

Tibolone inhibits leukocyte adhesion molecule expression in human endothelial cells

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Abstract

Tibolone is a synthetic steroid with mixed estrogenic and progestogenic/androgenic activity used for post-menopausal hormone replacement therapy. Since its cardiovascular effects are still not clear, and no data have been published on possible direct actions on the vessel wall, we studied the effects of tibolone and its metabolites on lipopolysaccharide (LPS)-induced expression of leukocyte adhesion molecules on human endothelial cells. Tibolone and its two estrogenic 3α -OH and 3β -OH metabolites, but not the progestogenic/androgenic Δ^4 -isomer, concentration-dependently decreased LPS-induced vascular cell adhesion molecule-1 protein expression. This effect was estrogen receptor dependent, since it was completely blocked by the pure estrogen receptor antagonist ICI 182780. Furthermore, only tibolone, the 3α -OH and the 3β -OH metabolites decreased endothelial expression of E-selectin, while none of the compounds changed the levels of intercellular adhesion molecule-1. These findings were associated with parallel changes in mRNA levels for the three adhesion molecules. Our data show that tibolone and its estrogenic metabolites exert direct actions on the vascular wall, decreasing the expression of endothelial–leukocyte adhesion molecules, thus producing potentially important direct anti-atherogenic effects. © 2000 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Hormone replacement therapy; Tibolone; Endothelial–leukocyte adhesion molecules; Atherosclerosis; Endothelial cells

1. Introduction

Coronary artery disease is the principal cause of morbidity and mortality in Western countries; however, strong gender differences in risk rates are well documented. Hormone replacement therapy (HRT) in post-menopausal women is associated with better cardiovascular prognosis (Nabulsi et al., 1993; Grodstein et al., 1997). Estrogen cardioprotective effects are partially related to positive changes in the lipid profile (Wahl et al., 1983) and to effects on the hemostatic/fibrinolytic system such as the reduction in serum fibrinogen and plasminogen activator-inhibitor-1 levels (The Writing Group for the PEPI Trial, 1995; Koh et al., 1997). However, most of the beneficial effects of estrogen are probably due to its local action on the

vascular wall (Farhat et al., 1996; Mendelsohn and Karas, 1999). In particular, estrogen therapy has been shown to improve endothelial function in post-menopausal women (Lieberman et al., 1994) and non-human primates (Williams et al., 1990), and to reduce the development of atherosclerotic lesions in the apolipoprotein E-deficient mice (Bourassa et al., 1996). These effects are probably exerted through various molecular mechanisms, such as the enhancement of nitric oxide release (Caulin-Glaser et al., 1997), the inhibition of endothelin-1 production (Akishita et al., 1998), as well as the blockade of the expression of endothelial–leukocyte adhesion factors like monocyte chemoattractant protein-1 (Pervin et al., 1998) and of different leukocyte adhesion molecules (Caulin-Glaser et al., 1996) by endothelial cells. The modulation of endothelial–leukocyte adhesion molecule expression by estrogen is a particularly important feature, since the derangement of this process has been shown to be one of the first events during atherogenesis, and has been

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demonstrated to be strictly involved in the pathogenesis of the early lesions (Cybulsky and Gimbrone, 1991).

In past years, pharmacological research has produced a series of new estrogenic compounds able to target selected tissues, while avoiding the undesirable side effects of natural estrogen on others, such as the endometrium. Some of these new molecules are now proposed for post-menopausal HRT, but only limited information is so far available about their possible action on the vessel wall.

Tibolone (Org OD14) is a synthetic steroid ((7 α ,17 α)-17-hydroxy-7-methyl-19-norpregn-5(10)-en-20-yn-3-one) clinically effective for the treatment of climacteric symptoms, and for the prevention of post-menopausal osteoporosis (Pavlov et al., 1999), having no stimulatory effect on the endometrium (Genazzani et al., 1991). In vivo tibolone is rapidly metabolized to the 3 α -OH and 3 β -OH metabolites, which have estrogenic effects, and to the Δ^4 -isomer characterized by progestogenic/androgenic action, explaining its mixed hormonal activity. Regarding the actions on the cardiovascular system, no conclusive data have been published, and the observational studies have shown both potentially positive and negative effects for this compound. In fact, treatment of post-menopausal women with tibolone produces an undesirable decrease in seric high-density lipoprotein cholesterol concentrations, associated with other positive effects, such as the decrease of circulating very low-density lipoprotein cholesterol, tryglycerides and Lp(a) (Castelo-Branco et al., 1999; Farish et al., 1999). Clinical studies show that tibolone has anti-ischemic effects on coronary circulation, similar to natural estrogen (Prelevic et al., 1997; Lloyd et al., 1998). Moreover, animal studies have shown that tibolone prevents the development of atherosclerotic lesions in rabbits fed with a hypercholesterolemic diet (Zandberg et al., 1998), and human studies show significant reductions in endothelin-1 concentrations in post-menopausal women receiving tibolone (Haenggi et al., 1999). These findings suggest that this molecule may have direct effects on endothelial cells.

To test this hypothesis, the effects of this compound on cultured human endothelial cells have been studied, using an in vitro model of endothelial activation to determine the modification of leukocyte adhesion molecules expression. Since tibolone and its metabolites (3 α - and 3 β -hydroxy metabolites and the Δ^4 -isomer) show different patterns of hormonal activity, these different molecules were compared, in order to determine the relative contribution of the estrogenic, progestogenic and androgenic activities to the effects of tibolone on human endothelial cells.

2. Materials and methods

2.1. Cell cultures

Human saphenous vein endothelial cells were harvested enzymatically with Type IA collagenase (1 mg/ml) as described (Simoncini et al., 1999), and maintained in phenol red-free Medium 199 (Gibco BRL, Life Technologies, Gaithersburg, MD), containing HEPES (25 mmol/l), heparin (50 U/ml), endothelial cell growth factor (50 μ g/ml), L-glutamine (2 mmol/l), antibiotics, and 5% estrogen-deficient fetal bovine serum. Estrogens were removed from fetal bovine serum by activated-charcoal stripping. Cell number was assessed by direct cell counting, after trypsin detachment, in a Neubauer hemocytometer. The percentage of cells excluding trypan blue after staining with the compound was taken as a measure of cell viability.

2.2. Drugs and treatments

Tibolone (Org OD14), the 3 α - and 3 β -hydroxy metabolites and the Δ^4 -isomer were provided by N.V. Organon (Oss, The Netherlands). ICI 182780 was provided by Zeneca Pharmaceuticals (Macclesfield, UK). *Escherichia Coli* lipopolysaccharide (LPS) was purchased from Sigma (St Louis, MO).

For all experiments, confluent endothelial cells were pretreated for 48 h with the studied compounds before being incubated with LPS (100 ng/ml) overnight (18 h) (to activate vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) expression) or for 6 h (to activate E-selectin expression) for cell surface enzyme immunoassays (EIAs) and for 4 h for Northern analysis.

2.3. Cell-surface enzyme immunoassays

Endothelial cells were grown on 96-well culture dishes and treated according to the experimental conditions. Assay of cell surface VCAM-1 expression was carried out using a mouse anti-human monoclonal antibody against VCAM-1 or monoclonal antibodies versus ICAM-1 or E-selectin. EIAs were carried out by incubating confluent monolayers (with comparable cell densities and total cell numbers) first with saturating concentrations of specific monoclonal antibodies against the target molecules, then with biotinylated goat anti-mouse immunoglobulin G (Amersham Italia, Milano, Italy), and finally with streptavidin-alkaline phosphatase (Amersham). Cell layers were washed three times with phosphate-buffered saline with 1% bovine serum albumin between each incubation step. Phase contrast microscopy was used before starting the treatments and between each step of the assay to insure that integrity of the monolayers was conserved and that

well-to-well cell density was comparable. Protein surface expression was quantified spectrophotometrically, reading the optical density of the wells (at 450 nm wavelength) 20 min after the addition of the chromogenic substrate *para*-nitrophenylphosphate, as previously described (Simoncini et al., 1999). At least eight different wells were used for each experimental condition. All the experiments were repeated at least three times.

2.4. Northern analysis

Total RNA, extracted with the standard guanidinium thiocyanate–phenol–chloroform method (Chomczynski and Sacchi, 1986) was separated (20 µg/lane) on a 1.2% formaldehyde–agarose gel, transferred to a nylon membrane (Hybond-N; Amersham) and immobilized by short-wave ultraviolet illumination. VCAM-1, E-selectin and ICAM-1 cDNA probes were labeled by a random priming method (Boehringer Mannheim, Indianapolis, IN) with [α - 32 P] deoxycytidine 5'-triphosphate (Amersham) and Klenow fragment of DNA polymerase I (Boehringer Mannheim). The membrane was hybridized at 42°C overnight and washed twice in 0.2 × saline-sodium citrate buffer, 0.1% sodium dodecyl sulfate for 15 min at 60°C before autoradiography at –80°C for 12–24 h.

2.5. Statistical analysis

All values are expressed as the mean \pm S.D. Multiple comparisons were performed by one-way analysis of variance (ANOVA) and individual differences were tested by Fisher's protected least significance difference test after the demonstration of significant inter-group

differences by ANOVA. Two-group comparisons were performed by unpaired Student's *t*-test.

3. Results

In order to measure the effect of tibolone and its metabolites on LPS-induced adhesion molecule expression on the cell surface of cultured human endothelial cells, we performed enzyme immunoassays with specific monoclonal antibodies. As expected, treatment with bacterial LPS induced a strong and sustained surface expression of VCAM-1 (Fig. 1) and E-selectin (Fig. 3) and significantly increased the basal levels of ICAM-1 (Fig. 4). When cultured cells were exposed to increasing concentrations (from 10 nM to 10 µM) of tibolone prior to the stimulation with LPS, expression of VCAM-1 was significantly decreased in a concentration-dependent manner (Fig. 1). A similar result was obtained using tibolone's 3 α -OH as well as 3 β -OH metabolites, although the relative potency of these two molecules was higher (Fig. 1). On the contrary, pretreatment with the same range of concentrations with the tibolone's Δ^4 -isomer had no effect on LPS-induced VCAM-1 expression (Fig. 1).

In order to determine if the action on VCAM-1 expression was linked with the activation of the estrogen receptor, as we previously showed for other synthetic estrogenic compounds (Simoncini et al., 1999), the cells were pretreated with the four studied compounds (100 nM) in the presence or absence of an excess of the pure estrogen receptor antagonist ICI 182,780 (1 µM) and then stimulated VCAM-1 expression with LPS. The ICI compound completely blocked the decrease in VCAM-1 expression induced by ti-

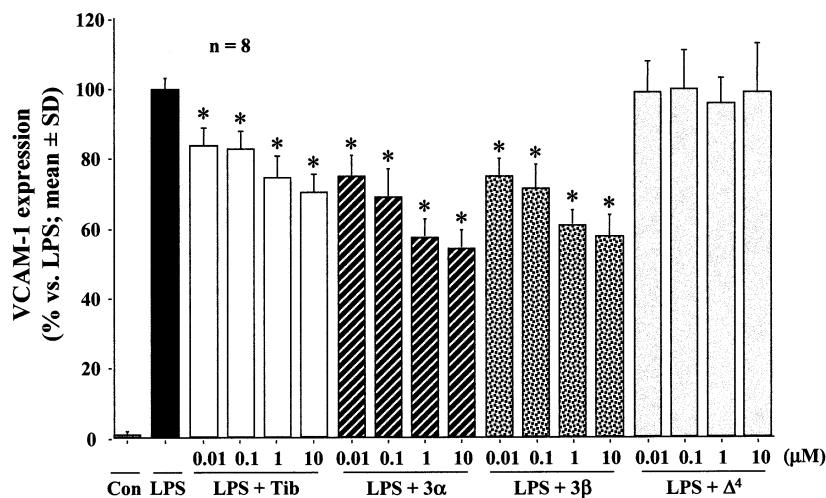


Fig. 1. Tibolone and its metabolites decrease LPS-induced VCAM-1 expression in human endothelial cells. VCAM-1 expression at EIA is shown on the Y axis for untreated cells (Con), for cells treated with LPS only (LPS), or with LPS + increasing concentrations of tibolone (LPS + Tib), LPS + 3 α -OH metabolite (LPS + 3 α), LPS + 3 β -OH metabolite (LPS + 3 β) or LPS + Δ^4 isomer (LPS + Δ^4). Values are expressed as the percentage of the OD value of cells treated with LPS only (taken as 100%). Asterisks denote statistically significant differences versus LPS-treated cells.

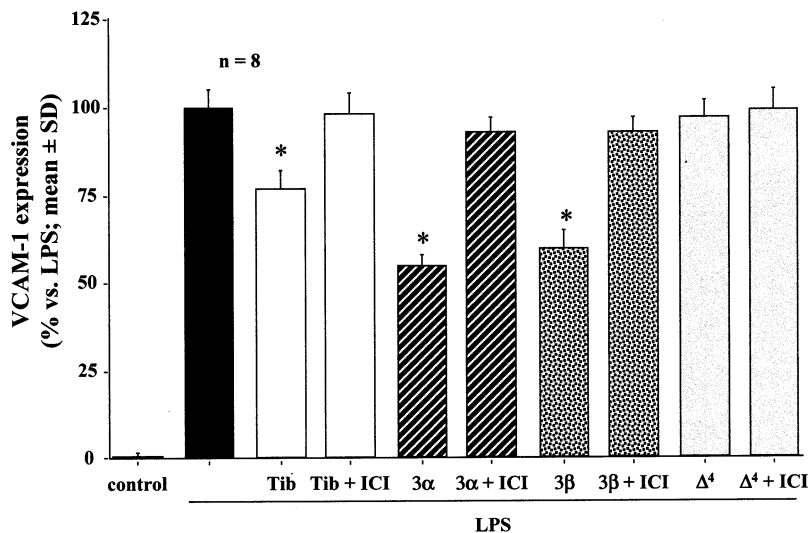


Fig. 2. The effects of tibolone and its metabolites on VCAM-1 expression are blocked by ICI 182780. VCAM-1 expression is shown for untreated cells (control), for cells treated with LPS only (LPS), or with LPS + 100 nM of tibolone (Tib), 3 α -OH metabolite (3 α), 3 β -OH metabolite (3 β) or Δ^4 isomer (Δ^4), respectively, or in the presence of 1 μ M ICI 182 780 (Tib + ICI, 3 α + ICI, 3 β + ICI, Δ^4 + ICI). Values are expressed as the percentage of the OD value of cells treated with LPS only (taken as 100%). Asterisks denote significant differences versus LPS-treated cells.

bolone and by the 3 α - and 3 β -OH metabolites, while it had no effect when added together with the Δ^4 -isomer (Fig. 2) or when used alone (not shown), suggesting that the demonstrated effects are indeed due to the estrogenic activity of the compounds.

Since 17 β -estradiol has been reported to have inhibitory effects on other adhesion molecules involved in the atherogenic process (Caulin-Glaser et al. 1996), we studied if tibolone and its derivatives also affect endothelial expression of E-selectin and ICAM-1.

E-selectin expression induced by LPS treatment was significantly reduced by both tibolone and the two

estrogenic metabolites (3 α - and 3 β -OH metabolites), but not affected by the Δ^4 -isomer (Fig. 3). The relative biological potency of the compounds (all used at 100 nM concentration) followed a similar pattern as for VCAM-1, with the two metabolites producing a stronger inhibition of E-selectin expression than the parent molecule.

On the contrary, the increase in ICAM-1 expression induced by LPS was not altered by the same treatments, suggesting that inhibition of endothelial-leukocyte adhesion molecule expression by tibolone is not a general phenomenon but, instead, is the result of a specific regulation.

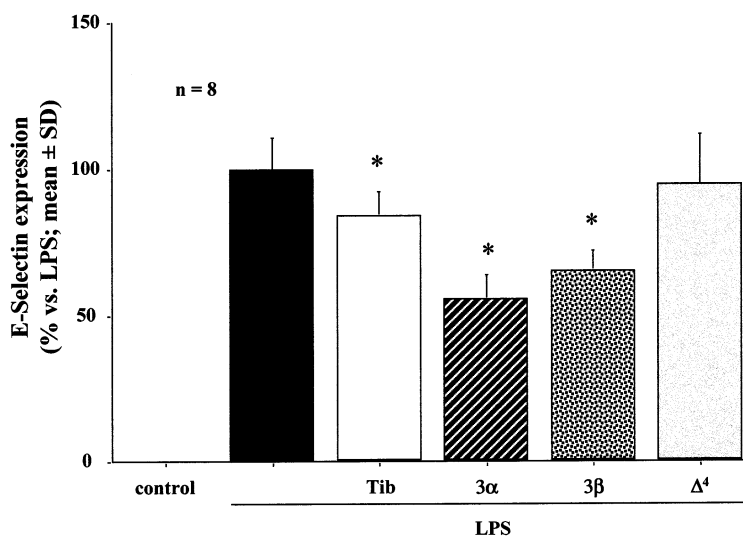


Fig. 3. Tibolone and its metabolites decrease LPS-induced E-selectin expression in human endothelial cells. E-Selectin expression is shown for untreated cells (control), for cells treated with LPS only (LPS), or with LPS + 100 nM tibolone (Tib), LPS + 100 nM 3 α -OH metabolite (3 α), LPS + 100 nM 3 β -OH metabolite (3 β) or LPS + 100 nM Δ^4 isomer (Δ^4). Values are expressed as percentage of the OD value of cells treated with LPS only (taken as 100%). Asterisks denote significant differences versus LPS-treated cells.

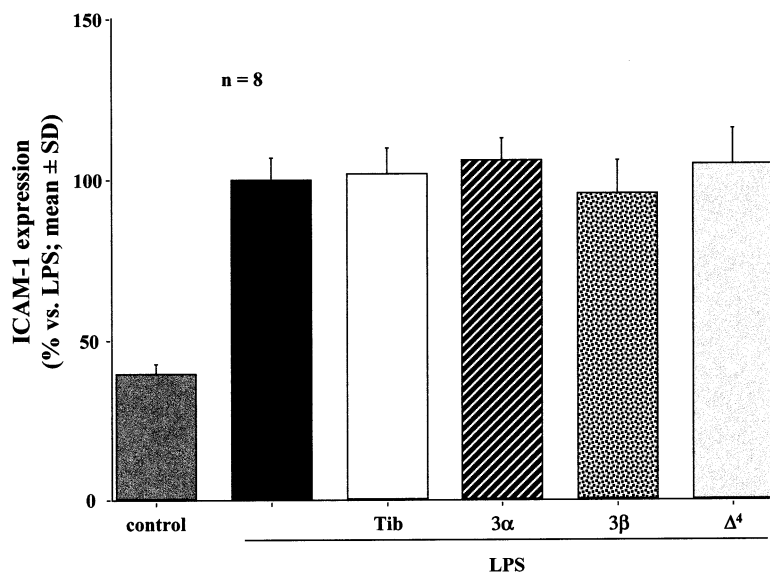


Fig. 4. Tibolone and its metabolites do not affect the LPS-induced increase in ICAM-1 expression in human endothelial cells. ICAM-1 expression levels are shown for cells with no treatment (control), for cells treated with LPS (LPS), or with LPS + 100 nM tibolone (Tib), LPS + 100 nM 3 α -OH metabolite (3 α), LPS + 100 nM 3 β -OH metabolite (3 β) or LPS + 100 nM Δ^4 isomer (Δ^4). Values are expressed as percentage of the OD value of cells treated with LPS only (taken as 100%).

Regulation of adhesion molecule expression in endothelial cells by estrogenic molecules has been previously shown to be a process involving gene transcription (Caulin-Glaser et al., 1996; Simoncini et al., 1999), so we studied if the effect of tibolone and its derivatives was possibly related to a control of messenger RNA (mRNA) levels for adhesion molecules with Northern technique. Parallely to that shown for cell surface molecule expression, VCAM-1 mRNA levels increased abruptly after LPS treatment of endothelial monolayers, and pre-treatment with either tibolone or the two estrogenic 3 α - and 3 β -OH metabolites was associated with a significantly lower increase, of an order of magnitude, compatible with the data obtained for the EIA analysis with the same dose of the compounds (100 nM), while the Δ^4 -isomer, again, had no effect (Fig. 5).

E-selectin mRNA levels induced by LPS were also reduced by the treatment with the two estrogenic 3 α - and 3 β -OH metabolites (Fig. 5) at a 100 nM concentration. Tibolone had a much lower inhibitory action, while the Δ^4 -isomer did not decrease E-selectin mRNA levels (Fig. 5).

Compatible with the protein data, ICAM-1 mRNA levels after LPS treatment were not affected by any of the studied compounds (Fig. 5).

4. Discussion

In recent years, increasing evidence has been produced showing that the cardiovascular system represents a non-classical target of female sex steroid

hormones, as well as of other steroids (Mendelsohn and Karas, 1999). In fact, sex steroid hormones have been found to actively regulate important biological func-

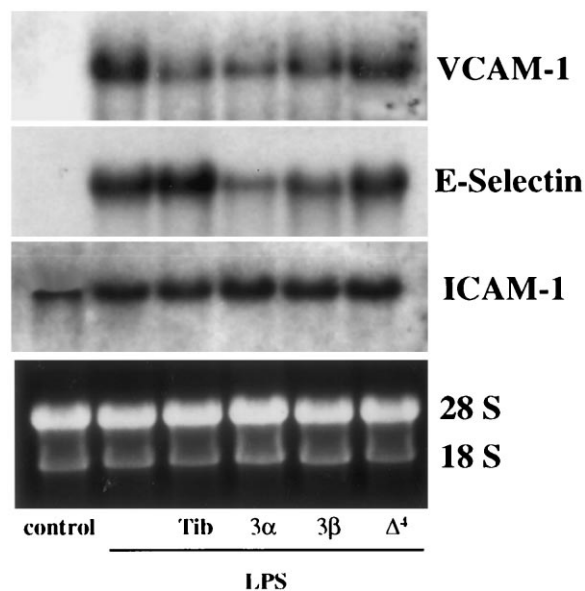


Fig. 5. Effects of tibolone and its metabolites on LPS-induced adhesion molecule mRNA accumulation in human endothelial cells. Messenger RNA levels for VCAM-1, E-selectin and ICAM-1 in untreated cells (control), in cells treated with LPS only (LPS), or with LPS + 100 nM tibolone (Tib), LPS + 100 nM 3 α -OH metabolite (3 α), LPS + 100 nM 3 β -OH metabolite (3 β) or LPS + 100 nM Δ^4 isomer (Δ^4) are shown in the upper boxes. In the lower boxes are shown the corresponding ethidium bromide-stained ribosomal RNA subunits, as a loading control. The same membrane was hybridized with the different labeled probes after stripping. The figure is representative of three different experiments that gave equal results.

tions of different cellular components of the vascular wall, such as the endothelial cell lining, the smooth muscle cells and the vascular stromal cells, as well as the different leukocytes species (Mendelsohn and Karas, 1999). Direct actions of estrogen on the vessel wall are actually considered to be extremely important, and have been estimated to explain the majority of the cardiovascular beneficial effects of these molecules (Grodstein et al., 1996).

Endothelial cells are a metabolically active cell layer and play a critical role in the regulation of the vascular tone as well as of hemostasis and fibrinolysis (Vane et al., 1990). Another pivotal function of endothelial cells is represented by the control of leukocyte recirculation between the vascular lumen and the subintimal space (Adams and Shaw, 1994). Atherosclerosis is actually considered an inflammatory disease (Ross, 1999), and the derangement of leukocyte–endothelial interactions is one of the earliest features of this process (Gimbrone, 1995). The molecular marker of this pathological event is represented by the increased expression on endothelial cells of adhesion molecules for circulating leukocytes (Cybulsky and Gimbrone, 1991), which leads to intimal accumulation of monocytes that soon become lipid-laden macrophages. VCAM-1 is an inducible adhesion molecule transcriptionally upregulated by cytokines and LPS (Cybulsky and Gimbrone, 1991). VCAM-1 is thought to play a central role during early atherogenesis, since its integrin ligand, VLA⁴, is expressed on monocytes and some lymphocytes but not on neutrophils (Cybulsky and Gimbrone, 1991), potentially providing an explanation for the selective monocyte recruitment during early atherogenesis. Other adhesion molecules like the E- and P-selectins and the integrin-binding intercellular adhesion molecule-1 (ICAM-1) also play a pathogenic role in the early atherogenic process (Gimbrone, 1995).

Natural estrogen has been shown to decrease cytokine-induced endothelial–leukocyte adhesion molecules and monocyte adhesion to the endothelium (Caulin-Glaser et al., 1996). In similar experimental settings, we have previously shown that two synthetic selective estrogen receptor modulators (LY117018 and tamoxifen) produced different effects on VCAM-1 expression and endothelial–leukocyte adhesion (Simoncini et al., 1999), suggesting that not all the synthetic estrogenic compounds may be capable of exerting direct actions on the vascular wall.

Our results also demonstrate that the synthetic steroid tibolone exerts direct effects on the vascular wall, selectively inhibiting adhesion molecule expression on human endothelial cells. Tibolone is *in vivo* rapidly metabolized to three stable molecules: the estrogenic 3 α -OH and 3 β -OH metabolites, and the progestogenic/androgenic Δ^4 isomer. The analysis of the effects of the metabolites enables to pharmacologically dissect the

relative contribution of the different hormonal activities on the action on adhesion molecules. As our data show, reduction of LPS-induced VCAM-1 and E-selectin protein, and mRNA expression on human endothelial cells is an exclusive feature of the compounds with estrogenic activity, and is not reproduced by the Δ^4 isomer. This is further demonstrated by the correspondence between the biological potency of the molecules and their relative binding affinity for the estrogen receptor (about 3-fold higher for the 3 α -OH and 3 β -OH metabolites than for tibolone) (Markiewicz and Gurbide, 1990), as well as by the blockade of the effect of the three active compounds on VCAM-1 expression by the pure estrogen receptor antagonist ICI 182780. Since the Δ^4 isomer did not exert any effect, it is possible to speculate that, given the present data on adhesion molecule expression and the previously published studies on vascular reactivity (Prelevic et al., 1997; Lloyd et al., 1998) and atherosclerotic lesion formation (Zandberg et al., 1998), the progestogenic/androgenic properties of tibolone may not be impairing the positive estrogenic effects on the vascular wall.

Tibolone and its two estrogenic metabolites were able to decrease LPS-induced endothelial expression of either VCAM-1 and of E-selectin in our system. This may be important, since these two molecules participate in two different steps of the process of leukocyte adhesion to the endothelium. In fact, E-selectin, together with other molecules, is responsible for the first, weaker, attachment of circulating leukocyte to endothelial cells (tethering), which is afterwards stabilized by the production by endothelial cells of chemotactic factors for leukocytes (such as monocyte chemoattractant protein-1) and of integrin-binding adhesion molecules like VCAM-1 and ICAM-1 (Springer, 1994). Thus, tibolone may produce modifications of the adhesion molecule expression profile on endothelial cells that may act in a synergistic way to reduce leukocyte accumulation in the intimal space. In agreement, rabbit arterial balloon-injury models of atherosclerosis show that treatment with tibolone is associated with a significant reduction of the post-injury intimal thickening, mainly due to decreased accumulation of lipid-laden macrophages (Zandberg et al., 1998). The mechanism for this reduction of intimal monocyte-derived cells may be represented by the decrease of endothelial–leukocyte adhesion molecules induced by tibolone.

The differential control of the expression of VCAM-1 and E-selectin versus ICAM-1, which was unaffected by all the compounds tested, may be due to different interactions of the liganded estrogen receptor with the LPS-inducible transcription factors that control the expression of these molecules. In fact, none of the promoter regions for the genes encoding for these molecules contain estrogen response elements; thus, although there are no available confirmations for this

hypothesis, it has been proposed that the estrogen receptor may directly or indirectly interact with one or more of the transcription factors that are activated after exposure to cytokines or LPS and that, afterward, transcriptionally activate the expression of these genes. Potential candidates may be represented by molecules such as nuclear factor- κ B, activator protein-1 or the various GATA-binding proteins, since the promoters for the considered endothelial–leukocyte adhesion molecules contain functional consensus elements for these transcription factors (Collins et al. 1995).

In conclusion, our paper provides novel evidence supporting the concept of the biological importance of the direct regulation of the vascular wall by steroid receptor-activating molecules, such as natural estrogen, as well as some (but not all) new synthetic estrogenic molecules, regarding the prevention of atherosclerotic lesion formation. By reducing leukocyte adhesion molecule expression on human endothelial cells, tibolone may have the intrinsic potential to exert additional, lipid-independent, cardiovascular protective effects.

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