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Medroxyprogesterone acetate attenuates estrogen-induced nitric oxide production in human umbilical vein endothelial cells☆

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Abstract

We report the novel observation that medroxyprogesterone acetate (MPA) attenuates the induction by 17β estradiol (E2) of both nitric oxide (NO) production and endothelial nitric oxide synthase (eNOS) activity in human umbilical vein endothelial cells. Although MPA had no effect on basal NO production or basal eNOS phosphorylation or activity, it attenuated the E2-induced NO production and eNOS phosphorylation and activity. Moreover, we examined the mechanism by which MPA attenuated the E2-induced NO production and eNOS phosphorylation. MPA attenuated the E2-induced phosphorylation of Akt, a kinase that phosphorylates eNOS. Treatment with pure progesterone receptor (PR) antagonist RU486 completely abolished the inhibitory effect of MPA on E2-induced Akt phosphorylation and eNOS phosphorylation. In addition, the effects of actinomycin D were tested to rule out the influence of genomic events mediated by nuclear PRs. Actinomycin D did not affect the inhibitory effect of MPA on E2-induced Akt phosphorylation. Furthermore, the potential roles of PRA and PRB were evaluated. In COS cells transfected with either PRA or PRB, MPA attenuated E2-induced Akt phosphorylation. These results indicate that MPA attenuated E2-induced NO production via an Akt cascade through PRA or PRB in a non-genomic manner.

Keywords: Nitric oxide; eNOS; Medroxyprogesterone acetate; Endothelial cells; Akt; 17β estradiol; Progesterone receptor; RU486; Actinomycin D; Non-genomic events

The risk of cardiovascular disease steeply increases after menopause. Many epidemiological and basic studies have been shown that estrogen has the significant function in the vasculature of preventing the primary development of cardiovascular disease in women [1,2]. We and others have reported that estrogen acts directly on endothelial nitric oxide synthase (eNOS) activity through a non-genomic mechanism [3], resulting in rapid dilatation of blood vessels [4].

In the Women's Health Initiatives (WHI) large prospective randomized controlled study, although women on the conjugated equine estrogen (CEE)-medroxyprogesterone acetate (MPA) arm had an increase in the relative risk of cardiovascular events and breast cancer [5], the more recent reports on women on CEE-only treatment arm did not show increased cardiovascular disease [6]. Thus, there is a possibility that progestin has an adverse effect on the cardiovascular system. In fact,

☆ *Abbreviations:* MPA, medroxyprogesterone acetate; NO, nitric oxide; eNOS, endothelial nitric oxide synthase; E2, 17β estradiol; PR, progesterone receptor; DAF-2,4,5,-diaminofluorescein.

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medroxyprogesterone acetate (MPA), commonly used as a progestine combined with estrogen, is reported to inhibit the endothelium-dependent vasodilatation induced by estrogen [7]. Thus, the mechanism by which progestin may interfere with the beneficial activities of estrogen should be determined. We therefore examined the effect of MPA on estrogen-induced eNOS activity and nitric oxide (NO) production in human umbilical vein endothelial cells (HUVECs). We report here the novel observation that MPA attenuates the induction by estrogen of both eNOS activity and NO production in HUVECs.

Materials and methods

Materials

17β-E2, medroxyprogesterone acetate, RU-486, and actinomycin D were purchased from Sigma Chemical (St. Louis, MO). ECL Western blotting detection reagents were obtained from Amersham Pharmacia Biotech (Arlington Heights, IL). Rabbit polyclonal anti-phospho Akt, anti-Akt, anti-phospho eNOS (ser-1177), and anti-eNOS antibodies were obtained from New England BioLabs (Beverly, MA).

Methods

Cell culture. HUVECs were isolated according to the method of Jaffe et al. [8], plated in gelatin-coated tissue culture wells, and grown in M199 medium containing 20% fetal bovine serum and 50 μg/ml endothelial cell growth supplement (Clonetics Corp., San Diego, CA). HUVECs were used at passage 2 or 3. COS cells were cultured at 37 °C in Dulbecco's modified Eagle's medium with 10% fetal bovine serum in a water-saturated atmosphere of 95% O_2 and 5% CO_2.

DNA transfection. COS cells were maintained in Dulbecco's modified Eagle's medium/2 mM glutamine (PAA Laboratories) supplemented with 10% fetal bovine serum (PAA Laboratories), 100 U/ml penicillin, and 100 μg/ml streptomycin. COS cells cultured in 100-mm dishes were transfected with 1 μg ERα expression vector (HEGO), 0.5 μg HEGO + 0.5μg progesterone receptor A expression vector (HEGO + PR A), or 0.5μg HEGO + 0.5 μg progesterone receptor B expression vector (HEGO + PR B) using LipofectAMINE plus as described previously [9]. Twenty-four hours after transfection, the cells were placed under serum-deprived conditions, and then the cells were treated with various ligands. The cells were harvested and assayed for reporter activity or used for Western blot analysis. Each experiment was performed a minimum of three times, and a representative example is shown.

Measurement of NO production in living cells. NO was measured by use of DAF-2 (Daiichi Pure Chemicals, Tokyo, Japan), an NO-sensitive fluorescent dye [10]. HUVECs were grown to 95% confluence and were serum-starved for 24 h. Then the cells were loaded with 10 μM DAF-2 for 60 min at 37 °C. The DAF-2 fluorescence was measured by use of a fluorescence microscope camera with a filter set permitting excitation at 490 nm and emission at 515 nm. It has been reported that DAF-2 fluorescence increases almost linearly with the NO concentration. Therefore, we expressed the intracellular NO production as the net increment of DAF-2 fluorescence in 15 min relative to its basal value.

Assay of eNOS activity. HUVECs were cultured in 100-mm dishes. The cells were serum-starved overnight in phenol red-free medium before eNOS activity measurements. eNOS activity was determined as the conversion of radiolabeled L-arginine to L-citrulline by a method described previously [11,12] with a minor modification. Briefly, 10 μl of a sample was incubated for 10 min at 37 °C in a solution consisting of 50 mM Hepes, 1 mM dithiothreitol, 1 mM $CaCl_2$, 0.1 mM tetrahydro-L-biopterin, 1 mM NADPH, 10 μg/ml calmodulin, 10 μM FAD, and 1.55 μM L-[guanidino-^{14}C]arginine (pH 7.8), in a final volume of 100 μl. The reaction was terminated by the addition of 200 μl buffer A (100 mM Hepes, 10 mM EDTA, pH 5.2). The whole reaction mixture was then applied to a 0.3-ml Dowex 50-WX column (Na^+ form, 200–400 mesh) that had been equilibrated with buffer A. [^{14}C]citrulline was eluted with 0.5 ml of buffer A, and then radioactivity was measured with a liquid scintillation counter. For activity assay, each data point was determined in triplicate and the average and standard deviation were calculated.

Western blot analysis. The cells were incubated in phenol red-free medium without serum for 24 h and then treated with various agents. They were then washed twice with phosphate-buffered saline and lysed in ice-cold HNTG buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EDTA, 10 mM sodium pyrophosphate, 100 μM sodium orthovanadate, 100 mM NaF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride) [13]. The lysates were centrifuged at 12,000*g* at 4 °C for 15 min, and the protein concentrations of the supernatants were determined using the Bio-Rad protein assay reagent. Equal amounts of proteins were separated by SDS–polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Blocking was done in 10% bovine serum albumin in 1× Tris-buffered saline. Western blot analyses were performed with various specific primary antibodies. Immunoreacted bands in the immunoblots were visualized with horseradish peroxidase-coupled goat anti-rabbit or anti-mouse immunoglobulin by using the enhanced chemiluminescence Western blotting system.

Statistics. Statistical analysis was performed using Student's *t* test, and $p < 0.01$ was considered significant. Data are expressed as means ± SE.

Results

MPA attenuates the E2-induced NO production and eNOS activity

NO production was examined by a fluorescence assay using an NO-sensitive dye, 4,5,-diaminofluorescein (DAF-2). HUVECs loaded with DAF-2 were treated with 10^{-7} M E2 for the indicated times. The increase in NO production induced by 10^{-7} M E2 reached a plateau at 15 min and rapidly declined thereafter (Fig. 1A). The cells were then treated with 10^{-7} M E2 for 15 min with or without 10^{-7} M MPA. Although MPA had no effect on the basal NO production, MPA significantly attenuated the E2-induced NO production (Fig. 1B).

We reported that E2 induces eNOS activity [3] which is critical in NO production in HUVECs. Therefore, we evaluated the effect of MPA on E2-induced eNOS activity. HUVECs were treated with 10^{-7} M E2 for the indicated times, and then the eNOS activity was examined by measuring the conversion of arginine to citrulline. As we reported previously [3], the increase in eNOS activity induced by 10^{-7} M E2 reached a plateau at 10 min and rapidly declined thereafter (Fig. 1C). The cells were then treated with 10^{-7} M E2 for 15 min with or without 10^{-7} M MPA. 10^{-7} M MPA significantly attenuated the E2-induced eNOS activity (Fig. 1D).

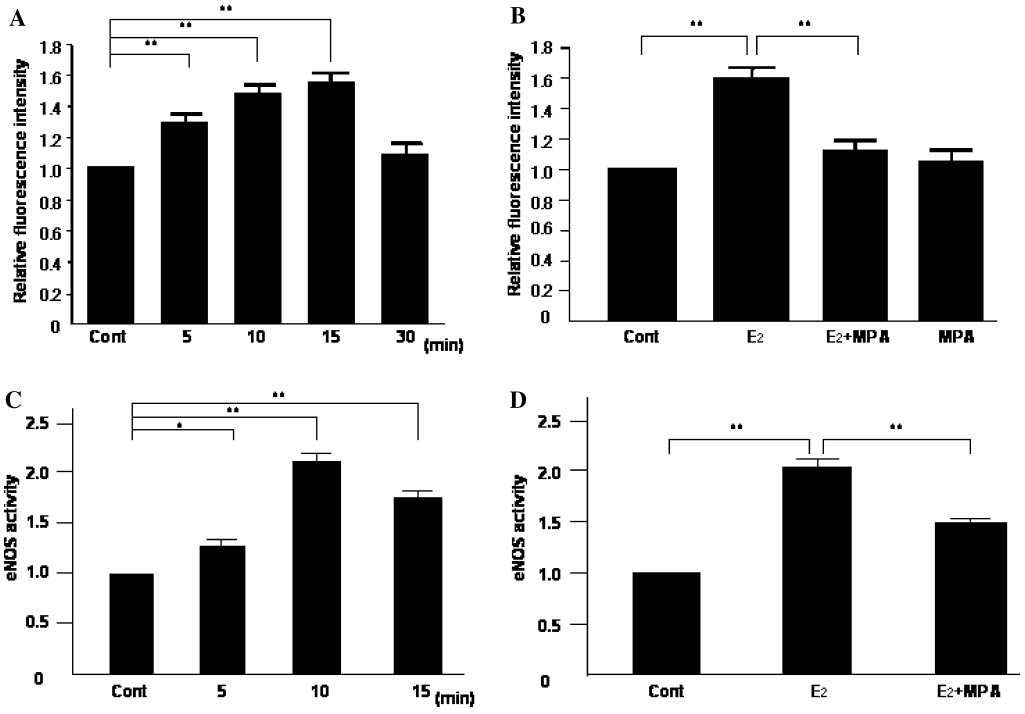

Fig. 1. MPA attenuates the E2-induced NO release and eNOS activation. To examine NO release, HUVECs were loaded with DAF-2 and then treated with 1 nM E2 for the indicated times (A), or treated with 1 nM E2 or 1 nM E2 + 10^{-6} M MPA for 15 min (B). To examine eNOS activity, HUVECs were treated with 1 nM E2 for the indicated times (C), or treated with 1 nM E2 or 1 nM E2 + 10^{-6} M MPA for 15 min (D). eNOS activity was measured by the conversion of L-[guanidino-^{14}C] arginine to L-[guanidino-^{14}C]citrulline, as described under Materials and methods. The basal activity of eNOS was arbitrarily set at 1.0. Data are expressed as the mean-fold activation ± SE of three separate experiments. Significant differences are indicated by asterisks. *$p < 0.05$; **$p < 0.01$.

MPA attenuates the E2-induced eNOS phosphorylation and Akt phosphorylation

eNOS activity is regulated by its phosphorylation via the PI3K-Akt cascade, and we reported that E2 induced eNOS phosphorylation via the PI3K-Akt cascade in HUVECs [3]. Therefore, we first evaluated the effect of MPA on E2-induced eNOS phosphorylation. Cells were treated with E2 for various times and then used to prepare lysates that were subjected to Western blotting using anti-phospho-eNOS or -eNOS antibody. Although E2 did not affect the expression of eNOS (Fig. 2A,

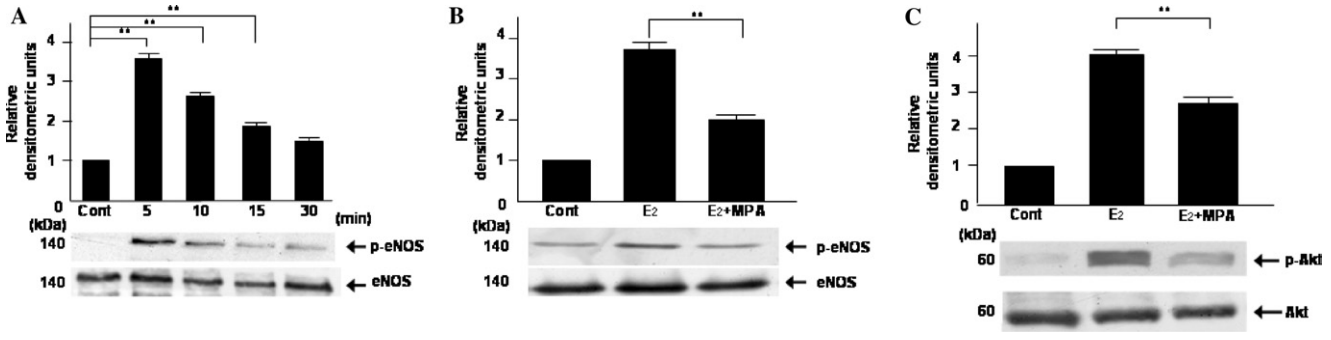

Fig. 2. MPA attenuates the E2-induced eNOS phosphorylation and Akt phosphorylation. HUVECs were treated with 1 nM E2 for the indicated times (A) or treated with 1 nM E2, 1 nM E2 + 10^{-6} M MPA for 15 min (B) and then harvested and used to prepare cell lysates. The lysates were subjected to Western blotting using anti-phospho-eNOS (middle panels) or anti-eNOS (bottom panels) antibody. The positions of molecular mass markers are noted on the left. Relative densitometric units of the p-eNOS bands are shown in the top panels, with the density of the control bands (0 min) set arbitrarily at 1.0. (C) HUVECs were treated with 1 nM E2 or 1 nM E2 + 10^{-6} M MPA for 15 min and then harvested and used to prepare cell lysates. The lysates were subjected to Western blotting using anti-phospho-Akt (middle panels) or anti-Akt (bottom panels) antibody. The positions of molecular mass markers are noted on the left. Relative densitometric units of the p-Akt bands are shown in the top panels, with the density of the control bands (0 min) set arbitrarily at 1.0. The values shown represent means ± SE from at least three separate experiments. Significant differences are indicated by asterisks. **$p < 0.01$.

lower panel), it significantly induced the phosphorylation of eNOS (Fig. 2A, middle and top panels). Cells were treated with 10^{-7} M E2 for 15 min with or without 10^{-7} M MPA. Although 10^{-7} M MPA had no effect on the basal eNOS phosphorylation, it significantly attenuated the E2-induced eNOS phosphorylation (Fig. 2B).

We next evaluated the effect of MPA on E2–induced Akt phosphorylation. Cells were treated with 10^{-7} M E2 for 15 min with or without 10^{-7} M MPA and then used to prepare lysates that were subjected to Western blotting using anti-phospho-Akt or -Akt antibody. Although 10^{-7} M MPA had no effect on the basal Akt phosphorylation, it significantly attenuated the E2-induced Akt phosphorylation (Fig. 2C).

Involvement of non-genomic PR activation

We further examined the mechanism by which MPA attenuated the E2-induced eNOS phosphorylation. To determine if this response involves rapid progesterone receptor (PR) activation, the effect of concomitant treatment with the pure PR antagonist RU486 was determined. RU486 completely abolished the inhibitory effects of MPA on E2-induced eNOS phosphorylation (Fig. 3A) and Akt phosphorylation (Fig. 3B).

Moreover, the effects of actinomycin D, an inhibitor of gene transcription, were tested to rule out the influence of genomic events mediated by nuclear PRs. Actinomycin D did not affect the inhibition by MPA of E2-induced Akt phosphorylation (Fig. 3C), indicating that the effects of MPA were independent of gene transcription regulation, and are thus termed ''non-genomic.'' These results suggest that MPA attenuated the E2-induced eNOS phosphorylation and Akt phosphorylation via non-genomic PR activation.

Effect of PRA or PRB expression on the inhibitory effect of MPA on E2-induced Akt phosphorylation

We also evaluated the potential role of PRA or PRB in the inhibitory effect of MPA on E2-induced Akt phosphorylation. We confirmed that both PRA and PRB were expressed in HUVECs (data not shown). Therefore, COS cells, which do not express PRA or PRB, were used to examine which of these receptors is involved in the inhibitory effect of MPA on E2-induced

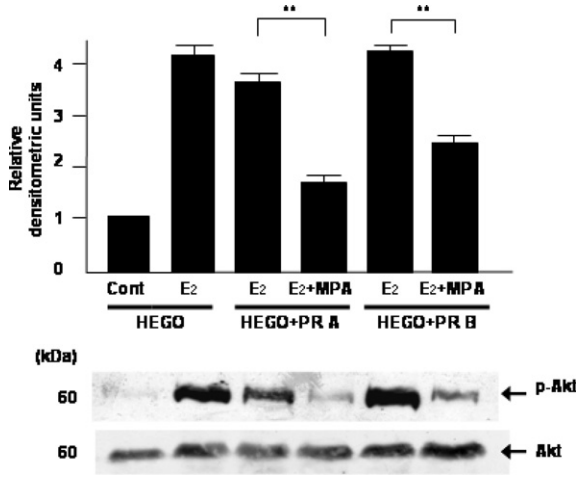

Fig. 4. Effect of PRA or PRB expression on the inhibitory effect of MPA on E2-induced Akt phosphorylation. COS cells were transfected with 1 μg wild-type estrogen receptor vector (HEGO) (lanes 1 and 2), and 0.5 μg progesterone receptor A vector and 0.5 μg HEGO (lanes 3 and 4), 0.5 μg progesterone receptor B vector and 0.5 μg HEGO (lanes 5 and 6). Then, transfected COS cells were treated with 1 nM E2 or 1 nM E2 + 10^{-6} M MPA for 15 min. Akt phosphorylation was measured as described in the legend for Fig. 2. Relative densitometric units of the bands are shown in the top panels, with the density of the control bands (0 min) set arbitrarily at 1.0. Values shown represent means ± SE from at least three separate experiments. Significant differences are indicated by asterisks. $^{**}p < 0.01$.

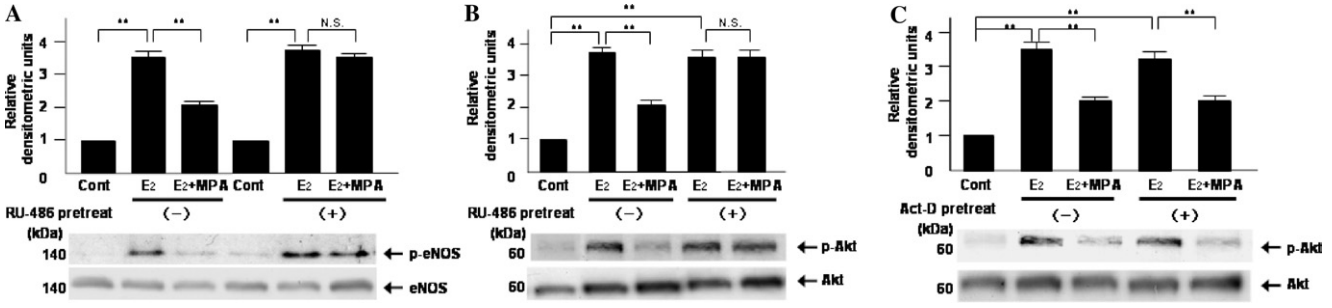

Fig. 3. Involvement of non-genomic PR. HUVECs were pre-incubated with or without 1 nM RU-486 for 1 h and then treated with 1 nM E2 or 1 nM E2 + 10^{-6} M MPA for 15 min and then harvested and used to prepare cell lysates. The lysates were subjected to Western blotting using anti-phospho-eNOS (A, middle panels), anti-eNOS (A, bottom panels), anti-phospho-Akt (B, middle panels), or anti-Akt (B, bottom panels) antibody (A). (C) HUVECs were pre-incubated with or without 25 μg/ml actinomycin D (Act-D) for 120 min and then treated with 1 nM E2 or 1 nM E2 + 10^{-6} M MPA for 15 min. The lysates were subjected to Western blotting using anti-phospho-Akt (middle panels) or anti-Akt (bottom panels) antibody. The positions of molecular mass markers are noted on the left. Relative densitometric units of the bands are shown in the top panels, with the density of the control bands (0 min) set arbitrarily at 1.0. Each experiment was repeated three times with essentially identical results. Significant differences are indicated by asterisks. $^{**}p < 0.01$.

Akt phosphorylation. In COS cells transfected with either PRA or PRB, MPA attenuated the E2-induced Akt phosphorylation (Fig. 4). These results indicate that MPA attenuated the E2-induced Akt phosphorylation through PRA or PRB.

Discussion

There are several mechanisms through which estrogen exerts cardio-protective effects [14]. In the WHI study, although women on the CEE-only treatment arm did not have an increase in the relative risks of cardiovascular events [6], women on the CEE-MPA arm had an increase in the relative risks of cardiovascular events [5]. These findings of the WHI study suggest the possibility that progestin has an adverse effect on the cardiovascular system. Does MPA have adverse effects on all of the cardio-protective functions of estrogen? In this report, we demonstrated that MPA attenuates the E2-induced NO production and eNOS activity in HUVECs, providing a molecular mechanism to account for the clinical findings that MPA inhibits the endothelium-dependent vasodilatation by estrogen [7]. Although it remains possible that MPA has adverse effects on cardio-protective functions of estrogen other than NO production and eNOS activation, MPA at least had no effect on estrogen-induced cell proliferation of HUVECs (data not shown).

How does MPA attenuate the E2-induced NO release and eNOS activity? It was reported that eNOS is one of the substrates of Akt [15] and that the activity of eNOS is regulated by its phosphorylation via the PI3K-Akt cascade [16]. In this report, we showed that MPA attenuates the E2-induced eNOS phosphorylation and Akt phosphorylation. Moreover, actinomycin D did not affect the inhibitory effects of MPA on E2-induced eNOS phosphorylation and Akt phosphorylation, indicating the involvement of a non-genomic response of PR, as reported previously [17–19].

There are two isoforms of PRs, PRA, and PRB. What is the different role of PRA and PRB in the biological actions of progestin? It was reported that an imbalance in the native ratio of the two isoforms can lead to alterations in PR signaling [20] and mammary gland development [21]. In this report, we demonstrated that both PRA and PRB are involved in the MPA-induced attenuation of E2-induced Akt phosphorylation. It was reported that PRB is required for the induction by MPA of cyclin D1 expression via the ERK cascade in MCF-7 cells [22]. Thus, the effects of MPA on the signaling cascades are different depending on the tissues. Although the involvement of non-genomic PRA and PRB in inhibiting E2-induced Akt phosphorylation is a novel finding, further investigations will be necessary to fully clarify the molecular mechanism of the adverse effect of MPA on the cardiovascular system.

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