

# Differential Signal Transduction of Progesterone and Medroxyprogesterone Acetate in Human Endothelial Cells

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The conjugated equine estrogens-only arm of the Women's Health Initiative trial, showing a trend toward protection from heart disease as opposed to women receiving also medroxyprogesterone acetate (MPA), strengthens the debate on the cardiovascular effects of progestins. We compared the effects of progesterone (P) or MPA on the synthesis of nitric oxide and on the expression of leukocyte adhesion molecules, characterizing the signaling events recruited by these compounds. Although P significantly increases nitric oxide synthesis via transcriptional and nontranscriptional mechanisms, MPA is devoid of such effects. Moreover, when used together with physiological estradiol (E2) concentrations, P potentiates E2 effects, whereas MPA impairs E2 signaling. These findings are observed both in isolated human endothelial cells as well as *in vivo*, in ovariectomized rat aortas. A marked difference in the recruitment of MAPK and phosphatidylinositol-3 kinase explains the divergent effects of the two gestagens. In addition, both P and MPA decrease the adhesiveness of endothelial cells for leukocytes when given alone or with estrogen. MPA is more potent than P in inhibiting the expression of vascular cell adhesion molecule-1 and intercellular adhesion molecule-1. However, when administered together with physiological amounts of glucocorticoids, MPA (which also binds glucocorticoid receptor) markedly interferes with the hydrocortisone-dependent stabilization of the transcription factor nuclear factor  $\kappa$ B and with the expression of adhesion molecules, acting as a partial glucocorticoid receptor antagonist. Our findings show significant differences in the signal transduction pathways recruited by P and MPA in endothelial cells, which may have relevant clinical implications. (*Endocrinology* 145: 5745–5756, 2004)

HORMONE REPLACEMENT THERAPY (HRT) has been assumed for a long time to have cardioprotective properties, based on a wealth of observational and mechanistic studies (1). However, recent clinical trials failed to show advantages for women receiving HRT for the prevention of primary (2) or recurrent (3) cardiovascular events. The reason for this is not clear, and studies are being carried out to identify whether genetic background, timing of HRT starting after the menopause, dosages, way of administration, or other variables may influence the cardiovascular effects of HRT.

One relevant issue raised from the Women's Health Initiative trial is the impact that the progestin used may have in altering estrogen's actions on the vessels. The clinical discrepancy between the recently published conjugated equine estrogens (CEE)-alone arm of the trial, where a trend toward benefit for coronary heart disease was observed in

Abbreviations: Ab, Antibody; CEE, conjugated equine estrogens; ds, double-stranded; E2, 17 $\beta$ -estradiol; eNOS, endothelial NO synthase; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GR, glucocorticoid receptor; HRT, hormone replacement therapy; HUVEC, human umbilical vein endothelial cells; ICAM-1, intercellular adhesion molecule-1; LPS, lipopolysaccharide; mAb, monoclonal Ab; MEK, MAPK kinase; MPA, medroxyprogesterone acetate; NF- $\kappa$ B, nuclear factor  $\kappa$ B; NO, nitric oxide; P, progesterone; PI3K, phosphatidylinositol-3 kinase; PR, P receptor; RT, reverse transcription; VCAM-1, vascular cell adhesion molecule-1; vWF, von Willebrand factor.

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younger individuals (4), as opposed to the CEE + medroxyprogesterone acetate (MPA) arm, which reported a nonsignificant increase in coronary heart disease (2), supports the hypothesis that the use of MPA may have some specific effect related to the development of cardiovascular disease, and calls for new research to understand the differences between the progestogenic compounds available for hormonal therapies.

Indeed, although synthetic progestins have been commonly assumed to have analogous clinical effects, there are circumstantial indications that each specific progesterone receptor (PR) ligand may have peculiar cellular effects. For example, detrimental effects of MPA on coronary vasomotion (5) and on arterial remodeling (6) have been described in female monkeys, where natural progesterone (P) has neutral effects (7). In addition, studies in postmenopausal women show that MPA does not share the positive actions of P on exercise-induced myocardial ischemia (8). Discrepancies in the biological actions of these two compounds have also been reported in other cell types, such as neurons (9, 10).

To tentatively explain these biological differences, it can be hypothesized that the distinct pharmacokinetics of the natural or synthetic progestogens as well as the different affinities for the PR may lead to recruit partially divergent signaling pathways in human vascular cells due to differential PR modulation, as previously observed with estrogen receptor ligands. Indeed, P concentration ranges between 2 and 10 ng/ml during the menstrual cycle (11), and MPA levels

have been estimated to vary between 3 and 4 ng/ml after a single oral administration of a 10-mg tablet (12). However, MPA's affinity for PR is about three times higher than that of P (13). An additional level of complexity is added by the fact that some progestins are able to interact with other steroid receptors and may therefore activate non-PR-dependent signaling pathways or compete with the natural ligands for these receptors (13).

Human endothelial cells are central for the function of human vessels in physiological and pathological conditions. Dysfunctional endothelial cells, as during the early phases of atherosclerotic degeneration, are characterized by impaired synthesis of vasodilatory molecules, such as nitric oxide (NO), and by the expression on the cell membrane of adhesion molecules to circulating leukocytes (14). Estrogens have been shown to preserve endothelial function *in vitro* and *in vivo*; however, the addition of P or other synthetic progestins, such as MPA, has been suggested to interfere to different extents with estrogen's actions (15–17).

The aim of this study was to compare the effects of natural P and of MPA on human endothelial cell function. To this aim, we comparatively investigated the effects on NO synthesis and on the expression of endothelial-leukocyte adhesion molecules, with the aim of characterizing the differences in signal transduction recruited by the two compounds.

## Materials and Methods

### Cell cultures and treatments

Human umbilical vein endothelial cells (HUVEC) were cultured as described (18). Before treatments, HUVEC were kept for 48 h in DMEM containing steroid-deprived fetal bovine serum (FBS). Before experiments investigating nontranscriptional effects, HUVEC were kept in DMEM containing no FBS for 8 h. Whenever an inhibitor was used, the compound was added 30 min before starting the treatments. P, MPA, hydrocortisone, RU486, PD98059, and wortmannin were obtained from Sigma-Aldrich (St. Louis, MO); ORG 31710 was a kind gift of Dr. Lenus Kloosterboer, from Organon Akzo Nobel (Oss, The Netherlands); and ICI 182,780 was obtained by Tocris Cookson (Avonmouth, UK).

### Animal treatments

Fertile female Wistar rats weighing 175–200 g were purchased from Harlan Nossan (San Pietro al Natisone, Italy). Animals were kept under 14 h of illumination per day (0600–2000h) and had free access to standard rat chow and tap water. After 14 d, the rats were either sham-operated or ovariectomized in the same estrus cycle stage. Ovariectomy was performed with a mini-laparotomic incision under pentobarbital anesthesia (20 mg/kg). After complete healing of the surgical wounds, ovariectomized rats as well as fertile animals were started on the different treatments. Estradiol valerate (E2; 0.05 mg/kg·d) was administered orally after suspension in pure ethanol and subsequent appropriate dilution in 0.9% NaCl solution for 14 d. P and MPA were dissolved in ethanol and subsequently suspended in sesame oil for daily subcutaneous administration. All fertile rats were in the same stage of the estrous cycle at the beginning of treatment. Animals were euthanized by decapitation under pentobarbital anesthesia (30 mg/kg), and the abdominal aorta was obtained. Aortas were snap-frozen in dry ice and stored at –80 C. Animals were maintained in accordance with the NIH Guide for the Care and Use of Laboratory Animals (available at: [www.nap.edu/readingroom/books/labrats](http://www.nap.edu/readingroom/books/labrats)).

### Endothelial NO synthase (eNOS) activity assay

eNOS activity was determined as conversion of [<sup>3</sup>H]arginine to [<sup>3</sup>H]citrulline in endothelial cell lysates. [<sup>3</sup>H]Citrulline was separated using an acidic ion-exchange resin, as described previously (19). Extracts

incubated with G-nitro-L-arginine-methyl ester (1 mM), served as blank (these samples were used to estimate the background).

### Nitrite assay

NO production was determined by a nitrite assay using 2,3-diaminonaphthalene (20). Fluorescence of 1-(*H*)-naphthotriazole was measured with excitation and emission wavelengths of 365 and 450 nm. Standard curves were constructed with sodium nitrite. Nonspecific fluorescence was determined in the presence of L-nitro-methyl-arginine (3 mM).

### Immunoblottings

Cell lysates were separated by SDS-PAGE. Antibodies used were: eNOS (Transduction Laboratories, Lexington, KY), wild-type or Tyr<sup>204</sup>-P-ERK1/2 (Calbiochem, San Diego, CA), Ser<sup>177</sup>-P-eNOS, wild-type and Thr<sup>308</sup>-P-Akt (Upstate Biotechnology, Lake Placid, NY), PR, glucocorticoid receptor (GR), von Willebrand factor (vWF), vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), and p65 (Santa Cruz Biotechnology, Santa Cruz, CA). Primary and secondary antibodies (Abs) were incubated with the membranes with the standard technique (21). Immunodetection was accomplished using enhanced chemiluminescence.

### Semiquantitative RT-PCR

RNA isolation, reverse transcription (RT), and semiquantitative PCR were performed with standard techniques. Primer pairs for eNOS (left primer: 5'-TAG CTG TGC TGG CAT ACA GG-3'; right primer: 5'-TGT CCT GCA CGT AGG TCT TG-3') and GAPDH (left primer: 5'-GTC AGT GGT GGA CCT GAC CT-3'; right primer: 5'-CCC TGT TGC TGT AGC CAA AT-3') were used to amplify in a single tube the relative cDNAs for 26 cycles. Each PCR cycle consisted of denaturing at 95 C for 30 sec, annealing at 53.5 C for 45 sec, and elongation at 72 C for 60 sec. The linear exponential phases for eNOS and GAPDH PCR were 35 and 30 cycles, respectively. Equal amounts of RT-PCR products were loaded on 2% agarose gels. Optical densities of ethidium bromide-stained DNA bands were quantitated using the Scion Image program (Scion Corp., Frederick, MD).

### Monocytoid cell adhesion assays

U937 monocytoid cells were obtained through American Tissue Culture Collection (ATCC, Rockville, MD) and grown in RPMI medium 1640 (Life Technologies, Inc.) containing 10% FBS. Confluent HUVEC were exposed to bacterial lipopolysaccharide (LPS; 100 ng/ml) for 18 h in the presence or absence of other treatments. Some monolayers were treated with blocking monoclonal Ab (mAb) *vs.* VCAM-1 or ICAM-1. Adhesion assays were performed adding 10<sup>6</sup> U937 cells to each monolayer under rotating conditions (63 rpm) at 21 C, as described (22). Nonadhering cells were removed by gentle washing, and monolayers were fixed with 1% paraformaldehyde. The number of adherent cells was blindly determined counting eight different fields, using an ocular grid and a 20× magnifying lens.

### Cell surface enzyme immunoassay

Endothelial cells were grown on 96-well culture dishes. LPS was used to induce the expression of VCAM-1 and ICAM-1 (100 ng/ml for 18 h). The assay of cell surface VCAM-1 or ICAM-1 expression was carried out using the mAb against VCAM-1 (Ab E1/6), ICAM-1 (HU 5/3), or the mAb E1/1 in control experiments, recognizing a constitutive and non-cytokine-inducible endothelial cell antigen (23) (all provided by Michael A. Gimbrone, Jr., Harvard Medical School). Enzyme immunoassays were carried out by incubating monolayers with the primary antibodies, then with biotinylated secondary Abs, and finally with streptavidin-alkaline phosphatase. Three washes with PBS + 1% BSA were performed between each incubation step, and integrity of the monolayers was monitored by phase contrast microscopy. Protein surface expression was quantified spectrophotometrically at 450-nm wavelength 20 min after the addition of the chromogenic substrate *para*-nitrophenylphosphate, as described elsewhere (22). Eight different wells were used for each experimental condition.

**Preparation of nuclear extracts and EMSA**

Nuclear extracts were prepared according to Dignam *et al.* (24), with the additional step of washing nuclear pellets in low-salt buffer before high-salt extraction of proteins to remove cytosolic contamination. The VCAM-1 promoter oligonucleotide was synthesized to encompass the two nuclear factor  $\kappa$ B (NF- $\kappa$ B) consensus repeats described at coordinates -77 and -63 of the human VCAM-1 promoter (25): 5'-TGCCTGGGTTTCCCCTTGAAGGGATTCCCTCC-3'. Double-stranded (ds)-oligonucleotides were radiolabeled with T<sub>4</sub> polynucleotide kinase with 50  $\mu$ Ci [ $\gamma$ -<sup>32</sup>P]deoxy-ATP (3000 Ci/mmol). The DNA binding reaction was performed at 20 C for 20 min in a buffer containing 2  $\mu$ g polydeoxyinosinic deoxycytidylic acid, 10  $\mu$ g BSA, 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, and 5% glycerol (total volume, 20  $\mu$ l), with or without the addition of cold competitor ds-oligonucleotide. DNA-protein complexes were resolved on 4% non-denaturing polyacrylamide gels. The dried gel was used to perform autoradiography.

**Cell immunofluorescence**

HUVEC were grown on coverslips and exposed for 2 h to LPS (100 ng/ml) in the presence or absence of other treatments. Cells were fixed and permeabilized with methanol at -20 C for 10 min. Blocking was performed with 3% normal goat serum for 20 min. Cells were incubated with a rabbit polyclonal Ab against the p65 NF- $\kappa$ B subunit for 1 h at RT.

A biotinylated goat antirabbit Ab was used as secondary Ab. After 45 min, streptavidin-fluorescein isothiocyanate was added for 45 min. Immunofluorescence was visualized using an Olympus BX41 microscope and recorded with a high-resolution DP70 Olympus digital camera (Olympus America, Inc., Melville, NY).

**Statistical analysis**

All values are expressed as mean  $\pm$  sd. Statistical differences between mean values were determined by ANOVA, followed by the Fisher's protected least significance difference.

**Results**

**Divergent signal transduction of P and MPA on NO synthesis**

Natural P and MPA differentially affect NO synthesis and eNOS activity in human endothelial cells. Steroid-deprived HUVEC were treated for 48 h with a physiological P concentration (10 nM) or with an equimolar amount of MPA (10 nM). Although P significantly increased the synthesis of NO, MPA was devoid of any stimulatory effect (Fig. 1A). NO induction by P was prevented by the P/GR antagonist RU486 (Fig. 1A).

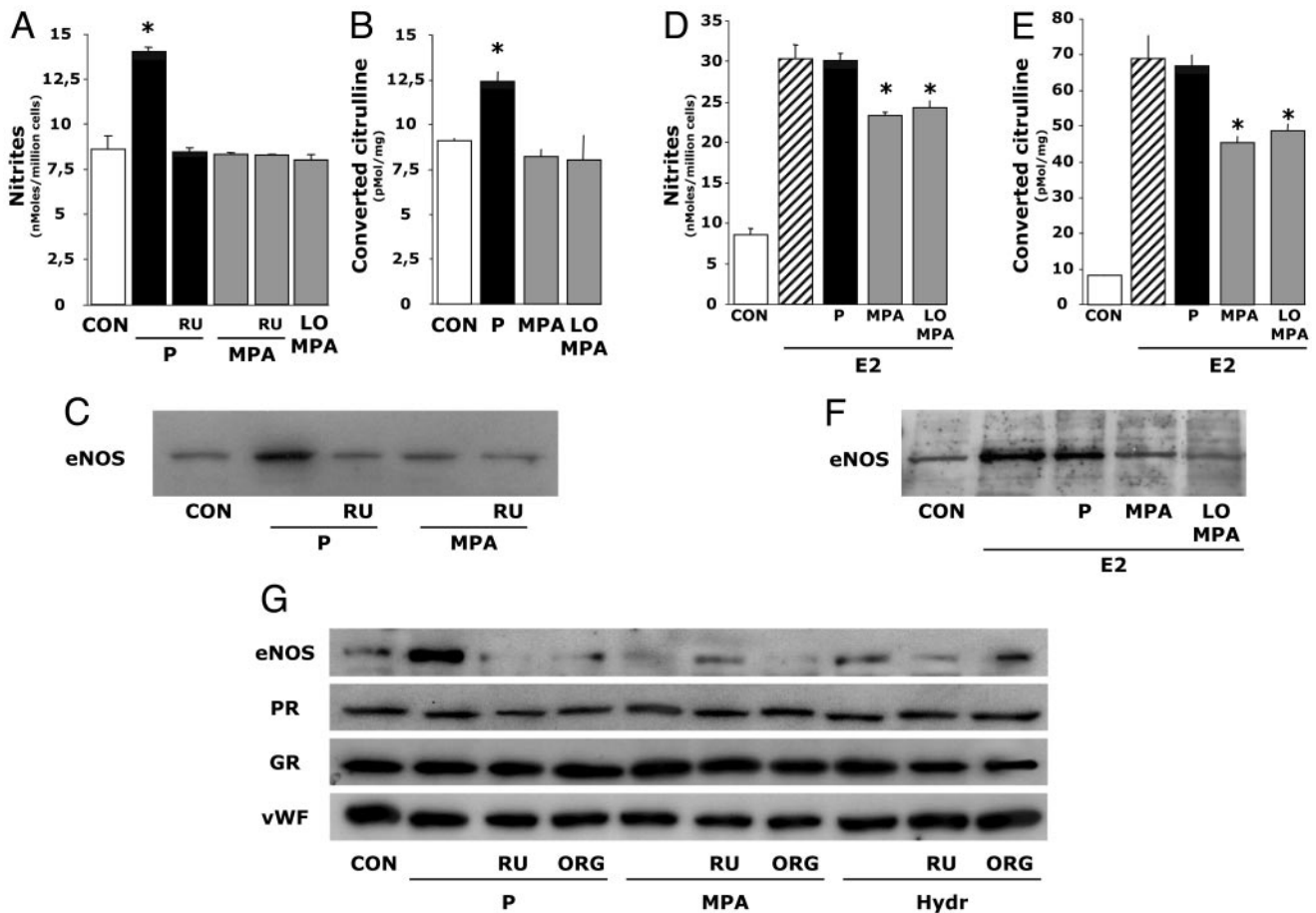


FIG. 1. Divergent transcriptional regulation of eNOS expression by P and MPA in human endothelial cells. Steroid-deprived HUVEC were treated for 48 h with 10 nM P, 10 nM MPA, 0.5 nM MPA (LO MPA), or 50 nM hydrocortisone (Hydr), in the presence or absence of the PR/GR antagonist RU486 (RU; 1  $\mu$ M), the pure PR antagonist ORG 31710 (ORG; 1  $\mu$ M), or 1 nM E2. A and D, Nitrites released in the cell culture medium were assayed. B and E, eNOS activity was tested in whole-cell lysates. C and F, eNOS protein amount was tested on whole-cell protein extracts. G, eNOS, PR, GR, or vWF protein amounts were assayed in cell lysates. A and B, \*,  $P < 0.05$  vs. control. D and E, \*,  $P < 0.05$  vs. E2. The experiments were repeated three times in quadruplicates (nitrite measurement) or in triplicates (eNOS activity), with comparable results. Western blot analyses were repeated three times, and representative blots are shown.



Because MPA is pharmacologically more potent than P, and it is used in clinical practice at an approximately 20-fold lower dose (*e.g.* 200 mg/d oral micronized P *vs.* 10 mg/d oral MPA in HRT), we also tested the effect of MPA when given at 0.5 nM, but we found comparable results (Fig. 1A).

P-dependent NO synthesis is due to increased enzymatic activity of eNOS (Fig. 1B).

*Diverging transcriptional actions of P and MPA on eNOS.* When cellular amounts of eNOS were assayed, increased protein levels were found upon treatment with P, whereas no induction of eNOS was found during treatment with MPA (Fig. 1C). Increased eNOS expression was prevented by RU486 (Fig. 1C).

*P and MPA differently alter estrogen-induced eNOS activity and expression.* When equimolar amounts of P or MPA were used together with E2 (1 nM), divergent effects were also noted. Although the cotreatment with P did not alter the strong NO synthesis induced by E2 (Fig. 1D), the addition of MPA significantly interfered with E2's effect (Fig. 1D). These modifications were accompanied by parallel changes in eNOS activity (Fig. 1E). When used at the 20-fold lower concentration, MPA equally altered E2-dependent NO synthesis and eNOS activity (Fig. 1, D and E).

*MPA decreases the E2-dependent transcriptional induction of eNOS.* E2 consistently increased eNOS expression (Fig. 1F). The addition of P to E2 did not modify the levels of eNOS, however; MPA visibly reduced eNOS expression both at 10 and 0.5 nM (Fig. 1F).

*Relative role of P and GR on eNOS modulation by P and MPA.* Because RU486 also binds to GR, we checked for the role of PR *vs.* GR relative to the effects of P and MPA on eNOS expression. P-induced expression of eNOS was completely prevented by both RU486 and by the pure PR antagonist

ORG 31710 (26) (Fig. 1G), indicating that PR mediates P effects on eNOS expression. MPA and hydrocortisone were each ineffective, showing that GR is not responsible for the induction of eNOS in this setting (Fig. 1G). In addition, we checked for eventual modifications of the content of PR and GR in HUVEC, but no significant change of either steroid receptors or the control protein vWF was seen in any of the different experimental conditions (Fig. 1G).

*In vivo effects of P and MPA on aortic eNOS expression in ovariectomized rats.* To check for the *in vivo* relevance of these observations, we treated ovariectomized Wistar rats with different concentrations of P (2, 4, or 8 mg/kg·d, corresponding to a dose of 100, 200, or 400 mg/d for a 50-kg woman) or MPA (0.02, 0.05, or 0.1 mg/kg·d, corresponding to 1, 2, or 5 mg/d for a 50-kg woman) for 14 d. Then, we measured the amount of eNOS expression as well as eNOS enzymatic activity in the abdominal aortas of the animals. As previously shown (27), ovariectomy is associated with a significant loss of eNOS expression in the abdominal aorta of Wistar rats, in the absence of modifications of the control protein vWF (Fig. 2A). Administration of increasing amounts of P was associated with partial, dose-dependent replenishment of eNOS expression, whereas MPA was completely ineffective (Fig. 2A). Administration of oral E2 (0.05 mg/kg·d, corresponding to 2.5 mg/d for a 50-kg woman) to ovariectomized animals induced fertile levels of eNOS expression in the abdominal aorta (Fig. 2B). Coadministration of P did not alter eNOS expression (Fig. 2B). However, when MPA was added to E2, a significant reduction of aortic eNOS expression was seen (Fig. 2B).

The modifications of eNOS expression were accompanied by parallel changes of eNOS enzymatic activity in aortic lysates (Fig. 2, A and B), indicating that the changes of eNOS expression resulted in modifications of vascular function.

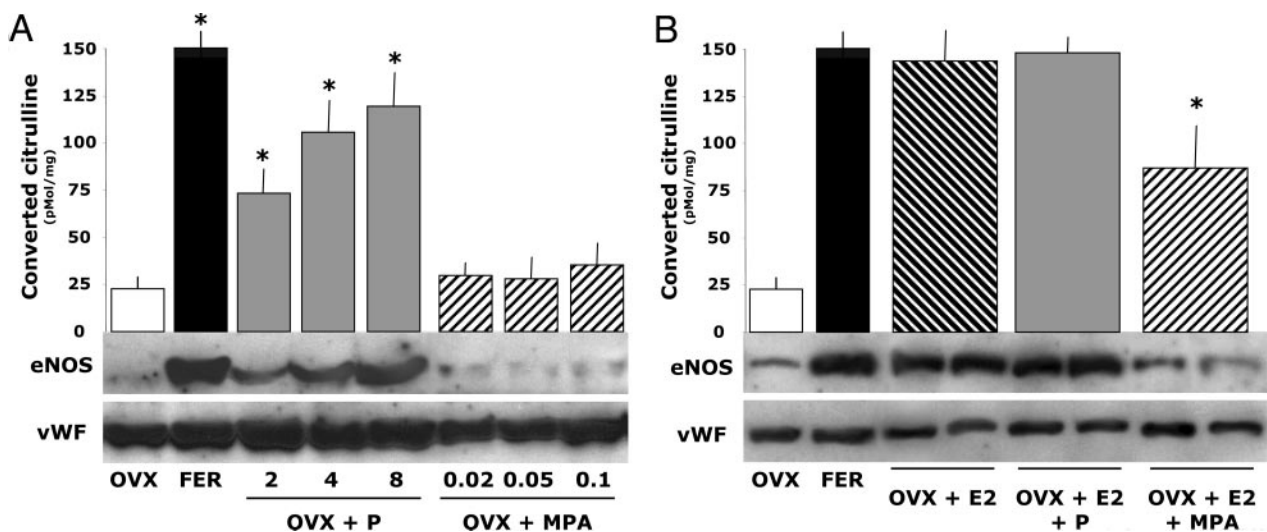


FIG. 2. Differential actions of P and MPA on aortic eNOS expression and activity in ovariectomized Wistar rats. Ovariectomized (OVX) adult Wistar rats were treated with vehicle or with oral E2 (0.05 mg/kg·d), subcutaneous P (2, 4, or 8 mg/kg·d), or subcutaneous MPA (0.02, 0.05, or 0.1 mg/kg·d) for 14 d. Aortic lysates were obtained and eNOS protein amount or eNOS activity was assayed. A, The blots show the amounts of eNOS or vWF in representative abdominal aorta samples (of 12 animals for each condition). B, The blots show the aortic amounts of eNOS or vWF in two of 12 different aortic lysates per condition. The Western analysis of the other samples provided equal results. The mean  $\pm$  SD eNOS activity of the 12 aortas assayed (per condition) is shown above the corresponding condition. A, Asterisks indicate a significant difference ( $P < 0.05$ ) *vs.* ovariectomized (OVX) rats. B, Asterisks indicate a significant difference ( $P < 0.05$ ) *vs.* fertile (FER) rats.

*Nongenomic modulation of eNOS by P and MPA.* NO production is modulated by steroids through nongenomic activation of eNOS (21, 22, 28, 29). When endothelial cells were exposed for 30 min to P, PR-dependent induction of NO synthesis and eNOS activity were observed (Fig. 3, A and B). On the contrary, MPA did not activate eNOS or NO synthesis at both tested concentrations (Fig. 3, A and B).

NO synthesis induced by P is triggered through nongenomic activation of eNOS, because no increase in eNOS protein was found (Fig. 3C). In parallel, the absence of NO induction by MPA is not due to altered eNOS expression (Fig. 3C). In agreement with the absence of gene induction during these short treatments, the absolute increase in eNOS activity and NO synthesis from baseline is lower than during longer exposures (Fig. 1, A, B, D, and E).

*Differential effects of P and MPA on nongenomic estrogen-dependent NO production.* E2 rapidly triggered NO synthesis (Fig. 3D) due to acute activation of eNOS (Fig. 3E). When P or MPA was added to E2, an additive effect on NO synthesis was observed for P but not for MPA (Fig. 3, D and E).

None of the steroid combinations used were associated with altered eNOS expression, indicating nongenomic actions (Fig. 3F). In addition, when semiquantitative RT and PCR (RT-PCR) were performed amplifying the cDNAs for eNOS and for the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH), no changes of eNOS mRNA were seen in cells exposed to E2, P, or MPA for 30 min (Fig. 3G). The absence of requirement of transcriptional modulation is also supported by the ability of a 30-min P treatment to induce NO synthesis and eNOS activation in the presence of a mRNA synthesis inhibitor,

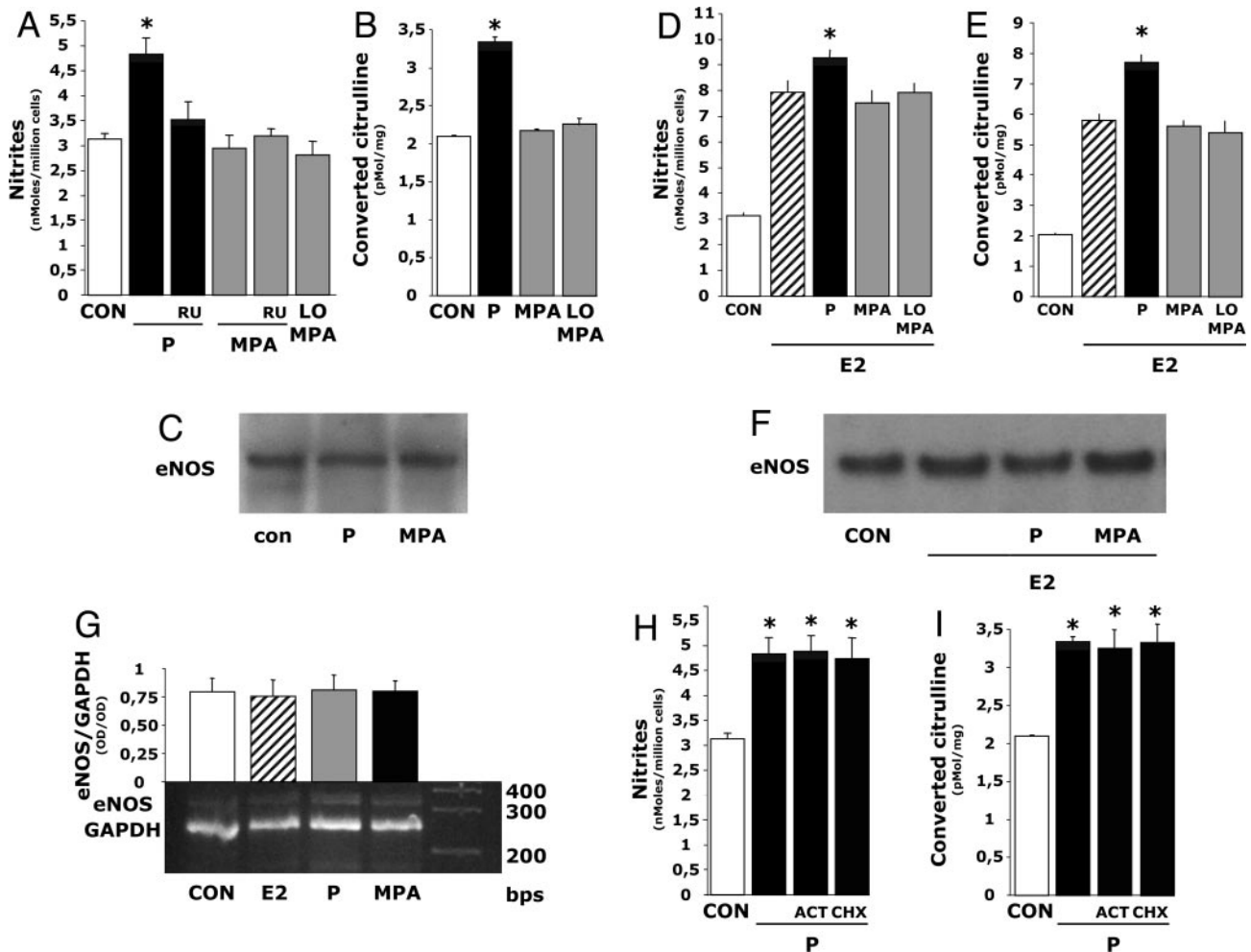


FIG. 3. Rapid eNOS activation by P (but not MPA) in endothelial cells. Steroid-deprived and serum-starved HUVEC were treated for 30 min with 10 nM P or 10 nM or 0.5 nM MPA (LO MPA), in the presence or absence of the PR/GR antagonist RU486 (RU; 1  $\mu$ M); 1 nM E2; or the RNA or protein synthesis inhibitors actinomycin-D (ACT; 5  $\mu$ M) and cycloheximide (CHX; 10  $\mu$ M). A, D, and H, Nitrites released in the cell culture medium were assayed. B, E, and I, eNOS activity was tested in whole-cell lysates. C and F, eNOS Protein amount was tested on whole-cell protein extracts. G, Total mRNA was used to perform semiquantitative RT-PCR, with parallel amplification of eNOS and GAPDH cDNAs. The lower box shows the amplified products, and the upper graph shows the eNOS/GAPDH ratio estimated after densitometric analysis of the amplified bands. A and B, \*,  $P < 0.05$  vs. control. D and E, \*,  $P < 0.05$  vs. E2. The experiments were repeated three times in quadruplicates (nitrites measurement) or in triplicates (eNOS activity), with comparable results. Western blot analysis was repeated three times, and a representative blot is shown. Semiquantitative RT-PCR was performed three times, and the graph shows the mean  $\pm$  SD of the eNOS/GAPDH ratio obtained from pooling the results of the three different experiments.

actinomycin D, or the protein synthesis inhibitor, cycloheximide (Fig. 3, H and I).

**Differential nongenomic signal transduction to eNOS of P and MPA.** P-dependent eNOS activation and NO synthesis were largely prevented by blocking MAPK signaling with the MAPK kinase (MEK) 1/2 inhibitor PD98059 (Fig. 4, A and B). Blockade of phosphatidylinositol-3 kinase (PI3K) with wortmannin resulted in a significant reduction of eNOS activity induced by P, although the decrease was not as large as with MAPK inhibition (Fig. 4, A and B). MPA (alone or with PD98059 or wortmannin) had no stimulatory effect on NO or eNOS (Fig. 4, A and B).

Together, p42 and p44 were phosphorylated upon rapid exposure of endothelial cells to P (Fig. 4C) in a PR-dependent manner. On the contrary, no phosphorylation of ERK 1/2 could be found after challenge with MPA (Fig. 4C), confirming the results obtained by other groups in different cell types (10).

**MAPK is the upstream component of the PR-to-eNOS nongenomic cascade.** To characterize the events between PR engagement and eNOS activation, we tested whether MAPK and PI3K are recruited sequentially or as parallel cascades. Blockade of PI3K did not result in reduced activation of MAPK after treatment with P (Fig. 4D). On the contrary, phosphorylation of Akt induced by P was significantly reduced when the MAPK inhibitor was coadministered (Fig. 4E), suggesting that ERK 1/2 activation occurs upstream of PI3K in the PR-to-eNOS cascade in human endothelial cells.

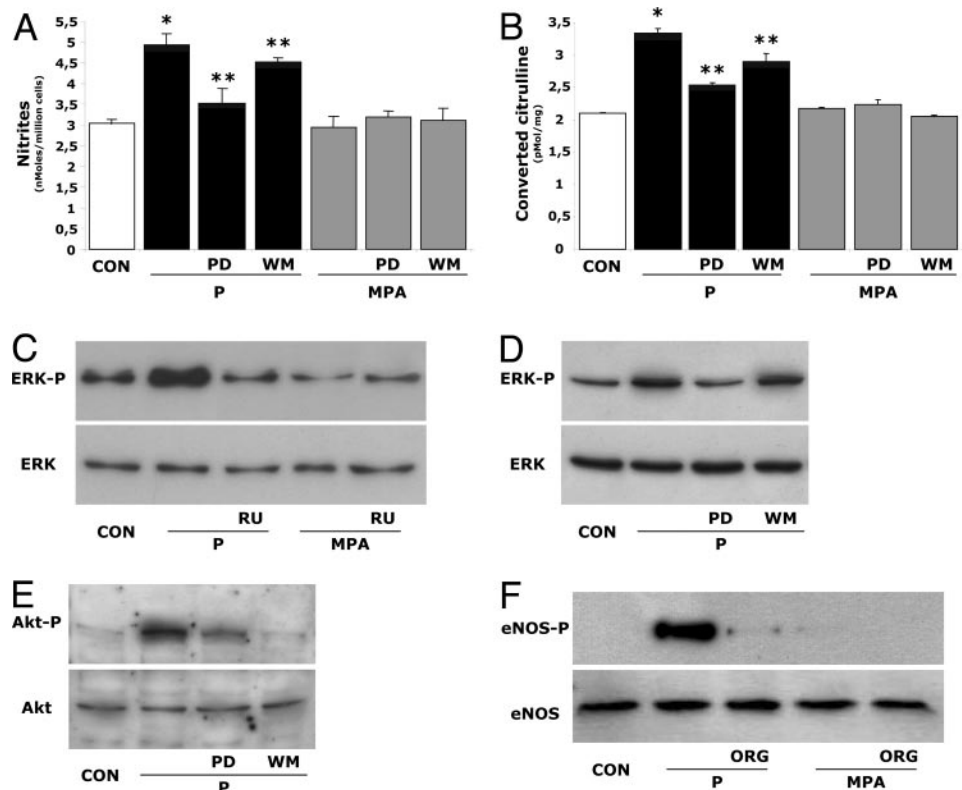
**Site-specific eNOS phosphorylation mediates the rapid PR-dependent activation by P.** Activation of the PI3K/Akt signaling

pathway leads to eNOS activation through an Akt-dependent phosphorylation of eNOS on Ser<sup>1177</sup> (30, 31). In addition, by using a phosphospecific Ab raised toward the phosphorylated form of eNOS Ser<sup>1177</sup>, we were able to show that a 30-min treatment with P results in rapid phosphorylation of eNOS on this residue, in the absence of changes of the overall amounts of wild-type eNOS in endothelial cells (Fig. 4F). ORG 31710 completely prevented this effect, showing that it depends on the recruitment of PR (Fig. 4F). In agreement with the absence of nongenomic actions, MPA did not induce eNOS phosphorylation (Fig. 4F).

**Differential interference of P and MPA with nongenomic eNOS activation induced by estrogen.** Treatment of endothelial cells with E2 induced a significant increase of eNOS activity and NO synthesis (Fig. 5, A and B). Addition of P to E2 resulted in an additive augmentation of eNOS activity (Fig. 5, A and B), and this action was not shared by MPA (Fig. 5, A and B). The addition of MAPK or PI3K inhibitors resulted in reductions of eNOS activity in E2 + P-treated cells, with wortmannin having a stronger effect (Fig. 5, A and B), consistent with PI3K being the major mediator of the nongenomic estrogen-dependent activation of eNOS (21, 29).

When the level of ERK 1/2 phosphorylation was assayed, a potentiation of estrogen-induced p42/p44 activation was observed when P was added (Fig. 5C), whereas no such effect was seen with MPA, which actually resulted in a slight reduction of E2-dependent ERK 1/2 phosphorylation (Fig. 5C). The additional effect associated with P was dependent on PR, as shown by RU486 (Fig. 5C). Moreover, PD98059 prevented the increased Akt phosphorylation induced by P in E2-

FIG. 4. Differential recruitment of MAPK and PI3K/Akt nongenomic signaling pathways by P and MPA. Steroid-deprived and serum-starved HUVEC were treated for 30 min with 10 nM P or MPA, in the presence or absence of the MEK 1/2 inhibitor PD98059 (PD; 5  $\mu$ M); the PI3K inhibitor, wortmannin (WM; 30 nM); the PR/GR antagonist RU486 (RU; 1  $\mu$ M); or the pure PR antagonist ORG 31710 (ORG; 1  $\mu$ M). A, Nitrites released in the cell culture medium were assayed. B, eNOS activity was tested in whole-cell lysates. C and D, Wild-type or phosphorylated ERK 1/2 was assayed. E, Wild-type and phosphorylated Akt were studied. F, Wild-type and phosphorylated eNOS were studied. \*,  $P < 0.05$  vs. control; \*\*,  $P < 0.05$  vs. P alone. The experiments were repeated three times in quadruplicates (nitrite measurement) or in triplicates (eNOS activity), with comparable results. Western blot analyses were repeated three times, and representative blots are shown.





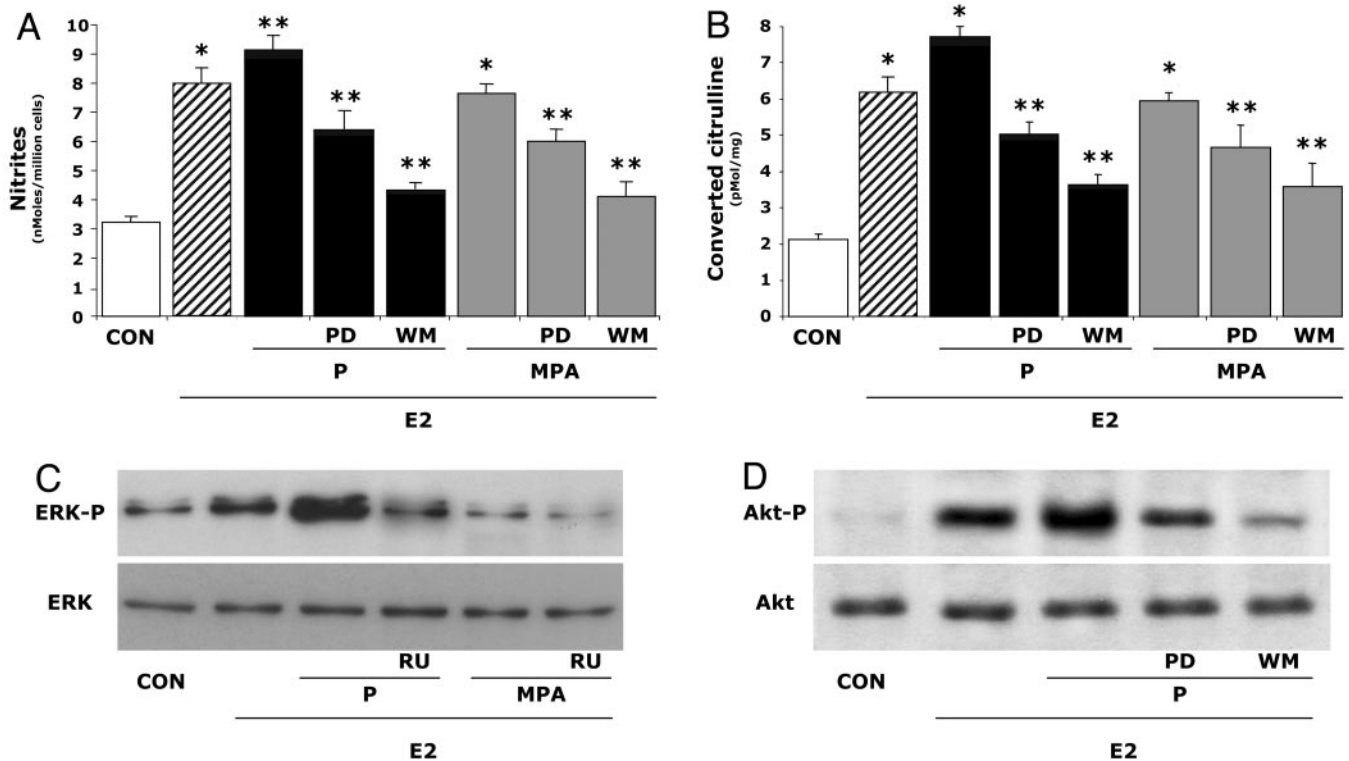


FIG. 5. Differential interference of P and MPA with the nongenomic signaling of E2 in human endothelial cells. Steroid-deprived and serum-starved HUVEC were treated for 30 min with 1 nM E2 alone or in the presence of 10 nM P or MPA, in the presence or absence of the MEK 1/2 inhibitor PD98059 (PD; 5  $\mu$ M); the PI3K inhibitor, wortmannin (WM; 30 nM); or the PR/GR antagonist RU486 (RU; 1  $\mu$ M). A, Nitrites released in the cell culture medium were assayed. B, eNOS activity was tested in whole-cell lysates. C, Wild-type or phosphorylated ERK 1/2 was assayed. D, Wild-type and phosphorylated Akt were studied. \*,  $P < 0.05$  vs. control; \*\*,  $P < 0.05$  vs. E2 alone. The experiments were repeated three times in quadruplicates (nitrite measurement) or in triplicates (eNOS activity), with comparable results. Western blot analyses were repeated three times, and representative blots are shown.

treated cells (Fig. 5D), suggesting that P and E2 signaling converged on Akt to exert additive activation of eNOS.

#### Divergent signal transduction of P and MPA on endothelial-leukocyte interaction

P and MPA affect monocyte adhesion to endothelial cells differently. Proinflammatory and atherogenic factors induce endothelial expression of adhesion molecules that are responsible for leukocyte adhesion to the vessel wall and migration toward the intima. Estrogens and glucocorticoids antagonize this process through partially distinct signaling pathways (22). MPA, as opposed to P, binds the GR and could therefore affect endothelial expression of adhesion molecules differently. We tested the effects of the two compounds on U937 monocytoid cell adhesion to human endothelial cells. With rotational assays, we found that P significantly decreases the number of monocytoid cells that stick to the endothelial monolayer due to treatment with bacterial LPS (Fig. 6A). When an equimolar concentration of MPA was used, a significantly stronger reduction of adherent monocytoid cells was observed (Fig. 6A). When used at the 20-fold lower concentration, MPA was still significantly more potent than P (Fig. 6A). Both P and MPA augmented the effect of E2 on monocytoid cell adhesion, but MPA was more effective than P (Fig. 6A). In parallel experiments, we added blocking Abs toward the proatherogenic endothelial-leukocyte adhesion

molecules VCAM-1 and ICAM-1 to the cell culture medium of LPS-treated cells. The addition of the two Abs together prevented nearly completely the adhesion of leukocytes to endothelial cells induced by LPS (not shown), indicating that VCAM-1 and ICAM-1 are responsible for LPS-induced adhesion of leukocytes to endothelial cells in this setting.

P and MPA affect endothelial expression of leukocyte adhesion molecules differently. We tested the effects of P and MPA on the expression of VCAM-1 and ICAM-1. Treatment with LPS effectively induced the expression of VCAM-1 and ICAM-1 in human endothelial cells, and the addition of P markedly decreased LPS effect (Fig. 6B). MPA was a more potent inhibitor of VCAM-1 and ICAM-1 expression than P was (Fig. 6B). In addition, when combined treatments with E2 and P or MPA were performed, both progestogens acted in an additive manner with E2, potentiating the inhibition of VCAM-1 and ICAM-1 expression induced by LPS (Fig. 6C). However, MPA was markedly more effective than P, even when used at 0.5 nM (Fig. 6C).

MPA alters endothelial expression of leukocyte adhesion molecules through both PR and GR. P-dependent inhibition of VCAM-1 and ICAM-1 was completely reversed by the cotreatment with both the mixed PR/GR antagonist, RU486, as well as by the pure PR antagonist, ORG 31710 (26) (Fig. 6B). On the contrary, MPA effect was only weakly decreased by the pure

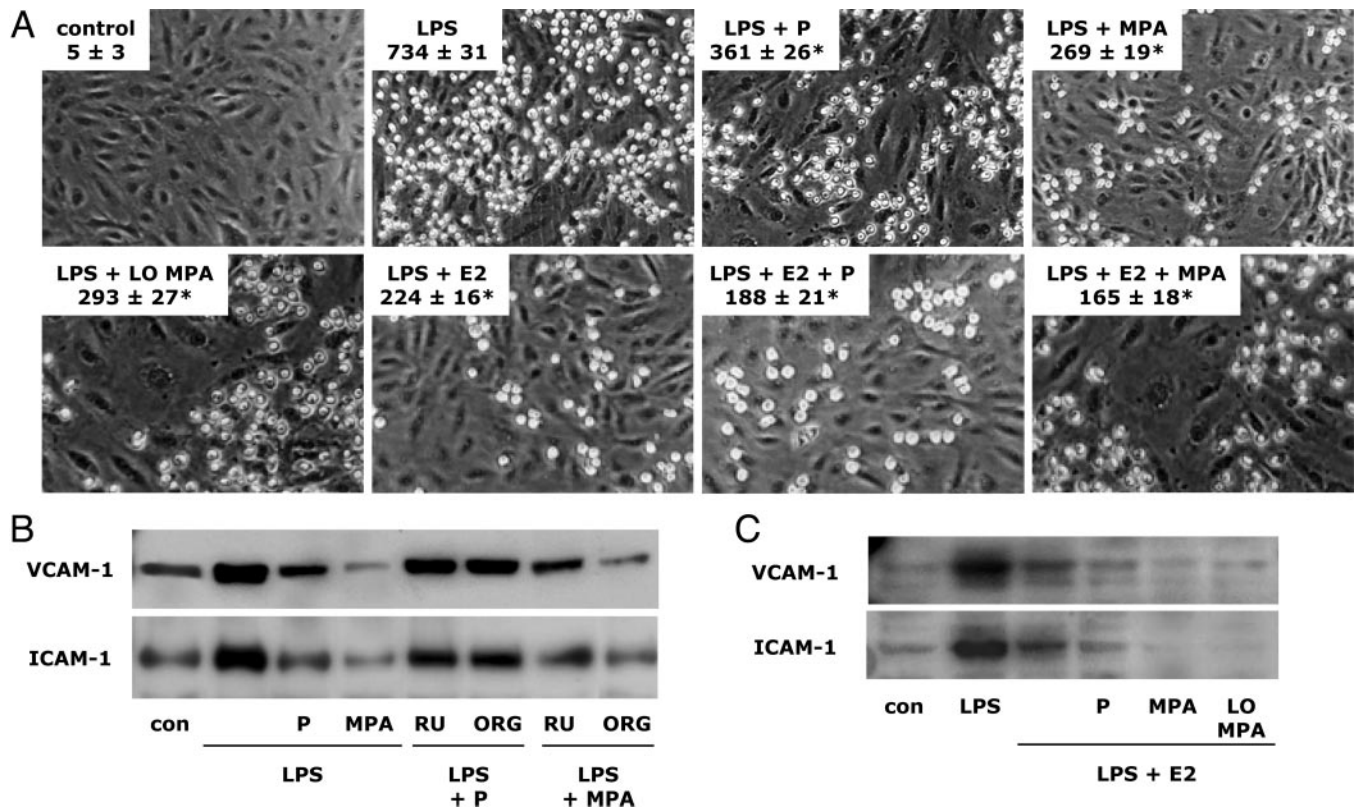


FIG. 6. P and MPA inhibit leukocyte adhesion to human endothelial cells through a reduction of endothelial adhesion molecule expression. Steroid-deprived HUVEC were treated for 16 h with 100 ng/ml bacterial LPS or LPS + 1 nM E2, in the presence or absence of 10 nM P or 10 nM or 0.5 nM MPA (LO MPA); the mixed PR/GR antagonist RU486 (RU; 1  $\mu$ M), or the pure PR antagonist ORG 31710 (ORG; 1  $\mu$ M). A, U937 monocytoid cells were added to endothelial monolayers under rotating conditions, and adherent cells were counted after washing. Numbers express the mean  $\pm$  SD of adherent cells counted in eight different optical fields within the dish. \*,  $P < 0.05$  vs. LPS. Pictures show representative optical fields. B and C, VCAM-1 and ICAM-1 protein amounts were assayed in whole-cell protein extracts. All experiments were repeated three times with comparable results.

PR antagonist, whereas it was nearly completely reversed by RU486 (Fig. 6B), suggesting combined signaling through PR and GR.

*Divergent effects of P and MPA on endothelial-leukocyte adhesion molecules in the presence of physiological glucocorticoid concentrations.* Because human endothelial cells are physiologically exposed to glucocorticoids, we studied the effect of P or MPA on endothelial-leukocyte adhesion molecule expression in the presence of physiological concentrations of hydrocortisone (50 nM). Hydrocortisone strongly reduced the LPS-dependent expression of VCAM-1 and ICAM-1 (Fig. 7A). The cotreatment with hydrocortisone and P did not result in significant changes compared with hydrocortisone alone (Fig. 7A). However, when MPA was added to hydrocortisone-treated cells, a net impairment of hydrocortisone effect on VCAM-1 and ICAM-1 expression was seen (Fig. 7A). Equal effects were seen with the lower dose of MPA (0.5 nM; data not shown).

In addition, by using a cell surface enzyme-linked immunoassay technique that allows the study of expression of adhesion molecules on endothelial cell membranes, we observed that P, MPA, and hydrocortisone effectively reduce the LPS-induced amount of VCAM-1 (Fig. 7B), as well as of ICAM-1 (not shown), on endothelial cell surface. Although the effects of P were completely blocked by both the mixed

GR/PR antagonist RU486 as well as by the pure PR antagonist ORG 31710, MPA and hydrocortisone actions were only affected by RU486 (Fig. 7B). Additionally, a marked reduction of hydrocortisone's effect was seen when MPA was added, but not with the addition of P (Fig. 7B).

*Divergent signal transduction of P and MPA on NF- $\kappa$ B in human endothelial cells.* Because endothelial-leukocyte adhesion molecule expression is driven by the transcription factor NF- $\kappa$ B and steroid hormones regulate NF- $\kappa$ B activation and nuclear translocation (22), we studied the subcellular distribution of NF- $\kappa$ B during the exposure to P or MPA. During challenge with LPS, NF- $\kappa$ B translocated into the nucleus (Fig. 8A), where it recognizes specific consensus sequences on the promoter regions of the genes encoding for endothelial-leukocyte adhesion molecules (22). When P or MPA were added to LPS, a significant reduction of NF- $\kappa$ B nuclear translocation was found (Fig. 8A). However, MPA was a more potent inhibitor than P (Fig. 8A). Cells exposed to hydrocortisone and LPS showed a marked reduction of NF- $\kappa$ B nuclear translocation (Fig. 8A); however, although the addition of P to hydrocortisone did not alter this finding, the addition of MPA reversed the action of hydrocortisone (Fig. 8A). Equal effects were seen with 0.5 nM MPA (data not shown).

Immunofluorescent studies of the subcellular translocation of NF- $\kappa$ B confirmed this pattern, showing that MPA



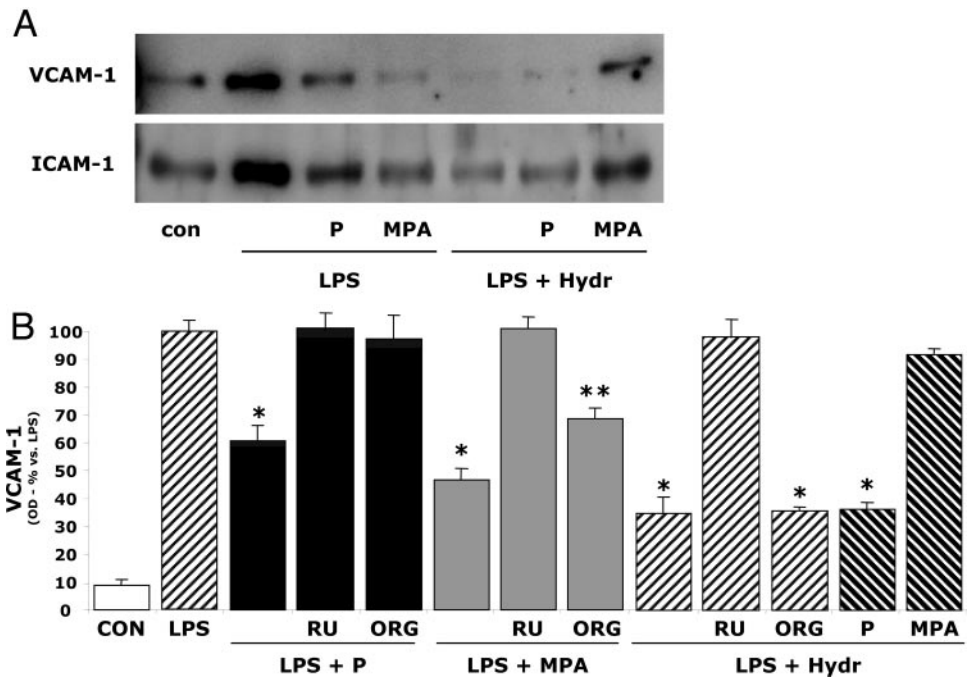


FIG. 7. Differential effects of P and MPA on adhesion molecule expression in the presence of physiological amounts of glucocorticoids. Steroid-deprived HUVEC were treated for 16 h with 100 ng/ml bacterial LPS or LPS + 50 nM hydrocortisone (Hydr), in the presence or absence of 10 nM P or 10 nM MPA; the mixed PR/GR antagonist RU486 (RU; 1  $\mu$ M); or the pure PR antagonist ORG 31710 (ORG; 1  $\mu$ M). A, VCAM-1 and ICAM-1 protein amounts were assayed in whole-cell protein extracts. B, Cell surface expression of VCAM-1 was assayed with enzyme-linked immunoassay on live cells. \*,  $P < 0.05$  vs. LPS; \*\*,  $P < 0.05$  vs. LPS + MPA. All experiments were repeated three times with comparable results.

efficiently prevents NF- $\kappa$ B nuclear translocation during LPS treatment of endothelial cells (Fig. 8B). The addition of the pure PR antagonist, ORG 31710, prevented only partially this action, whereas the mixed PR/GR antagonist, RU486, effectively reverted MPA action on NF- $\kappa$ B (Fig. 8B). Also, although P did not alter hydrocortisone-induced NF- $\kappa$ B stabilization, MPA markedly interfered with hydrocortisone (Fig. 8B).

Finally, we tested the interaction of NF- $\kappa$ B with the specific consensus sequence on VCAM-1 promoter with gel-shift technique. Purified nuclear protein extracts were challenged with radiolabeled ds-oligonucleotides reproducing the NF- $\kappa$ B consensus sequences on VCAM-1 promoter, and NF- $\kappa$ B-DNA interaction was visualized as a retardation in the electrophoretic run of the labeled probes. In agreement with the previous studies, treatment with P, MPA, or hydrocortisone resulted in significant reduction of the interaction of NF- $\kappa$ B with its consensus sequence (Fig. 8C). However, when MPA was added to hydrocortisone, a visible reduction of the antiinflammatory effect of hydrocortisone was observed (Fig. 8C).

### Discussion

The understanding of steroid receptor signal transduction has significantly advanced in the past 2 decades. Differently from other receptors, steroid hormone receptors recruit specific signaling pathways depending on the conformation induced by different ligands (32). This concept extends to PR, where the actions of different agonists and antagonists are more and more found to be compound specific (33). In addition, steroid hormones can bind with other steroid receptors with variable affinity. This particularly applies to synthetic progestins, which often interact with and transactivate androgen, mineralocorticoid, or GRs (34). Due to this variety of actions, it can be foreseen that each progestin will have

specific effects as well as peculiar interferences with other steroid hormone signaling pathways, therefore resulting in unique clinical effects. Although these concepts are well established, little research has been devoted to understanding the basis of the differential signaling of progestins.

The main finding of this paper is the demonstration that P and MPA trigger significantly different signaling events in controlled *in vitro* systems.

Indeed, we observed that natural P stimulates NO synthesis via transcriptional and nontranscriptional pathways in human endothelial cells as well as *in vivo* in ovariectomized rat abdominal aorta. In addition, when added to E2, P does not impair the estrogen-dependent induction of eNOS expression, and it even potentiates the effects of estrogen during rapid stimulations. In contrast, MPA does not trigger eNOS expression either *in vitro* or *in vivo*, and it does not induce rapid increases of eNOS activity nor does it potentiate E2-dependent nongenomic eNOS activation. Even more interestingly, when prolonged exposures of endothelial cells with E2 and MPA were performed, reductions of estrogen-dependent eNOS overexpression were seen, implying some sort of interference with estrogen receptor-dependent transcriptional signaling. These effects were confirmed *in vivo* in ovariectomized rats treated with clinically relevant doses of MPA or P, which supports the possible clinical interest of these results.

The clarification of the mechanism of eNOS gene modulation by progestogens is beyond the aim of this paper, but it may be hypothesized that PR, depending on the specific ligand engaging the hormone binding pocket, may be differently able to interfere with transcription factors regulating eNOS expression, such as Sp1 and GATA (35). Indeed, PR has been recently shown to interact with Sp1 in breast cancer cells (36), and up-regulation of GATA-1 has been reported in P-treated erythroid cells (37). Such a mechanism would also

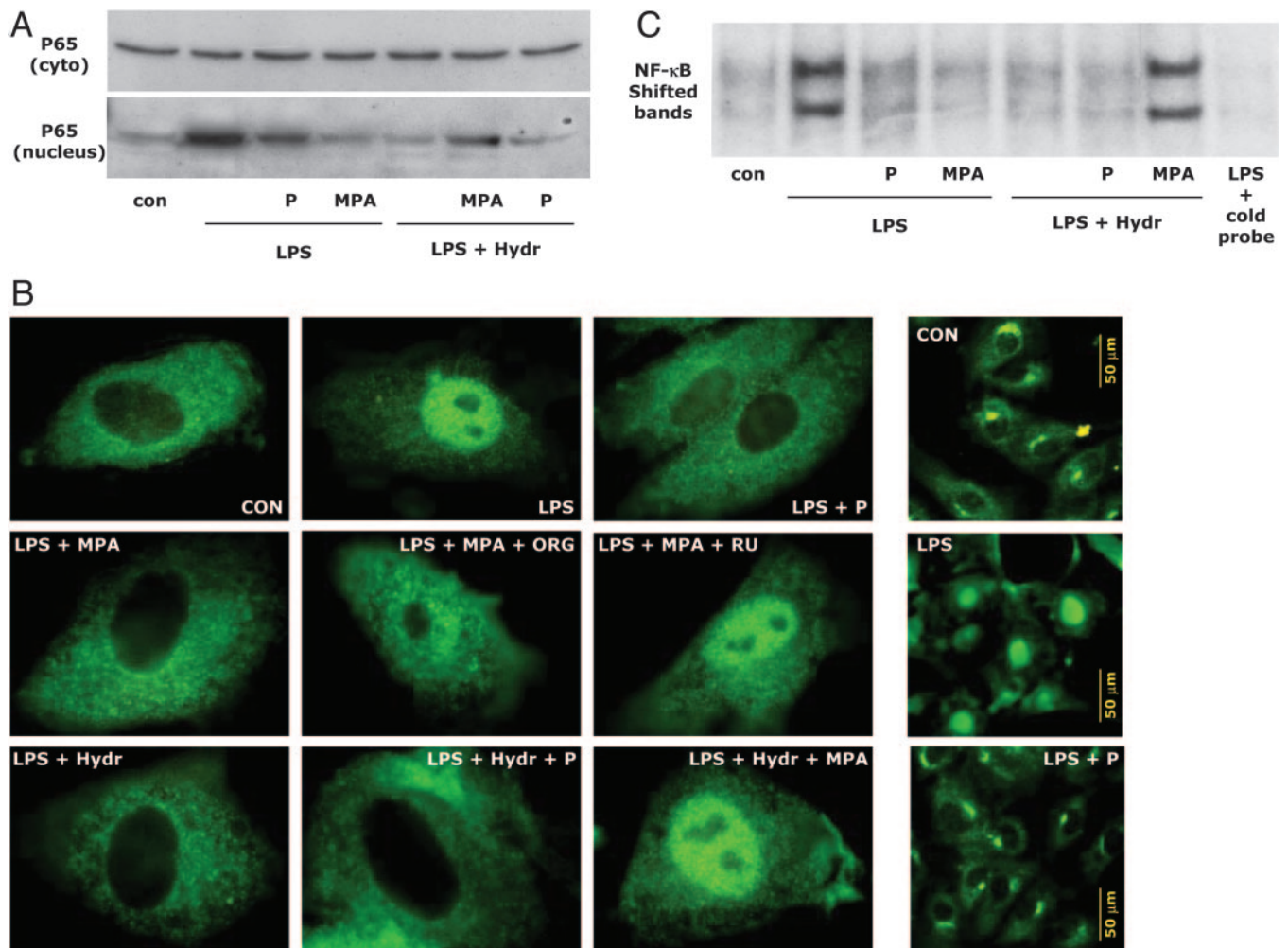


FIG. 8. Differential effects of P and MPA on activation, nuclear translocation, and DNA binding of the proatherogenic transcription factor NF- $\kappa$ B. Steroid-deprived HUVEC were treated for 2 h with 100 ng/ml bacterial LPS or LPS + 50 nM hydrocortisone (Hydr), in the presence or absence of 10 nM P or 10 nM or 0.5 nM MPA (LO MPA); the mixed PR/GR antagonist RU486 (RU; 1  $\mu$ M); or the pure PR antagonist ORG 31710 (ORG; 1  $\mu$ M). A, p65 NF- $\kappa$ B subunit amounts were assayed in purified cytoplasmic or nuclear extracts. B, Subcellular localization of the p65 NF- $\kappa$ B subunit was assayed with cell immunofluorescence technique. Shown on the *left* are details of the cells at high magnification (100 $\times$ ). Shown on the *right* are sample fields of cells at 20 $\times$  magnification. C, Gel-shift assay was performed using purified nuclear protein extracts incubated with radiolabeled ds-oligonucleotides reproducing the NF- $\kappa$ B consensus sequences contained in the VCAM-1 gene promoter. Shown are the shifted bands indicating NF- $\kappa$ B/DNA complexes. All experiments were repeated three times with comparable results.

provide a possible basis for the interference of MPA with estrogen-dependent eNOS induction, because estrogen activates eNOS expression through regulation of Sp1 (38, 39). The relevance of our results is supported by the recent observation that chronic administration of P but not of MPA to ovariectomized rats restores the endothelial control of vascular tone in the mesenteric artery through a presumable enhancement of endothelial NO synthesis (40).

In analogy to other steroid hormones (21, 28, 29, 41, 42), P activates eNOS rapidly via nongenomic mechanisms, but this action does not extend to MPA. Our results show that the basis for this difference in human endothelial cells is the incapacity of MPA-bound PR (as opposed to P-bound PR) to activate the ERK pathway and therefore to subsequently recruit PI3K. Our data are consistent with recent observations in hippocampal neurons, where MPA, as opposed to P, is unable to induce the translocation of activated ERK toward

the cell nucleus and prevents ERK nuclear migration induced by E2 (10).

Activation of PI3K through the ERK cascade is a novel observation. PI3K and MAPK act coordinately in different cell types (43); however, the existence of a hierarchical activation of PI3K after MAPK had been identified only in *Xenopus laevis* after PR activation (44). This signaling pathway is now described also in human cells, and may represent an important mechanism of signal transduction for PR ligands.

One important issue to be clarified is the reason why the two progestogens induce such markedly different nongenomic signaling events in endothelial cells. Most of the nontranscriptional actions of steroid receptors are dependent upon protein-protein interaction at the cell membrane or inside the cytoplasm (28). As happens for estrogen ligands (45), it is likely that the structural conformation of PR induced by distinct progestins may differ and that only se-

lected conformations may activate the Src/ERK cascade. This may be also relevant for the different abilities of P and MPA to act additively to E2 in activating MAPK. Indeed, PR-B and estrogen receptor  $\alpha$  interact to activate c-Src in breast cancer cells (46), and the steric hindrance imposed to PR by P or MPA may be different enough to explain the divergent activation of the kinase cascade.

On the other hand, the ability to engage other steroid receptors shown by some progestins may alter their biological effects. To this aim, the relevance of the affinity of MPA for the GR becomes evident when the proinflammatory signal transduction to endothelial-leukocyte adhesion molecules is studied. In this study, MPA has more prominent antiinflammatory effects than P when used alone or with E2. However, when administered in the presence of physiological concentrations of glucocorticoids, which are potent inhibitors of NF- $\kappa$ B signaling (47), MPA markedly impairs the action of hydrocortisone, acting as a partial antagonist on GR and resulting in reduced efficacy of hydrocortisone to prevent NF- $\kappa$ B nuclear translocation and DNA binding.

In agreement with our findings, MPA prevents the expression of the adhesion molecule E-selectin on human endothelial cells to a similar extent like glucocorticoids (48) and shows more prominent antiinflammatory properties in selected *in vivo* inflammation models (49). However, when used in castrated rats who still retain endogenous glucocorticoids, MPA is found to have detrimental effects on vascular infiltration by leukocytes (15).

Differential antiinflammatory actions of MPA with respect to other progestins, such as norethisterone acetate, have been suggested previously in endothelial cells, where MPA antagonizes the reduction of monocyte chemoattractant protein-1 synthesis induced by E2, whereas norethisterone is neutral (50). In addition, differences between P and MPA have been described with respect to TNF- $\alpha$ -dependent expression of VCAM-1 in endothelial cells, although in the opposite direction with respect to our data, possibly due to the different inflammatory trigger or to the much higher ( $\approx$ 1000-fold higher) concentrations of steroids used (16).

In our study, as performed in the *in vitro* studies from other groups, we chose to have a pure pharmacological comparison, using the compounds at the same molar (physiological) concentration (10 nM) so as to have the same number of molecules reaching the cells in PR-saturating amounts. However, similar clinical effects (*e.g.* on the endometrium) are obtained using a 200-mg/d dose of oral P or 10 mg/d of oral MPA during therapeutic administration. Plus, each steroid differently associates with carrier proteins, such as sex hormone-binding globulin. It is therefore difficult to translate the *in vitro* comparison to a realistic *in vivo* situation. However, we consistently obtained the same results when using a 20-fold lower concentration of MPA, therefore confirming that our present observations reflect differences in the structure and biological activity of the two molecules, rather than on concentration-related effects.

Along the same lines, it is difficult to draw a parallel between our results and the available *in vivo* studies investigating the vascular effects of progestins. Indeed, although indications of markedly different actions of P and MPA on atherogenesis and vascular function in animals (5–7, 40) and

in humans (8, 51) have been reported, other studies have shown no difference between these gestagens (52–54).

Our findings provide a biological support for the studies, indicating differences between P and MPA (and more in general for synthetic progestins), and suggest that these two steroids divergently modulate endothelial function through differential signaling to MAPK, PI3K, and NF- $\kappa$ B pathways. This leads to distinct alterations of NO synthesis and of endothelial response to inflammatory stimuli and leukocyte adhesion. Unfortunately, no study is available that specifically investigates the clinical relevance of these findings, but the recent news on the striking difference between the two arms of the Women's Health Initiative trial, indicating a trend toward protection from heart disease in younger women receiving only CEE (4), as opposed to those receiving CEE + MPA (2), confirms the importance of the hereby reported differences between natural P and MPA and may suggest that each progestin may have peculiar indications and contraindications depending on the patient's characteristics.

In conclusion, we show that P and MPA are not equivalent in terms of molecular signaling in human endothelial cells and in vascular tissues of ovariectomized rats. These two compounds have clearly distinct features and differently affect estrogen and glucocorticoid signaling. Although there may be different reasons for this discrepancy, differential ability of PR engaged by different progestins to interact with cell membrane-based or cytoplasmic signaling intermediates, such as MAPK or PI3K, as well as different interferences with transcription factors, may explain the observed results. These findings may have important clinical implications, indicating that each progestin has specific actions on vascular cells and that different hormonal preparations may differ as to their clinical cardiovascular effects.

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