1 mM dithiothreitol] and allowed to equilibrate for 30 min. The reaction was initiated by addition of 1 μCi/ml [^{3}H]L-arginine (PerkinElmer Life Sciences, Inc., Boston, MA) in the presence or absence of VEGF (2.4 nM), BK (10 μM), or ACh (10 μM) with or without 0.1–10 nM 16K-PRL or PRL. Cell stimulation was for 1 h at 37 C and terminated by aspiration of the treatment medium and addition of ice-cold stop medium (50 mM HEPES, pH 5.5, and 4 mM EDTA), followed by chilling on ice for 10 min and sonication. Cell lysates were applied onto 1-ml columns of Dowex AG50WX8 (Na^+-form, Bio-Rad Laboratories, Hercules, CA), and [^{3}H]L-citrulline was eluted with 1 ml water and quantified by liquid scintillation counting.

Endothelial cell proliferation assay

The proliferative effects of VEGF and of 16K-PRL were determined in BUVECs. Briefly, BUVECs were seeded at 5000 cells/cm^2 in complete culture medium and allowed to attach for 3–4 h. Cells were then serum starved for 24 h with 0.5% FCS-culture medium, and the medium was then replaced with complete 10% FCS-culture medium with or without 10 μM (*Z*)-1-[2-(2-aminoethyl)-*N*-(2-ammonio-ethyl)amino]diazen-1-ium-1,2-diolate (DETANONOate) (Alexis Corp., San Diego, CA). After 30 min, 0.6 nM VEGF with or without 10 or 20 nM 16K-PRL was added to the cultures. Cells were allowed to proliferate for 24 h and were pulsed for the last 12 h with 0.6 μCi [^{3}H]thymidine per 15-mm well, as described (4).

Intracellular Ca^{2+} measurements

Ca^{2+} measurements were performed on an Aminco-Bowman Series-2 luminescence spectrometer using a 150-W xenon source (Rochester, NY) and the fluorescent calcium probe fura 2-AM (Molecular Probes, Eugene, OR) as described (34). For the studies with cell suspensions, endothelial cells were mechanically dispersed with a plastic pipette after a 2-min incubation with 0.01% trypsin solution, centrifuged, and resuspended in Ringer's solution to a final concentration of 10^6 cells/ml. For Ca^{2+} determination in perfused monolayers, cells were plated on glass coverslips coated with poly-D-lysine, placed in 60-mm dishes, and cultured until they reached confluence. Cell suspensions or monolayers were loaded for 1 h with 1 μM fura 2-AM in Ringer's solution at room temperature. They were then washed three to four times in PBS and placed in static (cellular suspensions) or perfused (cellular monolayers) incubation under constant agitation with Ringer's solution with or without BK (10 μM) in the presence or absence of 16K-PRL (10 nM). Recording used alternate excitation at 340 and 380 nm alternating at 1-sec intervals, and emitted light was measured with a photomultiplier. From the ratio of emission at 520 nm detected at the two excitation wavelengths and by comparison with a standard curve established for the same settings using buffers of known free [Ca^{2+}], the free [Ca^{2+}] was calculated in real time as described (35).

Relaxation of coronary vessels

Animal care and treatment were according to the Institutional Guidelines of the Neurobiology Institute of the National University of Mexico. Male English short-hair guinea pigs and Wistar rats (350–400 g) were anesthetized with an ip injection of pentobarbital (50 mg/kg) and heparin sodium salt (500 U). The animals were artificially ventilated, the chests were opened, and a loose ligature was passed through the ascending aorta. The hearts were rapidly removed, immersed in ice-cold physiological saline, and perfused in a retrograde manner via a nonrecirculating perfusion system at a constant flow, as previously indicated (36). Coronary flow was adjusted with a variable-speed peristaltic pump (Harvard Apparatus, model 55-1762, Holliston, MA). After an initial equilibration period, experiments were begun, and all hearts were perfused at a coronary flow of 10 ml/min. The perfusion medium was Krebs-Henseleit solution (pH 7.4) with the following composition (in mM): 117.8 NaCl, 6 KCl, 1.75 $CaCl_2$, 1.2 $MgSO_4$, 1.2 NaH_2PO_4, 24.2 $NaHCO_3$, 5 glucose, and 5 sodium pyruvate. The solution was equilibrated with 95% O_2/5% CO_2 and kept at 37 C. Because all experiments were performed at a constant coronary flow, the coronary vascular resistance was estimated from measurements of the coronary perfusion pressure, which was recorded continuously via a side arm of the perfusing cannula. Either 1 or 10 μM BK or ACh was applied in 30-sec intracoronary infusions, before or after a 30-sec infusion of 10 nM 16K-PRL, and the effects evaluated by perfusion pressure. In this system, perfusion pressure is an index of vascular tone (36).

Vascular relaxation of rat aortic segments

Sprague Dawley male rats (200–300 g) were killed by overdose injection of pentobarbital in accordance with animal protocols approved by the Animal Care and Use Committee of the University of California, Davis. Experiments were performed as previously described (37). Upon sacrifice, the aorta was excised, cleansed of adhering tissue, and cut in 3- to 4-mm-wide segments. Individual rings were suspended from a

Radnoti isometric transducer in oxygenated tissue baths containing bicarbonate-buffered Krebs-Henseleit solution (118 mM NaCl, 4.6 mM KCl, 27.2 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 1.75 mM $CaCl_2$, 0.03 mM Na_2EDTA, and 11.1 mM glucose, pH 7.4). A passive load of 2 g was applied, and the aortic segments were allowed to equilibrate for approximately 1 h with frequent readjustment of tension. KCl-induced (70 mM KCl) contractions were elicited in indomethacin (5 μM) to determine the maximal contractile capacity. Rings were washed and allowed to equilibrate for 40 min. Rat aortic vessels were incubated in the presence or absence of 10 nM enzymatically generated rat 16K-PRL or rat PRL for 30–45 min, and then vessels were contracted with phenylephrine (50 nM). When tension development reached a plateau, ACh (0.1 nM to 1 μM) was added to invoke endothelial cell-dependent relaxation. Sodium nitroprusside (0.1–100 nM) was used as a control for endothelium-independent relaxation. Real-time data were collected and analyzed using PowerLab software (ADI Instruments, Colorado Springs, CO).

Statistical analysis

All results were replicated in three or more independent experiments. Data are presented as means ± SEM. As appropriate, Student's unpaired *t* test or one-way ANOVA followed by Tukey's test to compare individual means was used for statistical comparisons. The significance level was set to 5%.

Results

16K-PRL but not PRL inhibits VEGF-induced NOS activity in endothelial cells

Treatment for 1 h with enzymatically generated rat 16K-PRL or with recombinant human 16K-PRL did not affect the morphology, number, or total protein concentration of BUVECs or HUVECs (not shown). However, both rat and human 16K-PRL, but not recombinant human PRL, inhibited the stimulation of NOS activity induced by a 1-h incubation with VEGF, as determined by the conversion of [^{3}H]L-arginine to [^{3}H]L-citrulline in both endothelial cell types (Figs. 1 and 2). 16K-PRL inhibition was dose dependent and did not occur on basal NOS levels in the absence of VEGF (Fig. 1). The activity of 16K-PRL was not caused by contaminants such as bacterial lipopolysaccharides, because 16K-PRL inhibition of NOS activity was not modified in the presence of the lipopolysaccharide inhibitor polymyxin-B, but it was abolished by heat denaturation of 16K-PRL (Fig. 2).

16K-PRL inhibits Ca^{2+}-dependent VEGF-induced NOS activity

The rapid, 1-h stimulation of endothelium-derived NO synthesis in response to VEGF is likely mediated by the activation of eNOS (22), because eNOS is the primary NOS isoform activated by VEGF. To provide additional evidence that 16K-PRL inhibits eNOS activity, which is Ca^{2+} dependent (38), we investigated whether 16K-PRL inhibited VEGF-induced Ca^{2+}-dependent NOS activity. Total and Ca^{2+}-independent conversion of [^{3}H]L-arginine to [^{3}H]L-citrulline was determined from incubations in the absence or presence of EGTA, respectively (Fig. 3A). Unlike total NOS activity, Ca^{2+}-independent NOS activity, determined after incubation with EGTA, was not modified by incubation with VEGF with or without 16K-PRL (Fig. 3A). However, Ca^{2+}-dependent NOS activity (eNOS activity), calculated by subtracting Ca^{2+}-independent NOS activity from total NOS activity, was increased by VEGF, and this increment was inhibited by 16K-PRL (Fig. 3B).

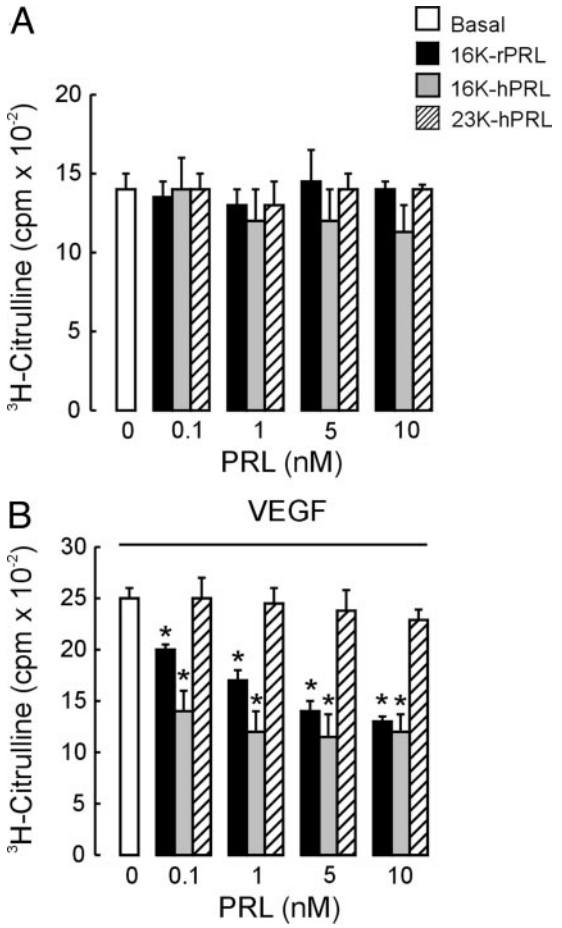

FIG. 1. 16K-PRL inhibits VEGF-induced NOS activity in bovine vein endothelial cells. BUVECs were incubated for 1 h in the absence (A) or presence of 2.4 nM VEGF (B) with increasing concentrations of enzymatically generated rat 16K-PRL (16K-rPRL), recombinant human 16K-PRL (16K-hPRL), or recombinant human 23K-PRL (23K-hPRL). NOS activity was determined by the [^{3}H]L-arginine conversion to [^{3}H]L-citrulline assay (33). Values are means ± SEM; *, $P < 0.05$ *vs.* control, VEGF without PRLs.

Exogenous NO blocks the 16K-PRL inhibition of VEGF-induced endothelial cell proliferation

To investigate whether a causal relationship exists between 16K-PRL-induced inhibition of eNOS activity and inhibition of endothelial cell proliferation, we assessed the effect of a NO donor on BUVECs incubated with or without VEGF in the presence or absence of 16K-PRL (Fig. 4). The NO donor DETANONOate stimulated the proliferation of BUVECs to levels similar to those following treatment with VEGF, confirming the *in vitro* angiogenic effect of NO (20). As shown for other endothelial cell types (4), 16K-PRL inhibited VEGF-induced proliferation of BUVECs. However, cells treated with a combination of VEGF, 16K-PRL, and the NO donor DETANONOate showed a mitogenic response comparable to that of cells treated with VEGF alone or with both VEGF and DETANONOate, indicating that the NO donor blocks the antimitogenic effect of 16K-PRL.

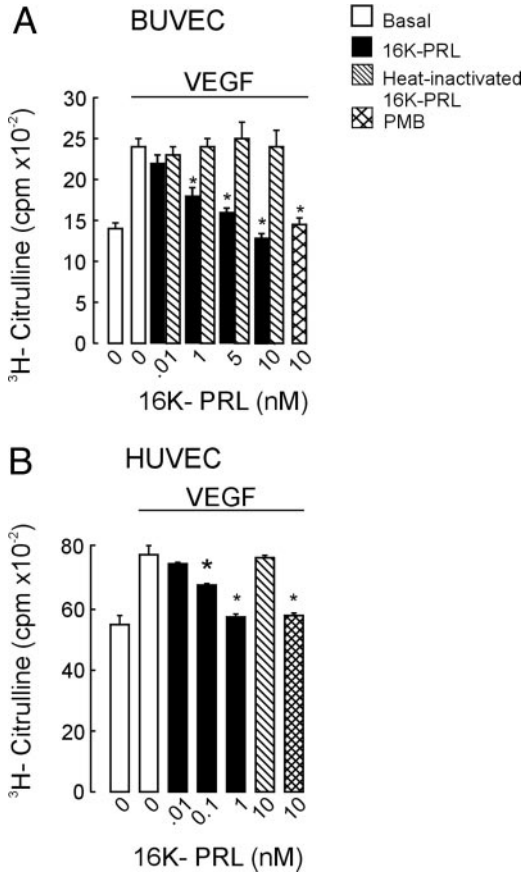

FIG. 2. Specificity of 16K-PRL inhibition of VEGF-induced NOS activity in bovine and human vein endothelial cells. BUVECs (A) and HUVECs (B) were incubated for 1 h in the absence or presence of 2.4 nM VEGF with increasing concentrations of enzymatically generated rat 16K-PRL before or after heat inactivation for 10 min or in the presence of polymyxin-B (PMB). NOS activity was determined by the [^{3}H]L-arginine conversion to [^{3}H]L-citrulline assay (33). Values are means ± SEM; *, $P < 0.05$ *vs.* control, VEGF without 16K-PRL.

16K-PRL inhibits BK-induced eNOS activity

We next asked whether 16K-PRL would also block the action of BK, a potent vasodilator and angiogenic agent that acts upon endothelial cells by activating eNOS and causing rapid production of NO (38–40). BUVECs were challenged with BK in the presence or absence of 16K-PRL for 1 h. As shown in Fig. 5, 16K-PRL significantly inhibited BK-induced eNOS activity in a dose-dependent manner.

16K-PRL inhibits BK-induced intracellular Ca^{2+} mobilization

Because eNOS is a Ca^{2+}-dependent enzyme and factors such as VEGF and BK activate eNOS through mobilization of intracellular Ca^{2+} (22, 24, 40), we speculated that 16K-PRL may inhibit intracellular Ca^{2+} mobilization induced by these eNOS activators. To explore this possibility, BUVECs and RRCECs in suspension were loaded with fura 2-AM and stimulated with BK before or after 16K-PRL. BK induced a transient elevation in free intracellular Ca^{2+} concentration in both cell types, and although 16K-PRL had no effect by itself, it prevented the increase in intracellular Ca^{2+} evoked by BK

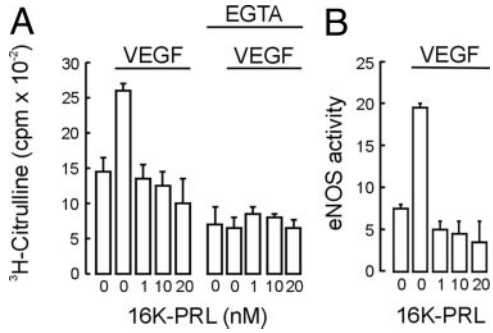

FIG. 3. 16K-PRL inhibits VEGF-induced Ca^{2+}-dependent NOS activity in bovine vein endothelial cells. A, BUVECs were incubated for 1 h with or without 2.4 nM VEGF and increasing concentrations of enzymatically generated rat 16K-PRL in the absence (total NOS activity) or presence of 2 mM EGTA (Ca^{2+}-independent NOS activity). NOS activity was determined by the [^{3}H]L-arginine conversion to [^{3}H]L-citrulline assay (33). B, eNOS activity (Ca^{2+}-dependent NOS activity) was calculated by subtracting Ca^{2+}-independent NOS activity from total NOS activity. Values are means ± SEM.

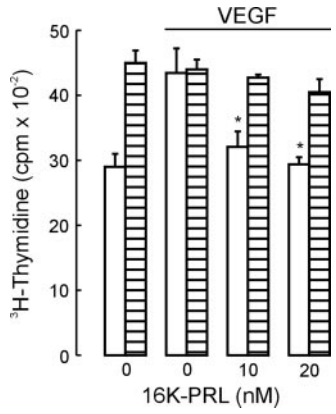

FIG. 4. Exogenous NO blocks 16K-PRL inhibition of VEGF-induced proliferation of bovine vein endothelial cells. BUVECs were serum starved for 24 h and then allowed to proliferate in complete medium for an additional 24-h period with or without 0.6 nM VEGF and two concentrations of recombinant human 16K-PRL in the absence (*white bars*) or presence (*hatched bars*) of the NO donor DETANONOate (10 μM). Values are means ± SEM; *, $P < 0.05$ *vs.* control, VEGF without 16K-PRL.

(Fig. 6A). Next, perfused monolayers of BUVECs and RRCECs were used to eliminate the previous treatment so that the same monolayer could serve as its own control. In the perfused monolayers, BK-induced Ca^{2+} transients were elicited at 5-min intervals, and pretreatment with 16K-PRL for 3–5 min prevented the effect of the subsequent BK administration (Fig. 6B).

16K-PRL inhibits BK-induced relaxation of coronary vessels in isolated perfused guinea pig hearts

Because VEGF and BK signal through eNOS to stimulate vasorelaxation (39–41), we reasoned that blockage of eNOS activation by 16K-PRL could lead to inhibition of vasodilation. To test this hypothesis, we used the isolated perfused heart model in which perfusion pressure is a conventional index of coronary vessel tone (36). Perfusion pressure was

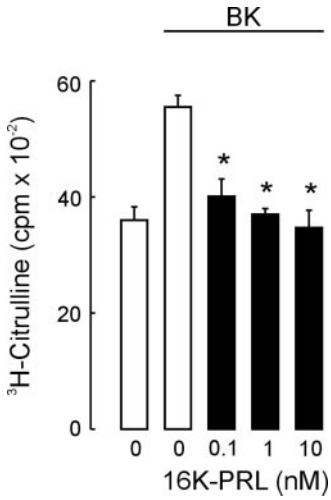

FIG. 5. 16K-PRL inhibits BK-induced NOS activity in bovine vein endothelial cells. BUVECs were incubated for 1 h with or without 10 μM BK and increasing concentrations of enzymatically generated rat 16K-PRL. NOS activity was determined by the [^{3}H]L-arginine conversion to [^{3}H]L-citrulline assay (33). Values are means ± SEM; *, $P <$ 0.05 *vs.* control, BK without 16K-PRL.

recorded from an isolated guinea pig heart subjected to 30-sec infusions of 10 μM BK every 15 min (Fig. 7). BK decreased perfusion pressure (coronary relaxation) to approximately 50% of the pressure developed under basal conditions (Fig. 7A, first trace recording, and Fig. 7B). Upon termination of BK infusion, the perfusion pressure returned gradually over a 1-min interval to baseline values. When administration of BK was immediately preceded by a 30-sec infusion of 10 nM 16K-PRL, the effect of BK was nearly abolished (Fig. 7A, second trace recording, and Fig. 7B). The inhibitory effect of 16K-PRL disappeared after 15 min, allowing BK to elicit a full-sized vasorelaxation response (Fig. 7A, third trace recording, and Fig. 7B).

16K-PRL inhibits ACh-induced eNOS activity and relaxation of coronary vessels

Because ACh is another potent vasodilator whose activity is mediated by eNOS activation and NO synthesis in endothelial cells (42), we wanted to determine whether 16K-PRL might inhibit eNOS activation and vasorelaxation in response to ACh. Consistent with this possibility, BUVECs incubated with ACh in the absence or presence of 16K-PRL showed that 16K-PRL significantly inhibited ACh-induced eNOS activity in a dose-dependent manner (Fig. 8). Moreover, experiments using the isolated perfused rat heart showed that 30-sec infusions of ACh (10 μM) evoked a significant 50% reduction in perfusion pressure that returned to basal values 0.5 min after termination of infusion (Fig. 9A, first trace recording, and Fig. 9B). Pretreatment with a 30-sec infusion of 10 nM 16K-PRL inhibited the effect of ACh when it was administered immediately (Fig. 9A, second trace recording, and Fig. 9B). However, the inhibitory effect of 16K-PRL was gone by 15 min, when a full-sized ACh vasodilatory effect could be observed (Fig. 9A, third trace recording, and Fig. 9B).

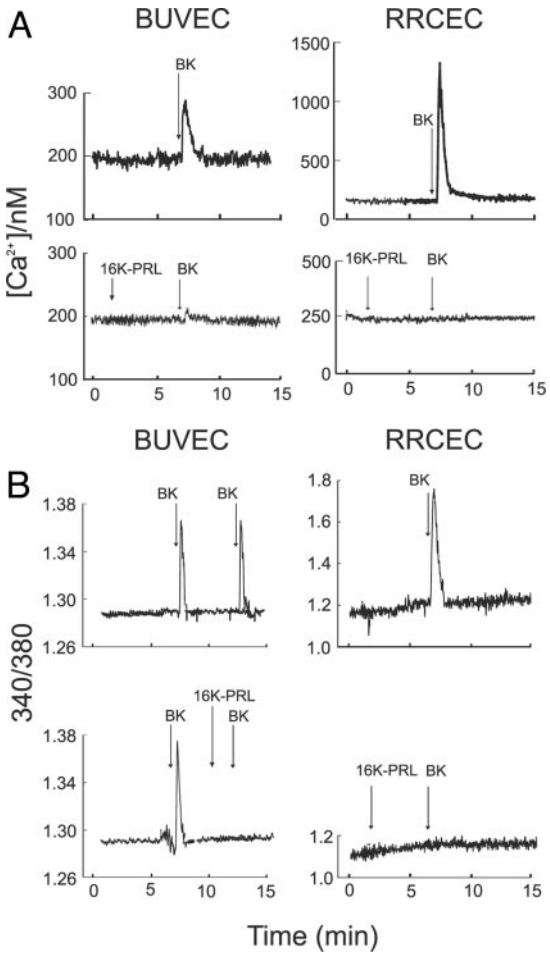

FIG. 6. 16K-PRL inhibits BK-induced Ca^{2+} transients in bovine vein and rat capillary endothelial cells. A, BUVECs and RRCECs in suspension were loaded with fura 2-AM and challenged with 10 μM BK before (upper traces) or after 10 nM enzymatically generated rat 16K-PRL. Changes in free calcium are shown as Ca^{2+} concentration. B, BUVECs and RRCECs in perfused monolayers were loaded with fura 2-AM and challenged with 10 μM BK alone or in combination with 10 nM 16K-PRL. Changes in free Ca^{2+} are shown as the fura 2-AM fluorescence ratio at the two excitation wavelengths, plotted as indicated in *Materials and Methods*. *Arrows* indicate the onset of substance administration. Results are representative of three independent experiments. The full scale was omitted in some y-axes to more clearly illustrate the responses.

16K-PRL inhibits ACh-induced relaxation of rat aortic segments

To further investigate the inhibition of vasorelaxation by 16K-PRL, rat aortic segments were submaximally contracted with phenylephrine and treated with 16K-PRL or PRL, followed by increasing concentrations of ACh to elicit NO-mediated relaxation (Fig. 10A). 16K-PRL, but not PRL, inhibited the vasodilatory effect of ACh. This effect is mediated through the inhibition of NO production by 16K-PRL, because vascular relaxation responses to the NO donor sodium nitroprusside (endothelium-independent relaxation) were similar in control, 16K-PRL-treated, and PRL-treated segments (Fig. 10B). These results indicate that 16K-PRL interferes with the endothelial NO signal to the smooth muscle.

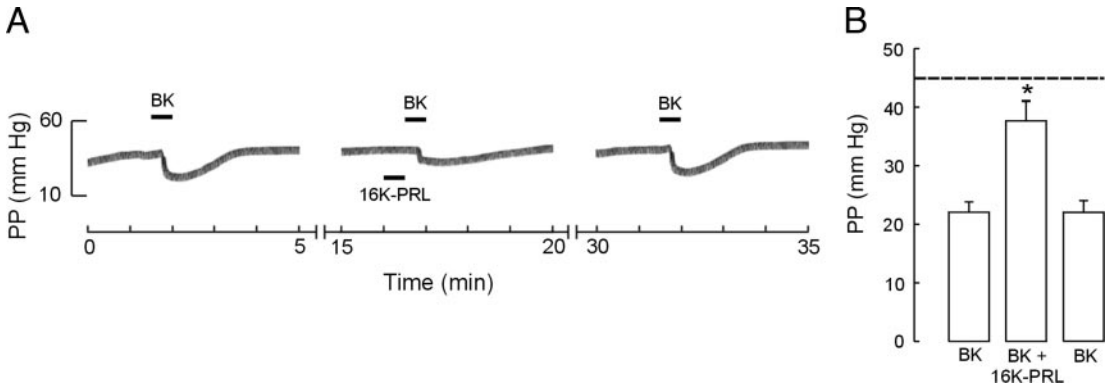

FIG. 7. 16K-PRL inhibits BK-induced relaxation of coronary vessels in isolated perfused guinea pig hearts. Perfusion pressure (PP), taken as an index of vascular tone, was recorded from isolated guinea pig hearts. A, PP representative trace recordings in response to three consecutive administrations of BK (10 μM) every 15 min. BK was infused for 30 sec alone (first and third trace), or immediately after (second trace) the 30-sec infusion of 16K-PRL (10 nM). *Bars* above and below the recordings indicate the time of infusion of the respective substances. B, Maximum inhibition of PP from trace recordings in response to BK obtained as indicated above from three independent experiments. *Horizontal line* represents PP under basal conditions. Values are means ± SEM; *, $P < 0.05$ *vs.* control, BK without 16K-PRL.

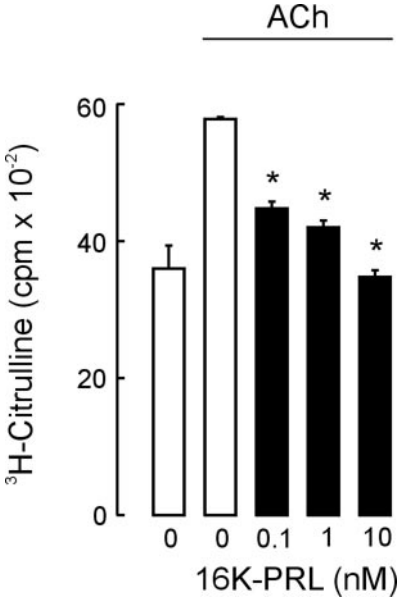

FIG. 8. 16K-PRL inhibits ACh-induced NOS activity in bovine vein endothelial cells. BUVECs were incubated for 1 h with or without 10 μM ACh and increasing concentrations of enzymatically generated rat 16K-PRL. NOS activity was determined by the [^{3}H]L-arginine conversion to [^{3}H]L-citrulline assay (33). Values are means ± SEM; *, $P < 0.05$ *vs.* control, ACh without 16K-PRL.

Discussion

In the vasculature, NO produced by eNOS activation is physiologically important for maintaining vascular homeostasis; it keeps the vessels dilated, protects the intima from platelet aggregates and leukocyte adhesion, and prevents proliferation and migration of smooth muscle cells (19). In fact, the loss or attenuation of NO production in the endothelium is one of the earliest biochemical markers of endothelial dysfunction found in many cardiovascular diseases such as hypertension and atherosclerosis (43). In addition, eNOS-derived NO is critical for the angiogenesis that develops in ischemic tissues (27, 44) and is up-regulated and down-regulated by angiogenic and antiangiogenic factors, respectively (25, 16, 17). Here, we report that 16K-PRL, a potent inhibitor of angiogenesis, blocks eNOS activation and that this action also results in novel vasomotor effects.

We demonstrate that 16K-PRL from different sources (recombinant or enzymatically generated) and species (human and rat) inhibits, within 1 h, the activation of NOS induced by VEGF, BK, and ACh in endothelial cells. This inhibition is mediated by blockade of eNOS activation because eNOS is the primary NOS isoform activated by VEGF, BK, and ACh in endothelial cells (22, 24, 40, 45), and the effect of 16K-PRL on NOS activity is abolished by EGTA.

In contrast to 16K-PRL, full-length 23K-PRL did not modify VEGF-induced eNOS activation. This observation is consistent with 16K-PRL signaling through a cell surface receptor in endothelial cells that is distinct from the 23K-PRL receptor (15). Endothelial cells are reported to have a high-affinity (K_d = 0.9 nM), saturable binding site that is specific for 16K-PRL and does not bind 23K-PRL (15). However, the 16K-PRL receptor is yet to be identified, and little is known about second messengers that might convey immediate intracellular signals triggered by the binding of 16K-PRL. Similarly, the receptors for other antiangiogenic fragments are unknown or remain poorly characterized. Although in some instances great efforts have been made to identify and characterize endothelial cell surface receptors, it is not known whether these binding molecules can transduce inhibitory signals (7, 14). Complexity is added by the fact that the antiangiogenic fragments do not share a common cleavage site motif, suggesting that several proteases are involved in their generation (14). Furthermore, it has become a common theme that several proteolytic fragments of the same protein inhibit angiogenesis and thus that various related ligands may activate the same receptor (14, 5). Advancing the knowledge about the intracellular signaling molecules mediating 16K-PRL angiostatic activity could help elucidate the nature of the specific receptor involved.

The mechanism by which 16K-PRL inhibits eNOS activation is unknown. Regulation of eNOS activity involves a range of posttranscriptional mechanisms, among which an increase in Ca^{2+}/calmodulin binding plays a predominant role (38). Blockage of intracellular Ca^{2+} mobilization could

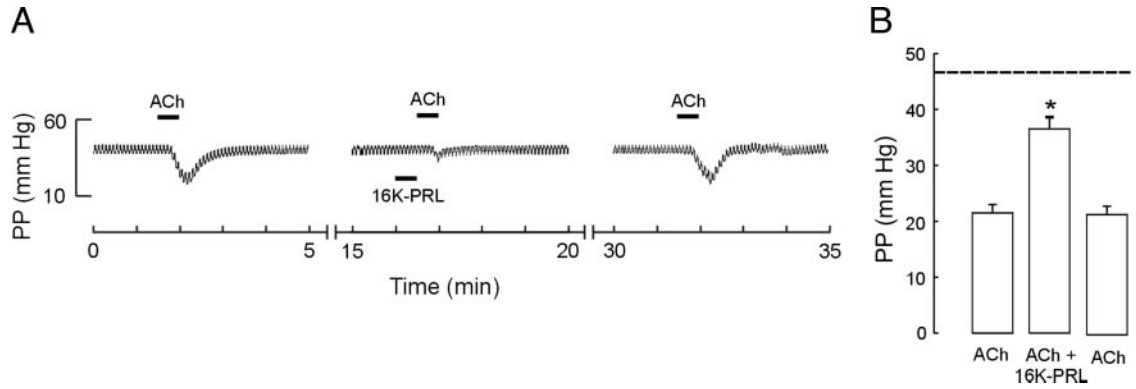

FIG. 9. 16K-PRL inhibits ACh-induced relaxation of coronary vessels in isolated perfused rat hearts. Perfusion pressure (PP), taken as an index of vascular tone, was recorded from isolated rat hearts. A, Representative trace recordings of PP in response to three consecutive administrations of ACh (10 μM) every 15 min. ACh was infused for 30 sec alone (first and third trace) or immediately after (second trace) the 30-sec infusion of 16K-PRL (10 nM). *Bars* above and below the recordings indicate the time of infusion of the respective substances. B, Maximum inhibition of PP from trace recordings in response to ACh, obtained as indicated above in three independent experiments. *Horizontal line* represents PP under basal conditions. Values are means ± SEM; *, $P < 0.05$ *vs.* control, ACh without 16K-PRL.

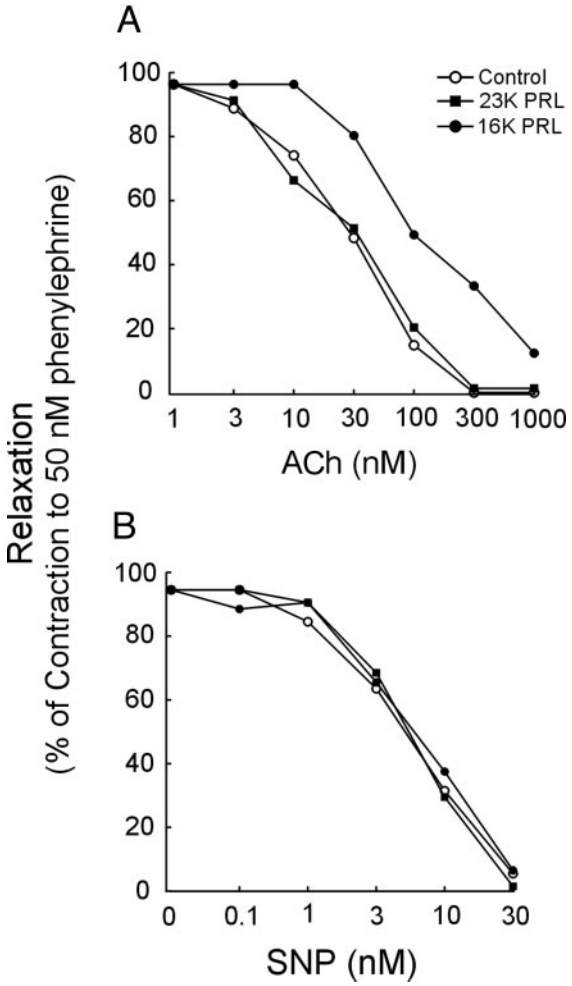

FIG. 10. 16K-PRL inhibits NO-mediated relaxation of rat aortic segments. Vessels submaximally contracted with phenylephrine were treated with 10 nM rat PRL or enzymatically generated rat 16K-PRL followed by increasing concentrations of ACh (endothelial NO-mediated relaxation) (A) or of the NO donor sodium nitroprusside (SNP; endothelial NO-independent relaxation) (B). Force is plotted as percent of that force developed before treatment with ACh or SNP. Results are representative of three independent experiments.

mediate 16K-PRL inhibition of eNOS activation, because 16K-PRL abolished BK-induced Ca^{2+} transients in both vein- and capillary-derived endothelial cells. Mobilization of Ca^{2+} by BK, as well as by ACh and VEGF, involves the activation of phospholipase C, followed by a transient increase in the formation of inositol 1,4,5-triphosphate and diacylglycerol, which in turn leads to Ca^{2+} release from intracellular stores, an influx of extracellular Ca^{2+}, and activation of protein kinase C (24, 38, 40, 41, 45). It remains to be determined whether 16K-PRL inhibits Ca^{2+} mobilization in response to VEGF and ACh, and how 16K-PRL interferes with the phospholipase C and protein kinase C signaling pathway and/or other potential target molecules, thereby inhibiting intracellular Ca^{2+} mobilization.

NO has been shown to promote angiogenesis and vascular permeability in wounds (46) and tumors (47). NO stimulates endothelial cell proliferation and migration, protects endothelial cells from apoptosis, and stimulates the production of VEGF (20, 26). VEGF is the most potent angiogenic and vasopermeability factor during wound healing and tumor progression, and its actions involve stimulation of NO synthesis (21–26). Here, we show that inhibition of VEGF-induced proliferation of endothelial cells by 16K-PRL is abolished by exogenous NO, indicating that the antimitogenic properties of 16K-PRL are mediated by NO-dependent mechanisms. Previous work showed that 16K-PRL blocks VEGF-induced proliferation of endothelial cells by inhibiting VEGF-induced Ras, Raf, and MAPK activation (11, 12). Actually, the eNOS pathway interacts with the MAPK pathway in mediating the mitogenic effect of VEGF. The following cascade has been proposed: NO stimulates cGMP production, which in turn activates cGMP-dependent protein kinase leading to Raf and MAPK activation (20). Because 16K-PRL inhibits activation of Ras and eNOS in response to VEGF, it is likely that 16K-PRL inhibition of the mitogenic properties of VEGF occurs at the level of the MAPK pathway through blockage of Raf activation by both Ras and cGMP-dependent protein kinase.

Considering the versatile nature of NO, other processes may be down-regulated by 16K-PRL inhibition of eNOS ac-

tivation. For example, eNOS mediates VEGF-induced stimulation of endothelial cell migration (23), inhibition of endothelial cell apoptosis (26), and stimulation of vascular permeability (25). In this regard, 16K-PRL promotes endothelial cell apoptosis (10) and up-regulates the expression of plasminogen activator inhibitor-1 (9), a specific inhibitor of urokinase, which activates proteases involved in endothelial cell migration. It needs to be determined whether these modulatory actions of 16K-PRL are mediated by inhibition of eNOS activation.

Here, the fact that 16K-PRL produces a rapid inhibition of intracellular Ca^{2+} mobilization and of eNOS activation prompted us to investigate the potential action of this peptide on vascular tone. The release of NO by the endothelium causes relaxation of vascular smooth muscle cells and consequent vasodilation. eNOS activators, including BK, ACh, and VEGF (38–42), act as vasorelaxant factors, and their action can lead to hypotension. In fact, eNOS knockout mice are hypertensive and lack NO-mediated, endothelium-dependent vasodilation (48, 49). By using two different experimental models, we demonstrate that 16K-PRL inhibits BK-and ACh-induced vasodilation. Also, exogenous NO abolished the inhibitory effect of 16K-PRL on relaxation of rat aortic segments, supporting the conclusion that 16K-PRL can act as a negative regulator of NO-dependent vasorelaxation.

Proof that the present observations are physiologically relevant would come from studies in which the effect of up-regulating or down-regulating the generation or action of 16K-PRL is tested on blood vessel growth and remodeling, vascular permeability, vasorelaxation, or blood pressure. 16K-PRL can be generated by proteolytic cleavage of PRL in the pituitary gland (50, 51), in the eye (52), and in peripheral cells, including vascular endothelial cells (31) and fibroblasts (53), and it can be found in the circulation (50, 51, 54). However, little is known regarding the generation of 16K-PRL and its regulation in angiogenesis-related conditions. Recent evidence indicates that cathepsin-D, the putative PRL-cleaving enzyme, is down-regulated by hypoxia in pituitary tumor cells, resulting in a reduced conversion of PRL to 16K-PRL (55). In addition, a higher production of 16K-PRL was recently reported in the eye of patients with advanced retinopathy of prematurity, where evidence suggests that 16K-PRL can promote apoptosis-mediated vascular regression of the newly formed blood vessels (52). Finally, neutralizing the action of PRL with anti-PRL antibodies in the cornea (8) or blocking the expression or action of PRL in the retina with small interfering RNA or anti-PRL antibodies, respectively (Aranda, J., and C. Clapp, unpublished observations), results in local neovascularization and vasodilation responses.

Although the physiological role of 16K-PRL remains unclear, the idea of PRL molecules affecting the cardiovascular system is not entirely unexpected. Blood volume and cardiac output increase during lactation, and tissues actively involved in the body's reaction to the demand of milk production receive a greater proportion of this output at the expense of other tissues (56). Thus, blood flow to the mammary glands, liver, and gastrointestinal tract is increased, whereas that of uterus, kidneys, and heart is reduced (56). The possibility that PRL contributes to some of these circulatory adaptations is suggested by the fact that these changes are strongly related to the suckling stimulus (57) and that the injection of PRL reduces urinary excretion of sodium, potassium, and water (58) and increases blood volume (59). Furthermore, redistribution of cardiac output away from less actively involved tissues and toward active secretory and absorptive tissues may be local and related to the production of vasoconstrictive and vasodilatory substances, respectively (60). Because 16K-PRL is shown to induce vasopressor responses, it would be of interest to compare the different tissues for their ability to generate 16K-PRL from PRL in lactating animals.

In summary, this work reports for the first time that 16K-PRL inhibits intracellular Ca^{2+} mobilization and activation of eNOS by important angiogenic and vasodilatory substances. Based on the fact that eNOS-derived NO is required for endothelial cell proliferation and vascular dilation, we have also demonstrated that inhibition of endothelium-derived NO mediates the antimitogenic effects of 16K-PRL and, more importantly, that 16K-PRL can function to up-regulate vascular tone. These antiangiogenic and vasomotor actions are unique to 16K-PRL and do not occur in response to PRL, which indicates that both the protease(s) responsible for processing PRL and the selective expression of receptors for 16K-PRL would critically influence these actions. Knowledge of the role of 16K-PRL in endothelial cell function may contribute to the understanding and control of angiogenesis-related diseases and cardiovascular pathologies, and further investigation is warranted.

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