Progesterone increases nitric oxide synthesis in human vascular endothelial cells through activation of membrane progesterone receptor-α

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Pang Y, Dong J, Thomas P. Progesterone increases nitric oxide synthesis in human vascular endothelial cells through activation of membrane progesterone receptor-α. Am J Physiol Endocrinol Metab 308: E899–E911, 2015. First published March 24, 2015; doi:10.1152/ajpendo.00527.2014.—Progesterone exerts beneficial effects on the human cardiovascular system by inducing rapid increases in nitric oxide (NO) production in vascular endothelial cells, but the receptors mediating these nongenomic progesterone actions remain unclear. Using human umbilical vein endothelial cells (HUVECs) as a model, we show that progesterone binds to plasma membranes of HUVECs with the characteristics of membrane progesterone receptors (mPRs). The selective mPR agonist Org OD 02-0 had high binding affinity for the progesterone receptor on HUVEC membranes, whereas nuclear PR (nPR) agonists R5020 and medroxyprogesterone acetate displayed low binding affinities. Immunocytochemical and Western blot analyses confirmed that mPRs are expressed in HUVECs and are localized on their plasma membranes. NO levels increased rapidly after treatment with 20 nM progesterone, Org OD 02-0, and a progesterone-BSA conjugate but not with R5020, suggesting that this progesterone action is at the cell surface and initiated through mPRs. Progesterone and Org OD 02-0 (20 nM) also significantly increased endothelial nitric oxide synthase (eNOS) activity and eNOS phosphorylation. Knockdown of mPRα expression by treatment with small-interfering RNA (siRNA) blocked the stimulatory effects of 20 nM progesterone on NO production and eNOS phosphorylation, whereas knockdown of nPR was ineffective. Treatment with PI3K/Akt and MAP kinase inhibitors blocked the stimulatory effects of progesterone, Org OD 02-0, and progesterone-BSA on NO production and eNOS phosphorylation and also prevented progesterone- and Org OD 02-0-induced increases in Akt and ERK phosphorylation. The results suggest that progesterone stimulation of NO production in HUVECs is mediated by mPRα and involves signaling through PI3K/Akt and MAP kinase pathways.

FEMALE REPRODUCTIVE HORMONES are widely considered to have beneficial effects on cardiovascular functions in women. The lower incidence of cardiovascular disease in middle-aged premenopausal women than in men and postmenopausal women has been attributed to the presence of female sex steroids in the circulation (22, 27, 41, 47). Blood pressure is typically lower in women than in men and often drops during pregnancy when circulating levels of estrogens and progesterone are elevated (30, 70, 71). Furthermore, synthesis of a major regulator of vasodilation, nitric oxide (NO), is greater in women than in men (14). These sex differences in the occurrence of cardiovascular disease are due partly to the well-known beneficial effects of estrogens, which include upregulation of NO production in human endothelial cells (7, 18, 28, 33). Numerous studies in animal models also support a role for estrogens in vasodilation and other cardiovascular protective functions (21, 53). In contrast, the potential beneficial effects of progesterone on cardiovascular functions in women have not been broadly acknowledged and have received little attention despite early evidence that progesterone exerts protective effects (9, 26, 63). The increased risk of coronary disease and stroke associated with medroxyprogesterone acetate (MPA) treatment observed in several large hormone replacement therapy trials (16, 50) is one likely reason why positive effects of progestins have not been investigated extensively. However, progesterone has divergent and often opposite effects to those of MPA on cardiovascular functions that are probably partially related to its antiandrogenic and antimineralocorticoid activities, which are not shared by MPA (20, 35, 60). Treatment with progesterone causes vasodilation and lowers blood pressure in normotensive as well as in hypertensive patients, including those with pregnancy-induced hypertension (35, 40, 48, 51). Improvements of cardiovascular functions such as decreased coronary artery hyperactivity and lower blood pressure have also been reported in primates and other animal models after progesterone treatment (2, 19, 45, 49). Collectively, these studies demonstrate that progesterone exerts important protective effects on the cardiovascular system, although its mechanism of action remains unclear.

There is mounting evidence that many of the protective actions of 17β-estradiol and progesterone on the cardiovascular system are rapid and nongenomic (7, 9, 32, 34, 46, 56). Vasodilation and declines in blood pressure in ewes and female monkeys were observed within a few minutes of progesterone treatment (34, 49). The finding that treatment with a cell impermeant progesterone-BSA conjugate mimicked these effects in monkeys (34) suggests that this progesterone action is initiated at the cell surface. The detection of a progesterone-binding moiety on the plasma membranes of human aorta endothelial cells and on human umbilical vein endothelial cells (HUVECs) (63, 69) is consistent with such a mechanism of action mediated through a membrane progesterone receptor. Nitric oxide, which is synthesized in vascular endothelial cells by the enzyme endothelial nitric oxide synthase (eNOS), is a principal regulator of vasodilation through its action on vascular smooth muscle cells, causing their relaxation (17, 41, 73). Studies in animal models suggest that nanomolar concentrations of progesterone induce rapid vasodilation of aorta and arteries through this mechanism (52, 53). Acute increases in eNOS activity and NO production have also been observed in HUVECs in response to 10 nM progesterone treatment (56). Both nuclear progesterone receptors (nPRs) in an extranuclear location and membrane progesterone receptors (mPRs) are candidates for the receptor(s) mediating this rapid, nongenomic
progesterone action because transcripts of both classes of progesterone receptors have been detected in HUVECs (62, 63). However, only limited evidence to date has been obtained from results with a few nPR ligand supporting a role for nPRs in rapid progesterone (10 nM) upregulation of NO synthesis (56). Similarly, only preliminary evidence has been obtained using an mPR-selective agonist for an involvement of mPRs in regulation by 20 nM progesterone of NO production (63). Moreover, there are no published reports to our knowledge that have compared the roles of these two classes of progesterone receptors in mediating rapid progesterone actions in cardiovascular endothelial cells.

The five mPR subtypes (mPRα, -β, -γ, -δ, and -ε) belong to the progestin and adipoQ receptor (PAQR) family (61, 65) but differ from other PAQRs in that they are only present in vertebrates and display high-affinity progesterone-binding characteristics of membrane steroid receptors (43, 65). The mPRα subtype is ubiquitously expressed in vertebrate tissues and is the intermediary in a variety of nongenomic progesterone actions in fishes, including induction of oocyte maturation and sperm motility (68, 74) and inhibition of granulosa cell apoptosis (13). The mPRα has also been implicated in progesterone modulation of gonadotropin-releasing hormone (GnRH) neuronal, T lymphocyte, breast cancer cell, and myometrial cell functions in mammalian cells (10, 11, 23, 36, 59). However, clear evidence that mPRα mediates distinct, physiologically important actions of progesterone in mammalian cells is currently lacking. Potential cross-talk between mPRα and nPR-B has been demonstrated in human myometrial cells (23), but there has not been a systematic evaluation of the relative importance of nPRs and mPRs as intermediaries in rapid progesterone actions in any mammalian cell models. In a preliminary study with HUVECs, in addition to detecting nPR mRNA, we also detected transcripts for mPRs and specific [3H]progesterone binding on plasma membranes prepared from these cells (63). However, it was not known from this pilot study whether the [3H]progesterone binding indicates the presence of a specific progesterone membrane receptor on HUVECs and its likely identity. Initial evidence, using specific agonists for nPRs (R5020) and mPRs (Org OD 02-0) (24, 63), that mPRs are likely candidates for receptors mediating the rapid induction of NO synthesis by progesterins in HUVECs was also obtained. In contrast, in a previous study, nPR has been implicated in progesterone (10 nM) regulation of NO synthesis in vascular endothelial cells (56). A detailed comparison of the potential roles of nPRs and mPRs in mediating this vascular protective effect of progesterone in endothelial cells will be required to resolve this issue. The results from such a study may also indicate whether mPRs have distinct, nPR-independent, important physiological functions in mammalian cells.

The relative importance of mPRs and nPRs in the regulation of eNOS activity and NO production in primary cultures of HUVECs by physiologically relevant progesterone concentrations (5–100 nM) was investigated in the present study. Cell surface initiation of progesterone signaling was investigated using a progesterone-BSA conjugate, and membrane localization of the different types of progesterone receptors was examined by immunocytochemistry. The presence of a functional progesterone receptor on HUVEC plasma membranes was demonstrated by saturation analysis and its likely identity by competitive binding studies with selective nPR and mPR agonists. The roles of nPRs and mPRs in progesterin regulation of NO synthesis and eNOS were explored using siRNA to knock down nPR and mPRα expression. Signal transduction through G proteins and the roles of the phosphatidylinositol 3-kinase (PI3K)/Akt and MAP kinase pathways in the regulation of eNOS and NO through nPRs and mPRs were investigated using specific kinase inhibitors and nPR and mPRα siRNA. The results show that the rapid protective actions of progesterone on HUVECs to increase NO production are mediated solely through mPRα.

**MATERIALS AND METHODS**

**Reagent and chemicals.** [2,4,6,7-3H]progesterone ([3H]P4; ~84 Ci/mmol) and 1-[(2,3,4,5-3H]arginine monohydrochloride (54.6 Ci/mmol) were purchased from American Radiolabeled Chemicals (St. Louis, MO). Nonradioactive steroids were purchased from Sigma-Aldrich (St. Louis, MO) or Steraloids (Newport, RI). Org OD-02-0 was obtained from Organon (Oss, The Netherlands). All other chemical reagents were obtained from Sigma-Aldrich unless otherwise stated. Antibodies against ERK, phosphorylated ERK (p42/44), Akt, and phosphorylated Akt were purchased from Cell Signaling Technology (Danvers, MA). Antibodies for eNOS and phosphorylated eNOS (Ser1177) were obtained from BD Biosciences (San Jose, CA). The PI3k inhibitor wortmannin and MAP kinase kinase (MEK) inhibitor PD-98059 were purchased from Enzo Life Sciences (Farmingdale, NY), and the specific ERK1/2 inhibitor AZD6244 was purchased from Selleckchem (Houston, TX).

**Collection of human placenta and culture of HUVECs.** HUVECs were harvested by enzymatic digestion, following previously published procedures (54), with some minor modifications. Umbilical cords attached to fresh deidentified human placenta were collected shortly after birth by cesarean section from subjects who had given informed consent to participate in the study. Ethical approval for the study was granted by the Institutional Review Boards (IRBs) of the hospital (Christus Spohn Health System, IRB no. 11-001) and the University of Texas at Austin (IRB no. 2010-10-0108). The tissues were kept in sterile PBS and transported to the laboratory within 2 h for processing. The umbilical cords were washed, and the umbilical vein was flushed several times with Hanks’ balanced salt solution (HBSS) medium without Ca2+ and Mg2+ (Sigma-Aldrich) to remove the blood cells prior to enzymatic treatment. The umbilical vein was incubated with 0.2% collagenase in HBSS at 37°C for 30 min. After the digestion, the endothelial cells were washed out of the umbilical vein with HBSS, and the cell suspension was centrifuged at 3,000 g for 15 min. The cells were then collected and incubated in TC-M-199 medium (Sigma-Aldrich) supplemented with 10% FBS and endothelial cell growth supplement (Sigma-Aldrich). The medium was changed on the 2nd day, and the floating blood cells and other cell debris were removed. The HUVECs were then cultured in culture flasks until they were 90% confluent for periods ranging from 2 days to 2 wk. The culture medium was replaced with fresh HBSS without serum 2–3 h before experimentation. Cells were treated with steroids in the presence or absence of kinase inhibitors for various periods before collection for subsequent analysis. In some experiments, the kinase inhibitors were added to the media 30 min prior to the addition of the steroid treatments. The progesterone concentrations selected for the experimental treatments (5–100 nM) are in the physiological range because plasma progesterone levels in pregnant women range from <10 to >300 nM, depending on the stage of pregnancy (6, 29).

**Progesterone receptor binding assays.** HUVECs were subcultured in 15-cm culture dishes and harvested with a cell scraper. Plasma membrane fractions were prepared, and [3H]P4 receptor binding was measured as described previously (65). Briefly, cell homogenates were centrifuged at 1,000 g for 7 min to pellet the nuclear fraction,
Specific [3H]P4 binding was calculated by subtracting nonspecific percent over vehicle treatment. Branes were solubilized and cell lysates (were prepared as described for the progesterone receptor binding assays were performed on HUVECs after 15 days of culture, following previously published procedures (43). In brief, total RNA was extracted from HUVECs with Tri Reagent (Sigma) and treated with DNase using a Turbo-DNA-free Kit (Ambion, Grand Island, NY) to eliminate any genomic DNA contamination. Gene expression of mPRs, nPR, and progesterone receptor membrane component 1 (PGRMC1) was measured by qPCR using the Eppendorf RealPlex Mastercycler (Eppendorf, Hamburg, Germany) in a 25-μl one-step Brilliant II SYBR Green qPCR Master Mix (Stratagene, La Jolla, CA) containing 100 nM of specific sense and antisense primers. Human mPR primers were as follows: mPR: sense 5′-CTGAAGTTTG-GCCTGACACCA, antisense 5′-AATTAGAACGCCAACGTTCTGA; mPRβ: sense 5′-TACCTACCTGACGCTTCT, antisense 5′-GC-AACAGCAGCAAAAGATA; mPRy: sense 5′-ACTATGGTGCC-GTCAACCTC, antisense 5′-TGTAAGGATAAGCAGGAGCCG-3. Primers for nPR were 5′-GAGCTTAAATGTTGGTCTTG (sense) and 5′-GTGGTATCGCTTGGCGCCCT (antisense). PGRMC1 primers were 5′-GTGCACCAAGAGCAAGGAT (sense) and GGACAA-CAGTGCAGCCAGA (antisense).

A housekeeping gene, β-actin, was used for a loading control and normalization (sense 5′-AAGAGGACCTCCTACCCCT, antisense 5′-TACATGCTGGGGTTGTGGA).

PCR was conducted with a denaturation step of 94°C for 10 min, followed by 40 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min. A melting cycle was followed with the prome of 95°C for 15 s and 55°C for 15 s, and then the temperature was ramped up to 95°C over a 20-min period and maintained for 15 s to verify the specificity of the PCR products. The amplification efficiency of each pair of qPCR primers was validated using serially diluted RT samples, and the cDNA levels in the reactions were calculated from the equation of efficiency: (EFF) = 10 ((1/slope) – 1). All the tested primers had similar efficiencies of ~97–99%. qPCR data were analyzed using the comparative quantitation method. The relative expression level of each gene was calculated from 2−ΔΔCT (2−ΔCT gene − ΔCT actin) values, and the results were multiplied by 104 and defined as arbitrary units or percent over vehicle treatment.

Western blot analysis of progesterone receptors. Western blot assays were performed on HUVECs after 15 days of culture, following published procedures (44). Plasma membranes and cell lysates were prepared as described for the progesterone receptor binding assay and Western blot analysis of eNOS and kinases. Plasma membranes were solubilized and cell lysates (5±20 μg of protein) prepared for electrophoresis by incubation in 5% reducing sample buffer (Pierce, Rockford, IL) for 15 min at room temperature and then loaded onto a 10% polyacrylamide gel. The proteins were separated by PAGE. The proteins were transferred from the gels onto nitrocellulose membranes (Bio-Rad, Hercules, CA), washed three times with Tris-buffered saline (TBS), and blocked with blocking reagent (LI-COR Biosciences, Lincoln, NE). The membranes were then incubated overnight at 4°C in TBS + blocking reagent containing the previously validated polyclonal mPRs, β-, or γ-antibodies (1:2,000) (23, 38, 64) or a specific PGRMC1 antibody (1:2,000) (64) or nPR antibody (1:1,000) (SC-130071; Santa Cruz Biotechnology) (44). The membranes were subsequently incubated with fluorescent-conjugated secondary antibodies (1:2,500) (LI-COR Biosciences) in PBS for 1 h at room temperature. The membranes were washed three times and scanned with the Odyssey Infrared Imaging System (LI-COR Biosciences).

Western blot analysis of eNOS, Akt, and ERK phosphorylation. Progestin- and kinase inhibitor-induced changes in eNOS, Akt, and ERK phosphorylation were measured in HUVECs that had been cultured in 12-well plates for 15 days by Western blot analysis, following the general procedures described for progesterone receptors. The cells were serum-starved for 2 h prior to treatment for 15 min with the progestins or actinomycin D. The cells were pretreated with kinase inhibitors for 30 min before the addition of progestins. At the end of the treatment period, the cells were lysed by vortexing with 100 μl of IP RIPA buffer containing protease inhibitor cocktail (Thermo Scientific) for 30 min at 4°C, and the mixture was centrifuged at 14,000 g for 10 min at 4°C to remove the cell debris. The supernatant lysates were either used for Western blot analysis immediately or stored in a freezer at −80°C for later measurement. Western blots were conducted with antibody concentrations of 1:1,000 for phosphorylated eNOS and eNOS, 1:2,000 for phosphorylated ERK and ERK, 1:1,000 for phosphorylated Akt, and 1:1,500 for Akt. Phosphorylated eNOS, ERK, and Akt expressions were normalized to total eNOS, ERK, and Akt, respectively, using ImageJ software to estimate relative densitometries.

Immunocytochemical and immunohistochemical analysis of progesterone receptors. Immunocytochemical analysis was performed on HUVECs immediately after harvesting or after culture for 15 days, following previously published procedures using the mPR, nPR, and PGRMC1 antibodies used in the Western blot analyses (43, 65). HUVECs were grown on glass coverslips, fixed with 2% paraformaldehyde and 0.5% glutaraldehyde, and then permeabilized with 0.5% Triton-X. The cells were blocked with 2% bovine serum albumin for 1.5 h and then incubated with mPRα, β- and γ- (1:1,000), nPR (1:500), and PGRMC1 (1:500) antibodies overnight at 4°C. The cells were washed, followed by incubation with Alexa Fluor 488 secondary antibodies for 1 h and an additional three washes prior to mounting the coverslips on glass slides with ProLong Gold antifade reagent with DAPI (Invitrogen, Grand Island, NY). Fluorescent-labeled mPRs, nPR, and PGRMC1 proteins in the HUVECs were visualized using a Nikon inverted microscope with a MetaVue Research Imaging System. Nikon C2 plus confocal microscope and NIS image analysis software were used to obtain confocal images.

For immunohistochemical analysis, the paraffin-embedded tissue sections were deparaffinized and rehydrated with standard methods, and then the above immunocytochemical (ICC) procedures were followed.

Measurement of eNOS activity. The eNOS activity in HUVECs was measured using a NO synthase assay kit (Calbiochem, Billerica, MA), following the manufacturer’s instructions. Cell lysates were co- incubated with [3H]arginine and then filtered through resin columns to retain the unconverted [3H]arginine. The radioactivity of the [3H]citrulline product eluting from the column was measured in a scintillation counter. The relative eNOS activities of the steroid-treated samples were expressed as percentages of the mean activity of the vehicle-treated samples.

Measurement of cellular NO. The NO levels in the incubated and treated HUVECs were measured with DAF-FM (a NO indicator) assay, as described previously (63). Cells were subcultured in 96-well plates (black with clear flat bottom; Falcon) overnight and then serum-starved for 2 h with serum- and VEGF-free TCM-199 medium before experimentation. Cells were preincubated with 50 μl of 3 μM DAF-FM diacetate (Invitrogen) for 30 min as suggested by the manufacturer and incubated for another 30 min in fresh medium prior to incubation with the steroid or inhibitor treatments at 37°C for 20 min. In some experiments, cells were pretreated with kinase inhibitors.
for 30 min before the addition of steroid treatments. The NO levels were measured with a fluorescence plate reader (CLARIOstar; BMG Labtech, Cary, NC) at 495/515 nm.

The cAMP measurement. HUVECs were subcultured in 12-well plates, the medium was replaced with TC-199 without serum and VEGF, and HUVECs were incubated for 2 h before treatment with progesterone, Org OD 02-0, or R5020 for 20 min. The cells were lysed, and the cAMP levels in the samples were measured immediately with a cAMP EIA kit (Cayman Chemical, Ann Arbor, MI), following the manufacturer’s instructions.

Knockdown of mPRα and nPR with siRNA. Antisense siRNA oligos for mPRα (ON-TARGETplus, SMARTPool) and nPR (h2) were purchased from Dharmacon (Lafayette, CO) and Santa Cruz Biotechnology (Dallas, TX), respectively. HUVECs were transfected twice with siRNA or nontargeting oligos (100 nM) at 0 and 16 h, using Lipofectamine 2000 (Invitrogen) as transfection reagent. The siRNA mix was replaced with normal medium after 48 h of incubation, and the cells were cultured for an additional 24 h before their use in experiments.

Immunoprecipitation of G protein α-subunits. Activation of G proteins and immunoprecipitation of their α-subunits was performed as described previously (65). Plasma membranes of HUVECs were extracted as described in the progesterone receptor binding assay and incubated with 100 nM progesterone for 30 min in the presence of [35S]GTPγS (1,250 Ci/mmol; GE Healthcare). The activated [35S]GTPγS-labeled G protein α-subunits were precipitated with specific α-subunit antibodies for inhibitory (Gi) and stimulatory (Gs) G proteins using protein A/G plus beads (Santa Cruz Biotechnology).

Statistics. Saturation curves of progesterone binding in radio receptor assays were analyzed by nonlinear regression, and the dissociation constants (Kd) and binding capacities (Bmax) were calculated using GraphPad Prism Software (version 3.02; GraphPad Prism, San Diego, CA). Results are expressed as means ± SE of 4–6 observations, and all experiments were repeated at least three times with different batches of cells from different donors. Student’s paired r-test was used for paired comparisons, and one-way ANOVA with Newman-Keuls’ multiple-comparison tests was used to determine differences between multiple experimental treatments using GraphPad Prism software.

RESULTS

Localization of progesterone receptors in HUVECs and vascular tissues. Western blot analyses with specific mPR and PGRMC1 antibodies showed the presence of strong immunoreactive protein bands of the predicted molecular mass for dimers of mPRα, -β, and -γ (~80 kDa) and for the monomers for mPRα (~40 kDa) and PGRMC1 (~28 kDa) in plasma membrane extracts prepared from HUVECs that had been cultured for 15 days (Fig. 1A). Western blotting with the nPR antibody detected a weak band of the predicted size for nPR (~120 kDa) in cell lysates (Fig. 1A). The sizes of these bands matched those of recombinant mPRα, -β, and -γ, PGRMC1 overexpressed in MDA-MB-231 breast cancer cells, and wild-type nPR highly expressed in T47DyB breast cancer cells (Fig. 1A). ICC analysis showed that the three mPR subtypes were localized on the cell membranes of HUVECs after 15 days of culture, whereas the PGRMC1 protein was distributed on the cell membranes of HUVECs (Fig. 1B). A similar pattern of significant mPRα expression and weak nPR staining was observed in freshly harvested HUVECs within 2 h of collection (mPRα initial, nPR initial; Fig. 1C). Localization of mPRα was also investigated in umbilical vein, umbilical artery, and pulmonary artery tissues. The mPRα protein was detected in the endothelial cells of all three tissues and also in the surrounding smooth muscle cells in the umbilical artery and vein (Fig. 1E).

The expression of mPRα, -β, and -γ, nPR, and PGRMC1 mRNAs in HUVECs did not change significantly from day 1 to day 15 of cell culture (Fig. 2, A and B), suggesting that these physiological characteristics of HUVECs were well-maintained during the 15-day cell culture period prior to experimentation. Progesterone treatment (20 nM) for 16 h caused a similar upregulation of mPRα and nPR mRNA expression in cells cultured for 1 and 10 days, suggesting that the progesterone responsiveness of HUVECs also did not change significantly during cell culture (Fig. 2C). The progestin activities of the lots of Org OD 02-0 and R5020 used in this study were tested in an assay of mPRα and nPR mRNA upregulation in human breast cancer T47DyB cells. Treatment with the selective mPR agonist Org OD 02-0 (20 nM) increased mPR expression, whereas the potent nPR agonist R5020 (20 nM) increased nPR expression in T47DyB cells (Fig. 2D), which confirms the biological activities of these progestins and suggests that mPR and nPR mRNAs are upregulated by progesterone through their own receptors.

[3H]progesterone binding to HUVEC membranes and G protein activation. Saturation analyses and Scatchard plots of [3H]P4 binding showed the presence of a high-affinity, limited capacity (Bmax = 1.63 nM/mg membrane protein), single binding site on plasma membranes of HUVECs with a dissociation constant (Kd = 9.43 nM) similar to that reported for mPRs (Fig. 3A). A single-point ligand competition assay showed that the progestin specificity of binding to HUVECs is characteristic of mPRs but differs from that of nPR in that the selective mPR agonist Org OD 02-0 caused significant displacement of [3H]P4 binding, whereas the nPR agonists R5020 and MPA were ineffective (Fig. 3B). Progesterone treatment caused G protein activation, which was shown by an increase in [35S]GTPγS binding to HUVEC plasma membranes, and was immunoprecipitated with an inhibitory G protein α-subunit (Gia) antibody, but not with a stimulatory G protein (Gsa) antibody or IgG control, indicating that progesterone activation of second-messenger pathways in HUVECs through mPRs is mediated through an inhibitory G protein (Fig. 3C). Treatment with 20 nM progesterone and Org OD 02-0, but not with R5020 and MPA, decreased cellular cAMP levels (Fig. 3D) presumably by reducing the activity of membrane adenylyl cyclase, which is consistent with progesterone activation of an inhibitory G protein (Gia) through mPRs in HUVECs.

Progesterone upregulation of NO production, eNOS activity, and eNOS phosphorylation in HUVECs. Production of NO by HUVECs was significantly elevated by 20 nM progesterone and Org OD 02-0 treatments, but not with R5020 treatment, after incubation for 30 and 60 min (Fig. 4A). Treatment with 20 and 100 nM progesterone and Org OD 02-0 significantly increased NO production after 30 min of incubation, whereas a lower concentration (5 nM) was ineffective (Fig. 4B). The stimulatory effect of 20 nM progesterone on NO production was mimicked by the cell-impermeable progesterone conjugate progesterone-bovine serum albumin, demonstrating that this progesterone action is mediated at the cell surface (Fig. 4C). In contrast, MPA and R5020 were ineffective at increasing NO production (Fig. 4C). NOS activity was significantly increased.
after 15 and 30 min of incubation with 20 nM progesterone (Fig. 4D). Both progesterone and Org OD 02-0 (20 nM) elevated cellular NOS activity significantly, whereas no increase was observed after treatment with R5020 (Fig. 4E). The role of eNOS in progesterone upregulation of NO production and NOS activity was investigated by assessing eNOS phosphorylation in HUVECs after progestin treatments. Treatment with 5, 20, and 100 nM progesterone and 20 and 100 nM Org OD 02-0 increased eNOS phosphorylation significantly compared with vehicle controls in the HUVECs, but treatment with 100 nM R5020 had no effect (Fig. 4, F and G), which suggests that progesterone phosphorylation of eNOS is mediated by mPRs.

Effects of knockdown of mPRα and nPR expression on progesterone-induced increases in NO production and eNOS phosphorylation. HUVECs were transfected with siRNA for mPRα to determine whether this mPR subtype is the primary intermediary in the progesterone-induced increase in eNOS phosphorylation and NO production. Transfection with mPRα siRNA knocked down mPRα protein expression on cell membranes and also abolished the 20 and 100 nM progesterone-induced increase in eNOS phosphorylation (Fig. 5A). The increase in NO production in response to progesterone and Org OD 02-0 treatments was also blocked in the mPRα siRNA-transfected cells (Fig. 5B). In contrast, knockdown of nPR expression by treatment with nPR siRNA did not attenuate progesterone (20 and 100 nM) stimulation of eNOS phosphorylation (Fig. 5C) or NO production (Fig. 5D). Pretreatment with pertussis toxin, which blocks activation of the inhibitory G protein Gαi, completely abolished the progesterone- and Org OD 02-0-induced increases in NO production (Fig. 5E), indicating an involvement of Gαi in this progestin action. Cotreatment with ICI-182,780, a selective nuclear estrogen receptor antagonist, with progesterone or Org OD 02-0 did not influence their effectiveness at 20 nM in stimulating NO production (Fig. 5F), suggesting that these progesterone effects
are not mediated through an estrogen receptor-dependent mechanism. Collectively, these data provide strong evidence that mPRs is the dominant receptor that mediates these rapid progesterone actions in HUVECs.

**Involvement of the PI3K/Akt pathway in progesterone stimulation of eNOS phosphorylation and NO production.** Treatment with progesterone and progesterone-BSA for 15 min increased the phosphorylation of Akt, and cotreatment with Actinomycin D, a transcription inhibitor, did not substantially attenuate this rapid progesterone action (Fig. 6A). Progesterone and Org OD 02-0 (20 and 100 nM) increased phosphorylation of Akt significantly, but 100 nM R5020 was ineffective (Fig. 6B). These results suggest that the cell membrane-initiated actions of progesterone through mPRs induce rapid PI3K/Akt signaling. Treatment with the selective PI3K inhibitors LY-294002 (25 μM) and wortmannin (100 nM) blocked phosphorylation of Akt and also completely blocked basal and progesterone- and Org OD 02-0-stimulated phosphorylation of eNOS (Fig. 6C). Wortmannin also blocked the stimulation of NO production by these progestins (Fig. 6D). Similarly, the in-
creases in eNOS phosphorylation and NO production induced by progesterone and Org OD 02-0 were also abolished by treatment with ML-9 (100 nM), an Akt inhibitor (Fig. 6, E and F). These results demonstrate that activation of the PI3K/Akt pathway is necessary for progesterone and Org OD 02-0 stimulation of eNOS phosphorylation and NO synthesis in HUVECs.

**Involvement of MAP kinase in progesterone stimulation of eNOS phosphorylation and NO production.** Both progesterone and progesterone-BSA (20 and 100 nM) treatments increased phosphorylation of ERK significantly, which was not markedly attenuated by cotreatment with actinomycin D (Fig. 7, A and B), suggesting that progesterone acts through mPRs on the cell surface to rapidly activate MAP kinase by a nongenomic mechanism. Treatment with PD-98059 (10 nM), a highly selective MAP kinase inhibitor, and AZD6244 (20 μM), a specific ERK1/2 inhibitor, in addition to blocking phosphorylation of ERK, also completely abolished progesterone- and Org OD 02-0-induced phosphorylation of eNOS (Fig. 7, C and E). In addition, these inhibitors blocked progestin upregulation of NO production (Fig. 7, D and F). These results demonstrate that activation of MAP kinase is necessary for progesterone and Org OD 02-0 stimulation of eNOS phosphorylation and NO synthesis in the HUVECs.

**Interactions between PI3K/Akt and MAP kinase signaling pathways activated by progesterone.** Treatment with the Akt inhibitor wortmannin (100 nM) completely blocked progesterone (20 nM) phosphorylation of ERK (Fig. 8A), suggesting that the PI3K/Akt pathway is necessary for progesterone activation of MAP kinase. Treatment with AZD6244, an ERK inhibitor,
The present results demonstrate clearly that mPRα is the primary receptor mediating the rapid, nongenomic actions of physiologically relevant concentrations of progesterone in human vascular endothelial cells to increase NO synthesis. This is the first comprehensive and unequivocal evidence for a unique role of mPRs distinct from that of nPRs in mediating an important physiological function of progesterone in mammalian cells. Knockdown studies with mPRα and nPR siRNAs provide definitive evidence that mPRα is the principal receptor regulating these rapid effects of progesterone (20 nM) on NO production and that the nPR is not involved. The stimulatory effects of 20 nM progesterone on NO production and phosphorylation of Akt and ERK were mimicked by the cell-impermeable progesterone conjugate progesterone-BSA, indicating that these progesterone actions are induced at the cell surface. The mPRs are the most likely candidates as intermediaries in these cell surface-initiated progesterone actions because the Western blot and ICC results show high expression of mPRα and the other mPR subtypes and their localization on the plasma membranes, whereas nPR expression is low and is detected only in the cytoplasm of HUVECs. Binding of [3H]progesterone is also characteristic of mPRs, and not of the nPR, with a Kd of 9.43 nM and displacement of progesterone binding with the specific mPR agonist Org OD 02-0 but not with the superactive nPR agonist R5020. The finding that Org OD 02-0 (20 nM) mimicked the stimulatory effects of progesterone on the entire signaling pathway regulating NO production, including activation of PI3K/Akt and MAP kinase, whereas R5020 was ineffective, further supports an exclusive role for mPRs in mediating these vascular protective effects. Collectively, these results demonstrate clearly that mPRs exert important protective functions of progesterone in the human vascular system.

Although similar rapid stimulatory effects of nanomolar concentrations of progesterone on NO production and eNOS activity in HUVECs and aortic strips have been reported previously, the receptor mechanisms mediating these progesterone actions and the possible role of mPRs were not inves-
tigated in these studies (52, 56). MPA was shown to be devoid of these activities (56), in agreement with the results of this study. In addition, it was demonstrated that this progesterone action involved activation of PI3K/Akt and MAP kinase, in accord with the present results (56). However, based on the results of their experiments showing that the nPR/GR antagonist RU-486 and the nPR antagonist Org 31710 blocked these progesterone actions, Simoncini et al. (56) proposed that they were mediated by the nPR. The present extensive negative results using knockdown of nPR with siRNA and the specific nPR agonist R5020 do not support such a role for nPR. Moreover, the ineffectiveness of MPA in inducing NO production is consistent with its low binding affinity for mPRs on HUVEC membranes and its inability to downregulate cAMP production, a mPRα-dependent progesterone response mediated through activation of an inhibitory G protein (Gii) that has been demonstrated previously in a wide variety of vertebrate cells (12, 23, 59, 65). Conversely, the lack of MPA activity is difficult to reconcile with a nPR-dependent mechanism because MPA has high binding affinity for the nPR and activates it (58). Finally, the finding that progesterone acts at the cell surface to increase NO production is not consistent with an involvement of the nPR because to date clear evidence that the nPR is expressed on the cell surface has been obtained for only a few types of vertebrate cells (5). Although Boonyaratana-kornkit et al. (4) unambiguously demonstrated that progesterone rapidly activates intracellular signaling through the nPR in an extranuclear location in breast cancer cells, they could not detect nPR by ICC on the plasma membranes of any of the cell types they examined (3). Equivocal ICC results on membrane nPR localization have been obtained in HUVECs (15, 69) because the antibodies used are either not specific for nPR or are directed against a different protein (15, 44, 63). Although the present results clearly show that progesterone rapidly upregulates NO production by an nPR-independent mechanism, the relative importance of mPRs and nPR as intermediaries in other rapid nongenomic actions of low concentrations of progesterone (10 nM) in HUVECs such as cyclooxygenase and meosin activation (15, 52) is unknown and warrants investigation. However, the nPR undoubtedly mediates longer-term genomic beneficial responses to progesterone, including upregulation of eNOS mRNA in endometrial endothelial cells (25), and also likely upregulates the receptor itself, as shown in the present study with breast cancer cells. It would be worthwhile to explore possible cross-talk between mPRα and the nPR in HUVECs since mPRα has been shown to influence nPR transactivation by downregulating steroid receptor coactivator 2 in human myometrial cells collected at the end of pregnancy (23).

We did not investigate directly the involvement of mPRβ and mPRγ in this nongenomic progesterone action because knockdown of mPRα expression alone completely blocked the progesterone- and Org OD 02-0-induced increases in NO production. Previous studies have shown mPRα is the principal

Fig. 6. Involvement of phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway in progestin upregulation of eNOS phosphorylation and NO production in HUVECs. A and B: effects of P4 (100 nM), P4-BSA (20 and 100 nM), and actinomycin D (AD; 1 μg/ml) (A) and 20 and 100 nM P4, 02, and 100 nM R50 (B) on Akt phosphorylation (pAkt) in HUVECs. C and D: effects of cotreatments with PI3K inhibitors LY-294002 (Ly) and wortmannin (Wm) on phosphorylation of Akt (56 kDa) and eNOS (C) and relative NO production (D) in HUVECs in response to 20 nM P4, 02, and R50 treatments. E and F: effects of cotreatment with the Akt inhibitor ML-9 on phosphorylation of Akt and eNOS (E) and NO production (F) in response to 20 nM P4, 02, and R50 treatments. EGF, epidermal growth factor positive control. Different letters denote significant differences between treatment groups (P < 0.05). Experiments were repeated 3 or more times, and similar results were obtained on each occasion; n = 6.
mPR mediating nongenomic progesterone actions in many reproductive and nonreproductive tissues (12, 59), and similarly to the current results, mPRα is highly expressed on the plasma membranes of murine vascular endothelial cells (72). However, mPRβ is frequently coexpressed with mPRα (10, 11) and is a partner in mediating membrane receptor binding and physiological functions of progesterone in diverse cell types, including human myometrial cells (23), a GnRH neuronal cell line (59), breast cancer cells (44), and fish oocytes (66). Therefore, a contribution of mPRβ to progesterone upregulation of NO synthesis remains a possibility. The nPR antibody used in this study does not distinguish between PR-A and PR-B proteins because it was generated to an amino acid sequence in the COOH-terminal of the receptor. The immunoreactive protein detected by the antibody most likely is PR-A, because only this nPR subtype was detected in HUVECs using nPR subtype-specific antibodies (67). The finding that a third distinct receptor involved in progesterone signaling, PGRMC1, is also ex-

Fig. 7. Involvement of MAP kinase signaling in progestin upregulation of eNOS phosphorylation and NO production in HUVECs. A and B: effects of P₄ (100 nM), P₄-BSA (20 and 100 nM), and AD (1 μg/ml; A) and 20 and 100 nM P₄, 02, and R50 (100 nM) (B) on ERK phosphorylation (pERK; 42/44 kDa) in HUVECs. C–F: effect of cotreatment with MAP kinase inhibitors PD-98059 (PD; C and D) and AZD6244 (AZD; E and F) on P₄- and 02-induced phosphorylation (20 and 100 nM) of ERK and eNOS (C and E) and NO production (D and F). Different letters denote significant differences between treatment groups (P < 0.05). Experiments were repeated 3 or more times, and similar results were obtained on each occasion; n = 6.

Fig. 8. Interactions between MAP kinase and PI3K/Akt signaling pathways activated by P₄ in HUVECs. A: effect of cotreatment with the PI3K inhibitor Wm on 20 nM P₄-induced ERK phosphorylation (pERK). B: effects of cotreatment with the MAP kinase inhibitor AZD on basal and P₄-induced phosphorylation of Akt (pAkt). Different letters denote significant differences between treatment groups (P < 0.05). Experiments were repeated 3 or more times, and similar results were obtained on each occasion; n = 3.

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pressed on the plasma membranes of HUVECs was not unexpected, because recent evidence suggests that membrane expression of PGRMC1 is a requirement for maintaining the receptor functions of mPRα (64). PGRMC1 has been shown to function as an adaptor protein for mPRα, closely associating with it and regulating its expression and functions on the cell membrane (64). PGRMC1 likely influences multiple receptor functions in HUVECs because it also regulates the expression of EGFR and ERβ on the membranes of human cells (1, 64). Finally, the fact that coinubcation with a nuclear estrogen receptor antagonist, ICI 182,780, did not modulate the NO response to the progestin treatments suggests that their effects are not mediated through a nuclear estrogen receptor-dependent pathway. It is noteworthy that the relative expression of all of these progesterone receptors did not change in HUVECs after long-term culture and also that mPRs and nPR remained responsive to hormonal upregulation, suggesting that this in vitro model is a physiologically relevant one for investigating the roles of the different progesterone receptors in progesterone regulation of NO synthesis in human vascular endothelial cells.

Progesterone and Org OD 02–0 induction of NO synthesis in HUVECs through mPR is dependent upon activation of an inhibitory G protein (Gi) since their stimulatory actions were blocked by pretreatment with pertussis toxin. Activation of G proteins has also been implicated in rapid progesterone (10 nM) activation of moesin in HUVECs (13), although the involvement of mPRs was not investigated in that study. The demonstration that 20 nM progesterone and Org OD 02–0 increased Akt and ERK1/2 phosphorylation and that pharmacological inhibition of PI3K/Akt and MAP kinase blocked upregulation of NO synthesis and eNOS phosphorylation by these progestins provides strong evidence that phosphorylation of these kinases is a critical event in the regulation of NO production through mPRα. These signaling pathways have been shown to mediate NO production in response to a variety of progestins and 20 nM dehydroepiandrosterone in human endothelial cells (55, 57). The discovery that the PI3K inhibitor wortmannin decreased both basal and progesterone-induced phosphorylation of ERK in HUVECs (13), although the mechanism of progesterone receptor activation of extra-nuclear signaling pathways in regulating gene transcription and cell cycle progression.

In conclusion, the results of the present study demonstrate clearly that the beneficial rapid, nongenomic effects of progesterone on NO production and eNOS phosphorylation in HUVECs are mediated primarily through mPRs and involve activation of the Akt/PI3K and MAP kinase pathways. Increased NO production by vascular endothelial cells induces relaxation of the surrounding vascular smooth muscle cells, resulting in vasodilation and a decrease in blood pressure. Therefore, the present findings raise the possibility that mPRα could be a useful therapeutic target for the treatment of hypertension. Studies are currently underway to further characterize progesterone actions through mPRα in vascular cells and evaluate whether activation of this novel signaling pathway would be an effective means of regulating blood pressure.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Y.P. and P.T. conception and design of research; Y.P. and J.D. performed experiments; Y.P. and J.D. analyzed data; Y.P. and P.T. interpreted results of experiments; Y.P., J.D., and P.T. prepared figures; Y.P. drafted manuscript; Y.P. and P.T. edited and revised manuscript; Y.P. and P.T. approved final version of manuscript.

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