Medroxyprogesterone Abrogates the Inhibitory Effects of Estradiol on Vascular Smooth Muscle Cells by Preventing Estradiol Metabolism

Raghvendra K. Dubey, Edwin K. Jackson, Delbert G. Gillespie, Lefteris C. Zacharia, Dorothea Wunder, Bruno Imthurn, Marinella Rosselli

Abstract—Sequential conversion of estradiol (E) to 2/4-hydroxyestradiols and 2-/4-methoxyestradiols (MEs) by CYP450s and catechol-O-methyltransferase, respectively, contributes to the inhibitory effects of E on smooth muscle cells (SMCs) via estrogen receptor–independent mechanisms. Because medroxyprogesterone (MPA) is a substrate for CYP450s, we hypothesized that MPA may abrogate the inhibitory effects of E by competing for CYP450s and inhibiting the formation of 2/4-hydroxyestradiols and MEs. To test this hypothesis, we investigated the effects of E on SMC number, DNA and collagen synthesis, and migration in the presence and absence of MPA. The inhibitory effects of E on cell number, DNA synthesis, collagen synthesis, and SMC migration were significantly abrogated by MPA. For example, E (0.1 μmol/L) reduced cell number to 51.3.6% of control, and this inhibitory effect was attenuated to 87.5 ± 2.9% by MPA (10 nmol/L). Treatment with MPA alone did not alter any SMC parameters, and the abrogatory effects of MPA were not blocked by RU486 (progesterone-receptor antagonist), nor did treatment of SMCs with MPA influence the expression of estrogen receptor-α or estrogen receptor-β. In SMCs and microsomal preparations, MPA inhibited the sequential conversion of E to 2–2/4-hydroxyestradiol and 2-ME. Moreover, as compared with microsomes treated with E alone, 2-ME formation was inhibited when SMCs were incubated with microsomal extracts incubated with E plus MPA. Our findings suggest that the inhibitory actions of MPA on the metabolism of E to 2/4-hydroxyestradiols and MEs may negate the cardiovascular protective actions of estradiol in postmenopausal women receiving estradiol therapy combined with administration of MPA. (Hypertension. 2008;51:1197-1202.)

Key Words: estradiol ☑ progestins ☑ cytochrome P450 ☑ metabolism ☑ cardiovascular disease ☑ vascular remodeling

Studies using pharmacological agents to block or induce the conversion of estradiol to hydroxyestradiols (mediated by CYP450s) and to methoxyestradiols (mediated by catechol-O-methyltransferase acting on hydroxyestradiols) and studies in knockout mice that cannot form methoxyestradiols provide strong evidence that methoxyestradiols mediate the inhibitory effects of estradiol on smooth muscle cell (SMC) DNA and collagen synthesis, as well as SMC proliferation and migration. Also, evidence supports the conclusion that methoxyestradiols mediate the inhibitory effects of estradiol on injury-induced neointima formation, as well as the inhibitory effects of estradiol on cardiac fibroblasts and glomerular mesangial cells, which are relevant for the cardiovascular system.

Medroxyprogesterone (MPA) is a synthetic progestin often given concomitantly with estrogens during hormone replacement therapy. However, because MPA is a substrate for CYP450s and is hydroxylated, it is feasible that MPA competes with estradiol for CYP450s and inhibits metabolism of estradiol to hydroxyestradiols and consequently to inhibitory methoxyestradiols.

Several observations support this hypothesis: (1) increased adverse cardiovascular events in postmenopausal women receiving estrogens plus MPA for hormone replacement therapy; (2) increased incidence of breast cancer in postmenopausal women taking estrogens in combination with MPA; (3) abrogation by MPA of the ability of estradiol to attenuate balloon injury–induced intimal thickening in animal models; and (4) attenuation by MPA of the beneficial effects...
of hormone replacement therapy on low-density lipoprotein and high-density lipoprotein levels and NO generation. In the present study we tested the hypothesis that MPA abrogates the inhibitory effects of estradiol on SMCs via a mechanism involving inhibition of hydroxyestradiol formation by CYP450s.

**Methods**

Female human aortic SMCs were obtained from Cascade Biologics and cultured as described by us previously. SMCs in the third passage were used for all of the studies. All of the chemicals for cellular studies were purchased from suppliers as described previously. SMCs were grown under standard tissue culture conditions in phenol red-free DMEM/F12 medium supplemented with 10% steroid-free FCS and antibiotics. Confluent SMCs were dislodged by trypsinization, washed, and plated for growth studies at required densities in multiwell plates.

In some experiments, SMCs were plated in 24-well plates, growth arrested for 48 hours using DMEM containing 0.4% albumin, and subsequently stimulated with 2.5% FCS in the presence and absence of 1 to 100 nmol/L estradiol containing or lacking MPA (1 to 100 nmol/L). Cell number was assessed by counting cells on day 6 (treatments changed every 48 hours), and DNA ([3H]thymidine incorporation) and collagen ([3H]proline incorporation) synthesis were assessed after 24 and 36 hours of treatment, respectively, as described previously.

To assess whether the modulatory effects of MPA were because of its direct effect on estrogen receptor (ER) expression, we analyzed the changes in the expression of ERα and ERβ in SMCs treated with MPA. Briefly, Western blot analysis was conducted in cell lysates prepared from SMCs treated for 48 hours with MPA. To determine the presence of CYP450 in SMCs, expression of CYP1A1 and CYP1B1 was assessed by Western blots in SMCs pretreated with 3-methylcholanthrene for 48 hours.

To assess the effects of MPA on the conversion of estradiol to 2-hydroxyestradiol, microsomal preparations (1 mg/mL) were incubated for 2 hours with 25 μmol/L of estradiol in the presence or absence of 0 to 100 μmol/L of MPA. Next, internal standard (160-hydroxyestradiol) was added, samples were extracted with methylene chloride, extracts were dried under vacuum, residues were reconstituted in mobile phase, and samples were analyzed by high-performance liquid chromatography with UV detection using gradient elution, as described previously. Because of decreased assay sensitivity, the metabolism of estradiol to 2-methoxyestradiol by SMCs was assessed in the presence of microsomes to facilitate the formation of 2-hydroxyestradiol (substrate for 2-methoxyestradiol) from estradiol. Briefly, microsomes (1 mg/mL) were incubated for 2 hours with 25 μmol/L of estradiol with or without 0 to 100 μmol/L of MPA, and the supernatants containing the metabolites were collected. Subsequently, confluent monolayers of SMCs were treated with 1 hour with supernatants collected from human microsomes incubated with estradiol or estradiol plus MPA (as described above). After the treatment, the samples were extracted and 2-methoxyestradiol levels analyzed by high-performance liquid chromatography.

We used (2-[^3]H)-estradiol to assess the metabolism of estradiol to 2-hydroxyestradiol in cultured SMCs. As shown previously, for every molecule of estradiol metabolized to 2-hydroxyestradiol, 1 molecule of [^3]H₂O is released. Hence, quantifying [^3]H₂O in the supernatant serves as a surrogate marker for 2-hydroxyestradiol formation. To study whether SMCs metabolize estradiol to 2-hydroxyestradiol and whether MPA inhibits this conversion, confluent monolayers of SMCs were treated for 12 hours with (2[^3]H)-estradiol, and formation of [^3]H₂O was assessed after extraction by liquid scintillation counting, as described previously.

**Results**

Treatment with 2.5% FCS stimulated [^3]H-thymidine and [^3]H-proline incorporation by 7- and 6-fold (P<0.001 versus 0.25% albumin), respectively. Treatment with estradiol (1 to 100 nmol/L) concentration-dependently inhibited [^3]H-thymidine incorporation (Figure 1A), [^3]H-proline incorporation (Figure 1B), and cell proliferation (Figure 2A and 2B). MPA, in a concentration-dependent manner, abrogated the concentration-dependent inhibitory effects of estradiol on [^3]H-thymidine and [^3]H-proline incorporation (Figure 1A and 1B) and abrogated the inhibitory effects of estradiol on SMC proliferation (Figure 2A and 2B). Treatment with MPA alone did not alter FCS-induced effects on [^3]H-thymidine or [^3]H-proline incorporation or cell proliferation (Figures 1 and 2). The effects of MPA were more potent than 1-aminobenzotriazole (ABT; a broad spectrum inhibitor of CYP450). For example, a concentration of 5 μmol/L of ABT was required to reverse the inhibitory effects of estradiol (100 nmol/L) on [^3]H-thymidine incorporation from 43±1% to 3±2.1%, whereas MPA at only 10 nmol/L reversed this inhibitory effect to 8±2.4%.

MPA also abrogated the inhibitory effects of estradiol on PDGF-induced SMC migration (Figure 2C), whereas MPA alone did not significantly influence PDGF-induced migration of SMCs. ABT also completely reversed the inhibitory effect of estradiol (100 nmol/L) on SMC migration; however,
as with DNA synthesis, higher concentrations of ABT (5 μmol/L) were required to achieve the same inhibition as observed for MPA (10 nmol/L).

As illustrated in Figure 3, 10 nmol/L of MPA blocked the inhibitory effects of 100 nmol/L of estradiol on cell proliferation and [3H]-proline incorporation, and the abrogatory effects of MPA were not diminished, but rather enhanced marginally, by 1 μmol/L of RU486. As shown in Figure 4, in contrast to estradiol, MPA did not block the inhibitory effects of either 2-hydroxyestradiol or 2-methoxyestradiol on FCS-induced [3H]thymidine and [3H]-proline incorporation. Treatment of human SMCs with MPA did not alter the expression of ERα and ERβ (Figure 5). Both CYP1A1 and CYP1B1 (involved in hydroxylation of estradiol) were highly expressed in SMCs stimulated with 3-methylcholanganthene, a broad-spectrum CYP450 inducer (Figure 5).

Microsomal preparations incubated with 25 μmol/L of estradiol efficiently metabolized estradiol to 2-hydroxyestradiol (Figure 6A). Cotreatment with MPA concentration-dependently inhibited the conversion of estradiol to hydroxyestradiol (Figure 6A). At concentrations of 25, 50, and 100 μmol/L, MPA inhibited the conversion of estradiol to 2-hydroxyestradiol by 12.0 ± 2.5%, 31.0 ± 2.0%, and 72.0 ± 5.0%, respectively. In SMCs treated for 1 hour with supernatants of microsomes incubated with estradiol, 2-methoxyestradiol was formed. Moreover, the formation of 2-methoxyestradiol was significantly inhibited by MPA (Fig-
The formation of 2-methoxyestradiol from estradiol was inhibited by MPA in a concentration-dependent manner (Figure 6B). At a concentration of 25, 50, and 100 \(\mu\)mol/L, MPA inhibited 2-methoxyestradiol formation by 24\%, 47\%, and 88\%, respectively (Figure 6B). SMCs also efficiently metabolized 2-hydroxyestradiol to 2-methoxyestradiol, and OR486 but not MPA inhibited the methylation of 2-hydroxyestradiol (Figure 6C). In SMCs incubated with \(^{2-}\)H-estradiol, the formation of \(^{1}\)H-H\(_2\)O, a surrogate marker of 2-hydroxyestradiol formation, was evident, and this conversion was concentration-dependently blocked by 1, 10, and 100 \(\mu\)mol/L of MPA (Figure 6D), as well as by 5 \(\mu\)mol/L of ABT (Figure 6D).

**Discussion**

This study demonstrates the following in human vascular SMCs: (1) physiological and pharmacological concentrations of estradiol inhibit DNA synthesis, collagen synthesis, cell proliferation, and cell migration; (2) these inhibitory effects of estradiol are abrogated by MPA; (3) the abrogatory effects of MPA are not reduced by RU486 (a progesterone receptor blocker); (4) treatment with MPA does not downregulate the expression of ERs in SMCs; (5) unlike estradiol, MPA does not block the inhibitory effects of either 2-hydroxyestradiol or 2-methoxyestradiol (metabolites of estradiol); (6) the abrogatory effects of MPA are mimicked by the broad-spectrum CYP450 inhibitor ABT; (7) SMCs metabolize estradiol to 2-hydroxyestradiol and 2-methoxyestradiol and express CYP450 enzymes responsible for converting estradiol to hydroxyestradiols; and (8) MPA blocks the conversion of estradiol to 2-hydroxyestradiol and 2-methoxyestradiol.

Taken together, our findings provide evidence that MPA blocks the inhibitory effects of estradiol on SMCs by inhibiting the sequential metabolism of estradiol to hydroxyestradiols and methoxyestradiols.

Progestins are known to induce their biological effects via progesterone receptors (PRs), and it is possible that the reversal of the effects of estradiol by MPA is mediated by PRs rather than by inhibition of estradiol metabolism. Our finding that the abrogatory effects of MPA are not blocked by RU486 provides strong evidence that MPA abrogates the effects of estradiol by inhibiting CYP450-mediated conversion of estradiol and not by activating PRs. Apart from PRs, MPA is also known to interact with glucocorticoid receptors; however, the role of glucocorticoid receptors in mediating the abrogatory effects of MPA can also be ruled out, because RU486 is also a glucocorticoid receptor antagonist and fails to reverse the abrogatory effects of MPA on the inhibitory effects of estradiol. The possibility that the effects of MPA are mediated via modulation of ER\(\alpha\) or ER\(\beta\) expression can also be ruled out, because the expression of both ERs is unaltered in SMCs treated with MPA. Taken together, the above findings provide evidence that MPA abrogates the antimitogenic effects of estradiol on SMCs via...
mechanisms independent of PRs, glucocorticoid receptors, and ERs.

Consistent with the current findings, we have shown previously that the inhibitory effects of estradiol on SMCs are blocked by CYP450 inhibitors and enhanced by CYP450 inducers.2 Because CYP450 isozymes are responsible for metabolizing estradiol to hydroxyestradiols, precursors of methoxyestradiols, we postulated, and subsequently demonstrated, that the sequential conversion of estradiol to hydroxyestradiols and methoxyestradiols is responsible for mediating the inhibitory effects of estradiol on vascular SMCs.1,2,5 Our finding that SMCs expressed CYP1A1 and CYP1B1 and that MPA blocks the conversion of estradiol to 2-hydroxyestradiol, a precursor of 2-methoxyestradiol, provides strong evidence that, in SMCs, MPA can block the inhibitory effects of estradiol by inhibiting the local conversion of estradiol to methoxyestradiols.

Circulating levels of MPA during combined hormone therapy are ≈1 to 2 nmol/L.19 In the present study, concentrations of MPA as low as 1 to 10 nmol/L significantly attenuated the inhibitory effects of 100 nmol/L of estradiol. Because the interaction between MPA and estradiol at the level of CYP450 is likely competitive, even lower levels of MPA would be expected to attenuate the inhibitor effects of lower levels of estradiol. These considerations imply that the levels of MPA under pharmacological conditions could effectively attenuate the inhibitory effects of estradiol on SMCs and abrogate the vasoprotective actions of estradiol.

Inasmuch as hydroxyestradiols and methoxyestradiols are formed by the sequential actions of CYP450 and catechol-O-methyltransferase, it is conceivable that exogenous substrates for CYP450 would abrogate the cardioprotective effects of estradiol. In this regard, MPA, a synthetic progestin used for hormone therapy in combination with estrogens, is a well-known substrate for CYP450 and is implicated in negating the beneficial effects of estrogens.8,9 It is possible, therefore, that MPA may abrogate the cardiovascular protective effects of estradiol by decreasing its sequential conversion to hydroxyestradiols and methoxyestradiols by SMCs. Our findings provide strong evidence that MPA could abrogate the cardioprotective effects of estradiol by inhibiting CYP450 activity and thereby reducing the sequential biosynthesis of hydroxyestradiols and methoxyestradiols. Our results also imply that concomitant treatment with MPA can negate the cardioprotective effects of estradiol in postmenopausal women receiving combined hormone therapy but not in women treated with estradiol alone.

Although our findings provide evidence that MPA blocks the antimitogenic effects of estradiol by inhibiting its metabolism to methoxyestradiols, other mechanisms likely participate in mediating the abrogatory effects of MPA on vasoprotective effects of estradiol. For example, MPA attenuates the beneficial effects of estrogens on endothelial-dependent relaxation and release of NO (antimitogenic molecule),10,17,18 increases vascular procoagulant activity by upregulating thrombin receptor PAR-1 expression,15 abrogates the cardioprotective and anti-inflammatory effects of estradiol,19 attenuates the favorable effects of orally administered estrogens on high-density lipoprotein,9 and abrogates the inhibitory effects of estradiol on injury-induced neointima formation.12 Finally, although the above findings suggest that MPA can abrogate the protective effects of estradiol, it should be noted that the estrogen alone arm of the Women’s Health Initiative Study did not show positive results, suggesting that factors other than MPA may also contribute to the lack of cardiovascular protective actions by estrogens in the Women’s Health Initiative Study.

Perspectives

Here we provide evidence that MPA, a synthetic progestin widely used with estrogens in combined hormone therapy in postmenopausal women, abrogates the ability of estradiol to inhibit DNA and collagen synthesis by SMCs and SMC proliferation and migration by inhibiting its sequential metabolism to hydroxyestradiol and methoxyestradiol. These results imply that progestins other than MPA should be used in combined hormone therapy in postmenopausal women with an intact uterus.

Sources of Funding

This work was supported by Swiss National Science Foundation grants 32-64040.00, 320000-117998, Oncosuisse OCS-01551-08-2004, and EMDO Forschung and National Institutes of Health grants HL69846 and DK068575.

Disclosures

None.

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Disclosures

None.


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Hypertension. 2008;51:1197-1202; originally published online February 7, 2008; doi: 10.1161/HYPERTENSIONAHA.107.106575

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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