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Effects of drospirenone on adhesion molecule expression and monocyte adherence in human endothelial cells

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ABSTRACT

Objective: A major concern in hormone replacement therapy is the associated increased risk of cardiovascular diseases. A progestogen without the unfavorable effects on cardiovascular disease should be explored. Monocyte adhesion to endothelial cells is an important initial event in atherosclerosis. In this study, the effects of the alternative progestogen drospirenone (DRSP) on monocyte adhesion in human umbilical venous endothelial cells (HUVECs) were examined.

Study design: In HUVECs treated with estrogens and progestogens, including DRSP and medroxyprogesterone acetate (MPA), the expression of the adhesion molecules E-selectin, P-selectin, ICAM-1, and VCAM-1 were examined by real-time PCR and using an enzyme-linked immunosorbent assay. A flow chamber system was used to investigate the effects of DRSP on U937 monocytoid cell adherence to HUVEC monolayers. All experimental data were compared using one-way Analysis of Variance.

Results: Upregulation of adhesion molecule mRNA or protein was not seen in HUVECs treated with DRSP alone or with 17β-estradiol + DRSP. DRSP alone, 17β-estradiol + DRSP or ethinylestradiol + DRSP did not increase the number of adherent monocytoid cells to HUVECs in the flow chamber system. However, MPA significantly enhanced the monocytoid cell adherence ($P < 0.05$).

Conclusions: DRSP did not increase the expression of adhesion molecules or monocytoid cell adherence to endothelial cells, indicating that DRSP could reduce the risk of atherogenesis caused by MPA. These results suggest that DRSP may be an alternative to MPA in hormone replacement therapy.

Introduction

In hormonereplacement therapy (HRT) for postmenopausal women, progestogens are co-administered with estrogen to reduce endometrial cancer risk caused by estrogen administration. Large-scale clinical trials, the Women's Health Initiative (WHI) study, demonstrated that HRT with conjugated equine estrogen (CEE) and the synthetic progestogen medroxyprogesterone acetate (MPA) enhanced the risk for coronary heart disease (CHD), stroke, and venous thromboembolic disease compared with estrogen alone [1,2]. Other trials have tested the benefit and risk of estrogen–progestogen therapy with MPA alternatives, e.g., norethisterone acetate, levonorgestrel (LNG), dydrogesterone, dienogest, and natural progesterone (P4) [3,4]. However, only a few basic studies have investigated the cellular development of arteriosclerosis caused by various types of progestogen [5,6].

Drospirenone (DRSP) is a progestogen derived from spirolactone with anti-mineralocorticoid and anti-androgenic effects [7]. DRSP-ethinylestradiol (EE) is widely used as an oral contraceptive (OC) [8,9]. However, the effect of DRSP on atherosclerosis risk in endothelial cells is currently unclear, as only a few clinical studies have investigated the impact of DRSP on atherosclerosis risk at the cellular or molecular level [10].

Adhesion of circulating monocytes to endothelial cells via specific receptor-adhesion molecule interactions is an important initial event in atherogenesis [11–13]. Such intercellular adhesion requires specific receptor–ligand interactions between endothelial cell adhesion molecules (e.g., E-selectin, P-selectin, intercellular adhesion molecule-1 [ICAM-1], and vascular cell adhesion molecule-1 [VCAM-1]) and adhesion receptors on the monocyte [11–13]. Various chemicals, such as inflammatory cytokines, are known to effect the expression of these adhesion molecules [13]. We previously reported that MPA, which is frequently used for HRT, modified the expression of adhesion molecules on human umbilical vein endothelial cells

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(HUVECs), possibly increasing the risk of CHD [5,14]. However, the association between other progestogens, notably DRSP, and the expression of these adhesion molecules remains elusive.

In the present study, we examined the effects of DRSP on the expression of cell adhesion molecules in HUVECs. Potential changes in monocyte adherence caused by progestogens were evaluated using a well-defined parallel plate flow chamber under flow conditions that mimic physiological interactions between monocytes to endothelial cells.

Materials and methods

Steroids

MPA, P4, and EE were purchased from Sigma–Aldrich (St. Louis, MO). DRSP was purchased from Bayer Yakuhin Ltd. (Osaka, Japan). Estradiol (E2) was purchased from Nacalai Tesque, Inc. (Kyoto, Japan).

Cell culture and treatments

Female infant umbilical cords were obtained from patients whounderwent normal delivery without complications. HUVECs were separated using the method described in our previous report [14]. Briefly, HUVECs were cultured at 37 °C under 5% CO_2 in 75 cm^2 tissue culture flasks until cells reached pre-confluence. The cells were re-plated onto 6- or 96-well plates until cells reached pre-confluence. The medium was changed to MEM (without phenol red) containing 4% charcoal-treated FBS, penicillin and streptomycin, and the cells were precultured for 12 h. The cells were then incubated for 24 h with medium containing recombinant human IL-1β (40 U/mL; Genzyme, Cambridge, MA) and one of the tested steroids. Each steroid was dissolved in ethanol to a final concentration of 0.1% per well. This protocol was approved by the Kyoto Prefectural University of Medicine Institutional Review Board.

Real-time PCR analysis

Total RNA was extracted from HUVECs using the RNeasy Mini kit (QIAGEN, Venlo, Netherlands) according to the manufacturer's instructions. RNA (1 μg) from each sample was reverse transcribed to cDNA and amplified using ReverTra Ace® qPCR RT Master Mix (TOYOBO, Osaka, Japan). Gene expression levels were analyzed using real-time PCR. The forward and reverse primers for E-selectin, P-selectin, ICAM-1, and VCAM-1 are listed in Supplementary Table 1. The cycle time (*Ct*) values obtained were used to quantify the relative expression of the genes of interest. The *Ct* values were first normalized to the internal control (GAPDH), and then the fold changes of target genes ($\Delta\Delta Ct$) in all the groups were calculated and represented as relative expression values.

Enzyme immunoassay (EIA)

Protein expression of adhesion molecules was examined by EIA as described previously [14]. Briefly, HUVEC monolayers in 96-well plates were fixed by addition of 4% paraformaldehyde. To create a standard curve, each 96-well plate was coated with diluted Capture Antibody (R&D systems, Minneapolis, MN) and standards were added according to the manufacturer's instructions. Mouse anti-human E-selectin monoclonal antibody (1.0 μg/mL), mouse anti-human P-selectin monoclonal antibody (1.0 μg/mL), mouse anti-human ICAM-1 monoclonal antibody (1.0 μg/mL), and mouse anti-human VCAM-1 monoclonal antibody (1.0 μg/mL) (R&D systems, Minneapolis, MN) were added to each plate. Biotinylated goat anti-mouse antibodies (Dako, Carpinteria, CA) diluted in 10% heat-inactivated normal human serum in Tris-buffered saline, pH 7.6, were added. The wells were washed twice with wash buffer, followed by addition of streptavidin-horseradish peroxidase complex (Dako, Carpinteria, CA). After 2 washes with wash buffer, *o*-phenylenediamine dihydrochloride in citric-acid phosphate buffer (pH 5.0) was added. The reaction was stopped by the addition of 1 M sulfuric acid. Absorbance was measured at 490 nm with an ELISA reader (Model 550; Bio-Rad, Hercules, CA).

Flow chamber system

We assessed the effect of shear stress on the adherence of U937 monocytoid cells to HUVEC monolayers induced by IL-1β. To produce well-defined shear stress, we used a flow chamber system previously described by Gerszten [15], with modification as previously described [16]. Briefly, the chamber consisted of a glass slide with a confluent HUVEC monolayer that was attached to a polycarbonate base. These 2 flat surfaces were held approximately 270 μm apart by a Silastic rubber gasket (Dow Corning, Midland, MI). Flow across the monolayer was controlled with a syringe pump (Harvard Apparatus, South Natick, MA). U937 monocytoid cell suspension (10,000 cells/mL) in HBSS was perfused through the flow chamber. Experiments were videotaped using a color camera mounted on an inverted microscope. Adherent cells were defined as cells that remained stationary for a period of ≥30 s. The number of adherent U937 monocytoid cells was counted 5 min after the perfusion of cells.

Statistical analysis

All data are expressed as mean ± SEM for 3 separate experiments. Differences in progestogen stimulation were analyzed using a one-way Analysis of Variance (ANOVA) followed by the Bonferroni–Dunn test for multiple comparisons. $P < 0.05$ was considered significant.

Results

DRSP did not increase mRNA expression of adhesion molecules in HUVECs

We first performed quantitative real-time PCR to examine how DRSP affects mRNA expression of cell adhesion molecules in HUVECs. MPA and E2 + MPA treatment yielded 1.4- to 2.5-fold increases in mRNA expression of E-selectin, P-selectin, ICAM-1, and VCAM-1 in HUVECs, compared with the control ($P < 0.01$, Fig. 1A–D). However, the results here show that DRSP and EE + DRSP did not increase mRNA expression of the same adhesion molecules in HUVECs (Fig. 1A–D).

DRSP did not increase protein levels of adhesion molecules in HUVECs

We next demonstrated the expression of cell adhesion molecules at the protein level in HUVECs treated with DRSP and MPA by EIA. DRSP, E2 + DRSP or EE + DRSP did not increase the protein levels of the cell adhesion molecules tested (Fig. 2A–D). In contrast, MPA increased the expression of E-selectin, P-selectin, and ICAM-1 compared with the control (E-selectin: 83.0 ± 0.7 vs. 72.3 ± 0.1 ng/mL, P-selectin: 34.8 ± 1.2 vs. 31.0 ± 0.1 ng/mL, and ICAM-1: 5.4 ± 0.1 vs. 4.9 ± 0.1 ng/mL, respectively) ($P < 0.05$, Fig. 2A–C). The E-selectin and ICAM-1 protein concentrations in HUVECs were significantly higher than that for the control (E-selectin: 76.3 ± 1.9 vs. 72.3 ± 0.1 ng/mL and ICAM-1: 5.1 ± 0.0 vs. 4.9 ± 0.1 ng/mL, respectively) when HUVECs were treated with E2+ MPA ($P < 0.05$, Fig. 2A and C).