Effects of dydrogesterone and of its stable metabolite, 20-α-dihydrodydrogesterone, on nitric oxide synthesis in human endothelial cells

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Objective: To investigate the effects of P, medroxyprogesterone acetate (MPA), and dydrogesterone (DYD) and its metabolite, 20-α-dihydrodydrogesterone (DHD) on endothelial synthesis of nitric oxide (NO) and characterize the signaling events recruited by these compounds. The Women's Health Initiative trial reports an excess of heart disease in postmenopausal women receiving MPA.
Design: Cell culture.
Setting: Research laboratory.
Patient(s): Human endothelial cells from umbilical vein.
Intervention(s): Treatments with P, MPA, DYD, or DHD.
Main Outcome Measure(s): Measure of NO release, endothelial nitric oxide synthase (eNOS) activity and expression, and activation of ERK 1/2 and Akt.
Result(s): The administration of DYD alone or in combination with estrogen to endothelial cells results in neutral effects on NO synthesis and on the activity and expression of eNOS. In parallel, the stable metabolite DHD acts similarly to natural P, enhancing the expression of eNOS and inducing rapid activation of the enzyme through the regulation of the ERK 1/2 mitogen-activated protein kinase cascade. 20-α-dihydrodydrogesterone and P also potentiate eNOS induction by E_2. On the contrary, MPA does not trigger eNOS enzymatic activation and decreases the extent of eNOS induction by E_2.
Conclusion(s): These findings support the concept that synthetic progestins act differently on vascular cells and that hormonal preparations may differ as to their cardiovascular effects. (Fertil Steril® 2006;86(Suppl 3): 1235–42. ©2006 by American Society for Reproductive Medicine.)

Key Words: Cardiovascular disease, endothelial cells, progestins, dydrogesterone, 20-α-dihydrodydrogesterone, nitric oxide

After the publication of the Women's Health Initiative trial, interest in the clinical effects of progestins suddenly has grown. Indeed, the unexpected report of a higher incidence of coronary heart disease in the cohort of women receiving conjugated equine estrogens plus medroxyprogesterone acetate (MPA) with respect to the group treated with conjugated equine estrogens only (1, 2) has revived the debate on the possible specific role of progestins on cardiovascular function in postmenopausal women (3).

Although the cardiovascular effects of P and of synthetic progestins in the clinical setting have been poorly investigated, there is suggestive evidence that the actions of the different compounds might not be the same. To this aim, a substantial number of publications have pinpointed that natural P and synthetic progestins often elicit significantly different effects on a number of processes in cells, animals, or humans (4), therefore raising interest in understanding the characteristics of each of the compounds available in clinical practice.

Dydrogesterone (DYD) is a synthetic progestin used together with E_2 for postmenopausal hormone replacement therapy. Because of its chemical structure DYD has a strong affinity to P receptor (PR) and does not significantly bind to other steroid hormone receptors. Dydrogesterone is closely related to natural P from a structural point of view, and after absorption it is largely converted to its stable 20-dihydrometabolite, 20-α-dihydrodydrogesterone (DHD) (5).

Clinical studies indicate that postmenopausal hormone replacement with estrogen plus DYD may result in a favorable cardiovascular risk profile (4). Indeed, increases in high-density lipoprotein cholesterol are found during administration of DYD along with either transdermal (6) or oral E_2 (7). Moreover, decreases of circulating Lp(a) lipoprotein (8) and homocysteine (9) have been reported in women receiv-

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ing such regimens. Slight improvements in insulin sensitivity have been reported in postmenopausal women receiving E_2 plus DYD (10, 11), which also could have an impact on cardiovascular risk. Moreover, the metabolic actions of HRT containing DYD significantly differ from those of hormonal associations containing MPA (12).

Human endothelial cells are central for the function of human vessels in physiologic and pathologic conditions, and the synthesis of the vasodilatory and anti-inflammatory molecule nitric oxide (NO) by endothelial cells is of paramount importance for vascular function (13). Estrogens preserve endothelial function in vitro and in vivo; however, the addition of P or other synthetic progestins can interfere to a different extent with the actions of estrogens (14).

We assessed the effects of the synthetic progestin DYD, as well as of its stable metabolite, DHD, on human endothelial cells' synthesis of NO, with the aim of testing the hypothesis that because of their chemical structures and receptor-binding properties they might show specific effects on endothelial cells when compared with natural P or MPA. We did so by characterizing both the transcriptional and nontranscriptional regulation of the endothelial nitric oxide synthase (eNOS), either by the progestins alone or in the presence of E_2 and by comparing the effects of DYD and DHD with those of P and of MPA in these conditions.

MATERIALS AND METHODS

Cell Cultures and Treatments

Human umbilical vein endothelial cells (HUVECs) were cultured as described (15). Before treatments, HUVECs were kept 48 hours in Dulbecco's minimum essential medium containing steroid-deprived fetal bovine serum. Before experiments investigating nontranscriptional effects, HUVECs were kept in Dulbecco's minimum essential medium containing no fetal bovine serum for 8 hours. Whenever an inhibitor was used, the compound was added 30 minutes before starting the treatments. Progesterone, MPA, and RU486 were obtained from Sigma-Aldrich (St. Louis, MO). Dydrogesterone and DHD were obtained from Dr. J. Alt, Solvay Pharmaceutical (Hannover, Germany).

Endothelial Nitric Oxide Synthase Activity Assay

Endothelial nitric oxide synthase activity was determined as conversion of [^{3}H]arginine to [^{3}H]citrulline in endothelial cell lysates. [^{3}H]Citrulline was separated with use of an acidic ion-exchange resin, as described (16). Extracts incubated with *N*-nitro-L-arginine methyl ester (1 mmol/L) served as blank.

Nitrite Assay

Nitric oxide production was determined by a nitrite assay with use of 2, 3-diaminonaphthalene (17). 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 NG-monomethyl-L-arginine (3 mmol/L).

Immunoblottings

Cell lysates were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Antibodies used were eNOS (polyclonal, catalog no. 610299; Transduction Laboratories, Lexington, KY), wild-type ERK 1/2 (polyclonal, catalog no. 442704; Calbiochem Merck Biosciences, GmbH, Darmstadt, Germany), Tyr^{204}-P-ERK 1/2 (polyclonal, catalog no. sc-7976; Santa Cruz Biotechnology, Santa Cruz, CA), and von Willebrand factor (polyclonal, catalog no. sc-8068; Santa Cruz). Primary and secondary antibodies were incubated with the membranes with standard technique (18). Immunodetection was accomplished with use of enhanced chemiluminescence.

Statistical Analysis

All values are expressed as mean ± SD. Statistical differences between mean values were determined by ANOVA, followed by Fisher's protected least significance difference.

Institutional Review Board

Institutional review board approval is not required by the University of Pisa for studies in isolated cells.

RESULTS

Dydrogesterone's Stable Metabolite, DHD, Enhances NO Synthesis and eNOS Activity in Human Endothelial Cells

Steroid-deprived HUVEC were treated for 48 hours with a physiologic P concentration (10 nmol/L) or with an equimolar amount of MPA (10 nmol/L), DYD (10 nmol/L), or DHD (10 nmol/L). As previously shown (14), P significantly enhanced NO synthesis whereas MPA was inactive. Like MPA, DYD did not modify NO synthesis (Fig. 1A);) however, DYD's active metabolite DHD elicited a consistent increase of NO synthesis in HUVECs (Fig. 1A). Nitric oxide induction by DHD was prevented by the PR antagonist RU486. The concentration used for DYD is in the range of the plasma concentrations found during a standard oral 20 mg/day administration, whereas DHD usually is found in the bloodstream at 25-fold higher concentrations (the area under the curve is 40 times higher and the c_{max} is 25 times higher for DHD than for DYD; information derived from the official Duphaston Summary of Product Characteristics, http://www.solvaypharmaceuticals.com). The increases in NO synthesis associated with P and DHD were due to increased enzymatic activity of the eNOS (Fig. 1A).

Diverging Transcriptional Actions of DYD, DHD, P, and MPA on eNOS

Consistent with our previous results (14), when eNOS protein amounts were assayed, increased eNOS expression was