Dydrogesterone exerts endothelial anti-inflammatory actions decreasing expression of leukocyte adhesion molecules

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ABSTRACT: Clinical observations and basic studies show that progesterone and progestins have a variable influence on endothelial function. Dydrogesterone (DG) is a widely used progestin, but its endothelial actions have not been thoroughly assessed. In this study, we investigated the effects of DG and its metabolite 20α-dihydro-dydrogesterone (DHD), natural progesterone as well as medroxyprogesterone acetate, on the expression of leukocyte adhesion molecules in human endothelial cells using an in vitro experimental endothelial inflammation system. Our findings show that all progestins significantly suppress endothelial expression of vascular cell adhesion molecule-1 (VCAM-1) and inter-cellular adhesion molecule-1 (ICAM-1) induced by bacterial lipopolysaccharide (LPS). These inhibitory effects of DG and DHD require activation of progesterone receptor. DG and DHD decrease adhesion molecule expression associated with LPS administration by preventing nuclear translocation of the pro-inflammatory transcription factor nuclear factor-κB. In addition, DG and DHD do not alter the anti-inflammatory effects of 17β-estradiol. In conclusion, DG and DHD decrease endothelial inflammatory responses induced by LPS, via reduced expression of the pro-atherogenic adhesion molecules VCAM-1 and ICAM-1. These actions may be relevant for the vascular effects of DG.

Key words: endothelial cell, dydrogesterone / 20α-dihydro-dydrogesterone / endothelial-leukocyte adhesion molecules / NF-κB

Introduction

Experimental and clinical rationale indicates that exposure to sex steroids beyond menopause in the form of hormone replacement therapy (HRT) should protect women from cardiovascular disease (Barrett-Connor and Bush, 1991). However, evidence form clinical trials in this area is conflicting (Manson et al., 2003). Even if the two parallel Women’s Health Initiative (WHI) trials cannot be directly compared, it is clear that HRT with conjugated equine estrogens (CEE) results in decreased cardiovascular risk in selected groups of women, while the combination of CEEs with medroxyprogesterone acetate (MPA) does not (Rossouw et al., 2002; Anderson et al., 2004). These findings have raised interest in the role of progestins in the vascular system and in particular, in endothelial cells.

Estrogen improves endothelial function in vitro and in vivo (Mendelsohn and Karas, 1999, 2005). However, the effects of progesterone or synthetic progestins on endothelial function are variable. For instance, natural progesterone increases endothelial nitric oxide (NO) production, while MPA is devoid of such action (Simoncini et al., 2004). In parallel, MPA interferes with the athero-protective effects of estrogens, which does not happen with natural progesterone (Adams et al., 1990, 1997). These discrepancies may be due to the different spectrum of binding abilities of these progestins to the progesterone receptor (PR) or to other steroid receptors (Simoncini et al., 2007). Indeed, binding of MPA to glucocorticoid receptor explains the anti-inflammatory actions found in endothelial cells when this progestin is used alone. However, it also determines a marked decrease of the powerful endothelial anti-inflammatory effects of hydrocortisone when the two compounds are used together (Simoncini et al., 2004).

Dydrogesterone (DG) is a synthetic progestin that is widely used for contraception, infertility, recurrent miscarriage, endometriosis and post-menopausal hormone therapy (HT). DG is metabolized by...
reduction at C20 to its pharmacologically active main metabolite, 20α-dihydroxy-dydrogestron (DHD). Both progestins show no clinically relevant androgenic, estrogenic or mineralocorticoid activities since they bind almost exclusively to PR (Schindler, 2009). Given their widespread clinical use, the effects of DG and DHD on the cardiovascular system is of interest. Clinical studies in this area indicate that HT containing DG results in beneficial effects on the lipid profile and ambulatory blood pressure in post-menopausal women (de Kraker et al., 2004; Godsland et al., 2004; Kaya et al., 2006).

Experimental studies indicate that both DG and its metabolite DHD also modulate endothelial cells, where they increase the synthesis of NO (Simoncini et al., 2006).

Expression of endothelial-leukocyte adhesion molecules induced by pro-atherogenic cytokines is one of the earliest detectable events during atherosigenesis. Estrogen exerts potent anti-inflammatory actions in vessels by decreasing expression of leukocyte adhesion molecules (such as vascular cell adhesion molecule-1 - VCAM-1— and inter-cellular adhesion molecule-1 (ICAM-1) — and by inhibiting the productions of inflammatory mediators (Simoncini et al., 2000; Jiang et al., 2010). The inhibitory effect on endothelial adhesion molecules has also been shown for natural progesterone and MPA. However, MPA acts through signaling avenues that largely differ from those elicited by progesterone (Simoncini et al., 2004). This supports the hypothesis that different progestins may have different endothelial effects, which may turn out to be relevant for their cardiovascular actions.

In this study, we compared the effects of DG, DHD, progesterone and MPA on leukocyte adhesion molecule expression in human endothelial cells and explored the underlying molecular mechanisms.

**Materials and Methods**

**Cell cultures and treatments**

Human umbilical vein endothelial cells (HUVECs) were cultured as described (Fu et al., 2008). Cells were grown to 85%–90% confluency and then kept 48 h in Dulbecco’s modified Eagle’s medium-containing steroid-deprived fetal bovine serum. Whenever an inhibitor was used, the compound was added 30 min before starting the treatments. Progesterone (10⁻⁸ mol/l), 17β-estradiol (E2) (10⁻⁹ mol/l), MPA (10⁻⁹ mol/l), Escherichia Coli lipopolysaccharide (LPS) (100 ng/ml) were obtained from Sigma-Aldrich (Saint-Louis, MO, USA). DG (10⁻⁸ mol/l) and 20α-hydroxy-derivative DHD (10⁻⁸ mol/l) were obtained from Dr J. Alt, Solvay Pharmaceutical (Germany). ORG 31710 (1 μM) was a kind gift of Dr Lenus Kloosterboer, from Organon Akzo Nobel, Oss, The Netherlands.

**Immunoblottings**

After the different treatments, endothelial cells were rinsed once with ice-cold phosphate-buffered saline (PBS) before addition of the lysis buffer (100 mM Tris—HCL, pH 6.8, 4% SDS, 20% glycerol, 1 mM sodium orthovanadate, 1 mM NaF and 1 mM phenylmethylsulfonyl fluoride) to the dishes on an ice tray. The cell lysates were scraped, boiled and centrifuged for 2 min at 28 340 g. Samples were normalized for protein concentration using Pierce bicinchoninic acid (BCA) method. Cell lysates (30 μg) were separated by SDS—PAGE. Antibodies used were: VCAM-1 [mouse immunoglobulin G (IgG), # sc-13160, Santa Cruz Biotechnology, Santa Cruz, CA, USA], ICAM-1 [mouse IgG, # sc-107, Santa Cruz] and p65 nuclear factor-κB (NF-κB; mouse IgG, # sc-71677, Santa Cruz). Primary (1: 1000) and secondary antibodies (rabbit anti-mouse IgG-horse-radish peroxidase, sc-358914, Santa Cruz) (1: 2000) were incubated with the membranes with standard technique. Immunodetection was accomplished using enhanced chemiluminescence. Chemiluminescence was acquired with a quantitative digital imaging system (Quantity One, BioRad, Hercules, CA, USA) allowing us to check for saturation. Overall emitted photons were quantified for each band, particularly for loading controls, which were homogeneously loaded.

**OD measurement for ICAM-1/VCAM-1 membrane immunostaining**

Endothelial cells were grown on 96-wells culture dishes. LPS (100 ng/l for 18 h) was used to stimulate the expression of VCAM-1 and ICAM-1. Assay of cell-surface VCAM-1 or ICAM-1 expression was carried out using the antibodies against VCAM-1 (Ab E1/6), ICAM-1 (HU 5/3) or the monoclonal antibody E1/1 (in control experiments) (De Caterina et al., 2001) (all antibodies were provided by Michael A. Gimbrone Jr, Harvard Medical School). Enzyme-linked immunosassays (EIA’s) were carried out by incubating endothelial monolayers with the primary antibodies, then with biotinylated secondary antibody, and finally with streptavidin-alkaline phosphatase. Three washes with PBS containing 1% bovine serum albumin 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. Eight different wells were used for each experimental condition.

**Preparation of nuclear extracts and cytosolic extracts for western blots**

Nuclear extracts were prepared as we previously described (Jiang et al., 2010). Cells were scraped in buffer containing 10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 10% glycerol, 0.5 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 2 μg/ml pepstatin A and 2 μg/ml leupeptin). Cells were collected at 4°C at top speed (150 000g) for 3 min and the supernatant was saved as cytosolic fraction. The lysate were resuspended into an equal volume of buffer, containing 20 mM HEPES, 0.5 mM NaCl, 1 mM EDTA, 10% glycerol and the protease inhibitors and shaken at 4°C for 2 h. The concentration of protein in the extracts was measured using the BCA assay (Pierce).

**Cell immunofluorescence**

HUVECs were grown on cover slips and exposed to different treatments. For the control, cells were treated with the same amount of PBS as LPS treatment. Cells were fixed with 4% paraformaldehyde for 30 min and permeabilized with 0.1% Triton X for 5 min. Blocking was performed with 3% normal serum for 20 min. Cells were incubated with a rabbit antibody against p65 NF-κB subunit (# sc-7151, Santa Cruz) for 2 h at room temperature. A Texas red goat anti-rabbit antibody was used 1 h as the secondary antibody. After washing, the nuclei were counterstained with or 4′-6-diamidino-2-phenylindole and mounted with Vectashield mounting medium. Immunofluorescence was visualized using an Olympus BX41 microscope and recorded with a high-resolution DP70 Olympus digital camera.

**Statistical analysis**

All values are expressed as mean ± SD. Statistical differences between mean values were determined by analysis of variance, followed by Turkey–Kramer test. All differences were considered significant at P < 0.05.
Results

DG and DHD decrease LPS-induced ICAM-1 and VCAM-1 expression in human endothelial cells

Bacterial LPS effectively increased whole-cell protein expression of VCAM-1 and ICAM-1 in HUVECs (Fig. 1A). Co-treatment with DG (10⁻⁸ M), DHD (10⁻⁸ M), progesterone (10⁻⁸ M) or MPA (10⁻⁸ M) resulted in a partial reduction of leukocyte adhesion molecules expression induced by LPS (Fig. 1A). Meanwhile, progesterone is most potent in reducing VCAM-1 expression, while DG is the strongest compound in reducing ICAM-1 expression (Fig. 1A). These actions of DG or DHD were prevented by the selective PR antagonist, ORG 31710 (Fig. 1A).

Effects of DG and DHD on LPS-induced ICAM-1 and VCAM-1 expression in the presence of E₂

When co-administrated with E₂, progestins under study did not result in further reductions of VCAM-1 and ICAM-1 expression beyond that associated with E₂ treatment (Fig. 1B). However, pretreatment with the PR antagonist ORG 31710 visibly modified the action of E₂ plus DG or DHD (Fig. 1B), suggesting that these progestins contribute a significant part of the anti-inflammatory effect induced by the hormonal combination.

DG and DHD decrease VCAM-1 and ICAM-1 endothelial cell membrane localization

Adhesion of circulating leukocytes to vascular endothelial cells is based on the localization of adhesion molecules on endothelial cell surface. As expected, LPS markedly increased VCAM-1 and ICAM-1 abundance on endothelial cell membrane (Fig. 2A and B). Progesterone, MPA, DG and DHD largely inhibited this effect (Fig. 2A and B). Intriguingly, the maximal inhibitory effect for VCAM-1 membrane expression was achieved by MPA, and not progesterone. The inhibitory effect of DG or DHD was prevented by the PR antagonist, ORG 31710 (Fig. 2A and B).

E₂ significantly decreased LPS-induced membrane localization of VCAM-1 and ICAM-1 (Fig. 3A and B). When HUVECs were treated with E₂ plus P, MPA, DG or DHD there were no significant differences between E₂ alone and E₂ plus each progestin (Fig. 3A and B). However, the inhibitory effect on VCAM-1 and ICAM-1 membrane localization was partially reverted when the PR antagonist ORG 31710 was added to the combination of E₂ plus DG or DHD (Fig. 3A and B).

DG and DHD decrease LPS-induced NF-κB nuclear translocation

Expression of VCAM-1 and ICAM-1 is under the control of the transcription factor NF-κB, which binds specific NF-κB consensus sequences in the promoter regions of these genes (Otsuki et al., 2001; Simoncini et al., 2004). Dissociation of the inhibitory subunit
IkB from NF-κB in the cytoplasm sets the p65 subunit free to translocate into the nucleus to start this process. Treatment with LPS for 2 h triggered a strong p65 nuclear localization, which was blocked by addition of DG or DHD. This was seen both with p65 protein analysis on purified cytoplasmic and nuclear fractions (Fig. 4A) as well as with visual identification of p65 cell localization with cell immunofluorescence (Fig. 4B).

E2 also prevented p65 nuclear translocation. Addition of DG or DHD to E2 did not change the effect of E2, as indicated by western analysis on separated cell fractions and cell immunofluorescence (Fig. 5A and B).

Discussion

Adhesion molecules play a crucial role in inflammation, immune response and in the early stages of atherogenesis, as they mediate attachment of leukocytes to the endothelium. Enhanced expression of multiple adhesion molecules, including VCAM-1, ICAM-1, E-selectin and platelet-endothelial cell adhesion molecule has been found in atherosclerotic lesions (Duplaa et al., 1996; Huo et al., 2000). We find that the progestins DG and DHD decrease LPS-induced VCAM-1 and ICAM-1 expression in an in vitro model of endothelial inflammation. This is similar to previous findings for natural progesterone and MPA. Furthermore, we find that combination of E2 with DG or DHD does not interfere with the anti-inflammatory effects of E2 in this setting. This manuscript adds to the previous identification of direct actions of DG and DHD on endothelial cells (Simoncini et al., 2006), pointing out that these compounds regulate these cells through multiple mechanisms.

Experimental work in this area has been partially conflicting, with reports indicating opposite effects for the synthetic progestin MPA, either found to efficiently decrease VCAM-1 and ICAM-1 expression (Simoncini et al., 2004) or being unable to do so (Otsuki et al., 2001). While these discrepancies may be related to the different stimuli used to create experimental inflammation (i.e. LPS versus tumor necrosis factor-α), these studies converge in the hypothesis that MPA may
differ from other progestins particularly in its endothelial actions because of its peculiar activity on glucocorticoid receptor (Arnal et al., 2009). To this extent, DG and DHD have no relevant androgenic, estrogenic, glucocorticoid or mineralocorticoid activities since they bind almost exclusively PR (Schindler, 2009). This is also supported in our endothelial experimental system by the evidence that the selective PR antagonist ORG 31710 blocks the effects of DG and DHD. Consistent with our data, long-term HRT with E2 and DG in healthy post-menopausal women is associated with reduced E-selectin serum concentrations (van Baal et al., 1999). In view of the available literature, our results bring new information suggesting that discrepancies in mechanisms of action of progestins may be explained by different binding affinities to PR and other steroid receptors (Simoncini et al., 2007).

Atherosclerosis is an inflammatory disease (Ross, 1999). At sites of inflammation, leukocytes are recruited to the vessel wall and transendothelial migration begins. This process is mediated by a number of adhesion molecules. ICAM-1 and VCAM-1 are important factors in this game. When expressed on endothelial cell membrane, adhesion molecules determine firm adhesion of leukocytes to the apical surface of endothelial cells through interactions with leukocyte antigens (Muller, 2009). The identification that DG and DHD decrease ICAM-1 and VCAM-1 expression and localization on endothelial cell membrane by controlling the pro-inflammatory transcription factor NF-κB may thus be valuable pre-clinical evidence suggesting a potential anti-atherogenic effect.

Figure 3 DG and DHD decrease VCAM-1 and ICAM-1 endothelial cell surface localization when combined with E2. HUVECs were treated for 18 h with 100 ng/ml LPS and E2 (10⁻⁹ M), together with progesterone (10⁻⁸ M), MPA (10⁻⁸ M), DG (10⁻⁸ M) and DHD (10⁻⁸ M), respectively. PR antagonist ORG 31710 (1 µM) was also added. Cell surface expression of VCAM-1 (A) and ICAM-1 (B) were assayed with EIA on live cells. Results (mean optical densities ± SD) are graphed after normalization toward control cells and visualized as ratio of each experimental condition versus control. The numbers in the figure represent the raw P values when they were compared with LPS treatment. All experiments were repeated three times with comparable results.
post-menopausal women receiving CEEs alone, as opposed to null to negative effects with combined HRT in the same age range (Manson et al., 2003).

However, different progestins seem to elicit diverse (and sometimes opposite) effects in human tissues (Adams et al., 1990, 1997; Simoncini et al., 2004), hence indicating that these compounds cannot be grouped into a single class of hormones, and emphasizing the need for a better characterization of each molecule.

In this study, 10 nM of each progestin was used because this concentration falls within the physiological range of natural progesterone in fertile life and pregnancy in women, which is 0.3–300 nM (Rousell et al., 1979). Moreover, this concentration was used to
compare each progestin’s potency on reducing VCAM-1/ICAM-1 expression, due to their proximal affinity for binding to PR (Kumar et al., 2000). Actually, the progestational potency of each compound is usually compared by identifying the dose associated with full endometrial transformation and ovulation inhibition in animals (Schindler et al., 2003). However, the real progestogenic activity depends on a variety of factors, including the route and timing of administration and the specific end-point tested, such that it is difficult to definitely establish the most appropriate concentration to compare two progestins.

We found that progesterone is the most potent compound in reducing total cellular VCAM-1 expression, while MPA exerts maximal inhibitory effect on membrane VCAM-1 expression. This apparent discrepancy, as we discussed above, may exist because of the different end-point tested. As a possible explanation, the relationship of increasing rates of expression of VCAM-1 or ICAM-1 between cytoplasm and plasma membrane are not linear. When endothelial cells are challenged with cytokines or LPS, de novo synthesis of VCAM-1/ICAM-1 happens in the cytoplasm. Of note, it will take several hours for these proteins translocating into the plasma membrane dependent on the different stimuli. For example, it was reported that new synthesis of VCAM-1 occurs at 1.5 h while transport granules for VCAM-1 to plasma membrane occurs at 4 h after interleukin-1 stimulation in human endothelial cells (Scholz et al., 1996). Hence, there is a time lag for these increasing protein levels between cytoplasm and plasma membrane, which raises the possibility that different progestins may have different impacts on the trafficking process of these proteins to plasma membrane.

In our work, progestins effects do not add to that of E2 on VCAM-1/ICAM-1 expression, whereas an alleged PR antagonist negates both effects. This is consistent with our previous work showing that ORG 31710 fully abolishes the combinatorial effect of E2 plus progestin. Similar observation has been reported in breast cancer cells, where the up-regulation of breast cancer resistance protein expression induced by the combination of E2 plus progesterone was abolished by the PR antagonist RU486 (Wang et al., 2006). One possible explanation of these observations could be that PR crosstalks with estrogen receptor (ER) to accomplish these functions, as indicated by recent findings showing that PR acts as an ER antagonist in certain circumstances, altering the ability of ER to interact with estrogen response elements and to trigger gene expression (Zheng et al., 2008). Thus, the presence of the PR antagonist may interfere with the ability of PR to serve as a scaffold to ER and antagonizes the function of both ER and PR.

Relevant to DG, the available clinical studies show that risk of breast cancer appears to be smaller with DG-containing HRT when compared with other formulations (Pasqualini, 2009). In addition, the clinical outcomes from the UK-based General Practice Research Database study found no increased risk of myocardial infarction or stroke with HT-containing DG (Schneider et al., 2009). However, no study currently allows conclusions on the possible superiority of any available progestin. This calls for new research and increases the relevance of pre-clinical models that allow systematic comparisons of progestins in controlled systems, providing tools for future clinical investigation.

In conclusion, we show that the synthetic progestin DG and its stable metabolite, DHD, decrease endothelial inflammation in vitro, resulting in reduced expression of the inflammatory/atherogenic leukocyte adhesion molecules VCAM-1 and ICAM-1. These findings support the concept that DG exerts direct actions on endothelial cells that may be relevant for its long-term cardiovascular effects.

Authors’ roles

All authors meet the qualification of Human Reproduction. X.-D.F. and S.G. designed and carried out the experiments, analysed the data, drafted and revised the manuscript; S.G., K.P., G.P., S.S. and P.M. carried out some experiments; A.R.G. and A.D.G. discussed the project and the results, reviewed the manuscript; T.S. raised funds for the project, designed the experiments, analysed the data, drafted and revised the manuscript.

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Conflict of interest

The authors of this manuscript have nothing to declare.

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