

In vitro effects of progesterone and progestins on vascular cells

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Abstract

The impact of progesterone on the cardiovascular system is relevant, but not as well characterized as the effects of estrogens. The recent early interruption of the conjugated equine estrogens (CEE)-medroxyprogesterone acetate (MPA) arm of the Women's Health Initiative trial, but not of the parallel CEE-only treatment arm, suggesting the possibility of harmful cardiovascular effects of the progestins, boosts the debate on the role of progesterone and progestins on the vascular tree. The data available up to now show the presence of important regulatory effects of progestagens on vascular cells. Additionally, the presence of a progestagen results in diverse modifications of the effects of estrogens, sometimes acting synergically, others being neutral or antagonizing estrogens' effects. Notwithstanding the availability of consistent observations on the functional effects of progestins on the cardiovascular system, the molecular mechanisms of progestins actions on vascular cells have been up to now only scarcely characterized. Novel mechanisms of signal transduction are being discovered for progesterone receptors in different tissues, some of which are independent of gene transcription regulation, and are therefore indicated as "nongenomic." Furthermore, the contribution to signal transduction of co-activators is currently widely investigated, in order to understand the ways to tissue-specificity and to engineer new progesterone receptor modulators. The understanding of the molecular basis of progesterone receptor signaling in vascular tissue is therefore of paramount importance for the development of hormonal agents with an optimal cardiovascular profile.

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1. Progesterone, progestins and the cardiovascular system

The cardiovascular system is a target for progesterone [1]. Indeed, although this has not been recognized for several years, the blood vessels contain functional progesterone receptors (PR) [2], and are functionally regulated by progesterone as well as by synthetic progestins [3].

Sex steroid hormones have major metabolic effects, resulting principally in modifications of the lipid profile, of carbohydrate metabolism and of the hemostatic system. Although these effects principally derive from direct actions on the liver, they have a profound effect on the function of the cardiovascular system.

2. Progestagens: metabolic effects on the lipid profile

Estrogen administration to postmenopausal women is associated with an improved lipid profile, with reductions

of total and LDL-cholesterol (LDL-C), increases of HDL-C and (at least for transdermal 17β -estradiol) unchanged triglyceride levels [4]. The co-administration of a progestin during hormone therapy (HT) has been shown to blunt, to some extent, this potentially atheroprotective lipid profile [3], however, different gestagens induce substantially different effects. For instance, androgenic progestins partially reverse the positive increase in HDL of estrogen [5,6], while natural progesterone and some 19 nor-progesterone derivatives, such as nomegestrol acetate (NOM Ac), do not blunt the HDL levels [5,6]. Particularly, the Postmenopausal Estrogen/Progestin Intervention (PEPI) trial clearly showed the different impact of sequential micronized progesterone or medroxyprogesterone acetate (MPA) on estrogen therapy (ET) with CEE. Indeed, while all women had increases in HDL-C and reductions in LDL-C while on estrogens, the addition of MPA was associated with a partial reversal of the positive effect on HDL-C (not on LDL-C), that was not obtained with micronized progesterone [5]. Similarly, levonorgestrel (LNG) significantly reverses the HDL-C increase induced by estrogen, but norethisterone acetate (NETA) given transdermally has much weaker effects than LNG. These pharmacological differences between progestins are particularly relevant, in light of the first results

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of the Women's Health Initiative (WHI) trial [7]. This is a randomized controlled primary prevention trial, in which 16,608 postmenopausal women aged 50–79 years with an intact uterus received conjugated equine estrogens (CEE), 0.625 mg/d, plus MPA, 2.5 mg/d or placebo. The primary outcomes were nonfatal myocardial infarction and coronary heart disease (CHD) death, with invasive breast cancer as the primary adverse outcome. While the planned duration of the treatment was 8.5 years, the CEE + MPA treatment arm was stopped prematurely after 5.2 years, due to apparent increases in cardiovascular events and breast cancer incidence [7]. The evaluation of the CEE-alone treatment arm (women without a uterus) is still ongoing, due to the absence of such apparent increases in hazard. This is strongly suggestive of a potentially harmful effect of the progestin used in the study (MPA). If this is the case, it would follow that such negative effects may not extend to other progestins with different pharmacological properties. Particularly, NOM Ac, a 19-norprogesterone derivative, seems to be ineffective in blunting estrogen effects on HDL [8], highlighting the importance of the molecular structure of the different progestins for the final effect in the clinical setting.

3. Progestagens: metabolic effects on haemostasis/coagulation

The use of combined HRT results in relevant effects on the hemostatic system. From the clinical point of view, postmenopausal women receiving ERT or HRT are at increased risk for deep venous thrombosis (DVT) and pulmonary thromboembolism (PE) [9]. Although the absolute numerical increase in the general population would not be large, due to the rarity of these diseases, the odds ratios to develop a DVT or PE are significantly increased, indicating important effects of the hormones on the hemostatic system [9]. The risk of thromboembolic events appears to be maximum in the first months after starting HRT, which suggests that there may be a particular genetic predisposition to develop DVT during hormonal therapies. This has been recently confirmed by the finding of a surprisingly high association of a prothrombin 20210 G → A variant with the incidence of myocardial infarction in women receiving HRT in a population-based, case-control study [10].

Notwithstanding the presence of increased risk for DVT and PE, there is no evidence of increased incidence of stroke in postmenopausal women receiving continuous combined HT with CEE + MPA [7,11,12].

The role of progesterone or of the synthetic progestins for the determination of hemostatic derangements is not clear, and it has not been extensively investigated in the setting of postmenopausal HRT. However, on the basis of the available studies, there is evidence for some modifying effects of progestins on estrogen-induced changes on fibrinogen, factor VII and the fibrinolytic system [13]. These effects appear to be different with different progestins, but no correlation

with the molecular structure of the drugs has been proposed. Since it is difficult anyway to link a particular pattern of haemostatic regulation to the risk of cardiovascular diseases, the available data do not support the notion of a superiority of certain progestins with regard to cardiovascular risks [13].

4. Effects of progestins on blood pressure

Hypertension is a significant burden in postmenopausal women [14]. The currently available data indicate that ERT or HRT have generally neutral effects on blood pressure [9]. Recently, this has been confirmed by the analysis of the WHI trial, that shows no effects of the administration of CEE + MPA on blood pressure in postmenopausal women [7]. Since the CEE-only arm of the study has not yet been disclosed, it is difficult to draw comparisons in order to discern the relative effect of MPA, but negative effects can be reasonably ruled out.

5. Progesterone and progestins signaling in the vascular wall

Notwithstanding the relevance of the metabolic modifications of sex steroid hormones for the determination of cardiovascular disease, it has been estimated that the major part of steroid hormones actions are exerted on vascular tissues, through receptor-mediated direct effects [4,15]. The effects of steroid hormones on vasodilation are particularly prominent, and have been extensively characterized for estrogens. In non-human primates, the contribution of progestins has also been investigated, showing the presence of significant modifying effects on estrogen-induced vascular effects [16]. Indeed, by using *Cynomolgus* monkeys, Adams' group in North Carolina has been able to show consistently that 17 β -estradiol modulates coronary arteries dilatation in estrogen-deprived animals [17]. Co-administration of natural progesterone did not alter the effect of estrogen. However, in the same model, the addition of cyclic or continuous MPA inhibited the vasodilatory effect of estrogen responses by 50% [18]. These data have been confirmed by other groups, showing that progesterone plus estradiol protects, but MPA plus estradiol does not, against coronary artery vasospasm [19], thus highlighting the difference between natural progesterone and MPA. However, other progestins may exert different effects on the vascular tone. Indeed, in contrast to MPA, the non-androgenic progestin nomegestrol acetate, does not impair the beneficial effects of estrogen on the coronary dilator response in monkeys [20,21].

The vasoactive properties of estrogens are well known also in humans, where the acute administration of 17 β -estradiol induces rapid increases in blood flow in peripheral [22] as well as in coronary arteries [23]. The changes observed in the vasomotion of postmenopausal women appear to be as rapid as those observed in monkeys [24], therefore

suggesting the presence of a direct effect on the vessel wall. Indeed, most of estrogens' vasodilatory effects are mediated by an increased synthesis of nitric oxide in endothelial cells [25].

As for the effects of progestins on vascular tone, the data are more limited, but some inferences can be drawn from the available data. Recent work looking at the additional effects of natural progesterone or MPA on coronary blood flow and myocardial ischemia in postmenopausal women shows that progesterone has synergistic vasodilatory effects when added to estrogens [26]. In contrast, MPA does not share this action, therefore indicating that, on this particular target, all progestins are not the same [26].

While the concept of deleterious effects of androgenic molecules on the blood vessels is long-standing, this is far from being ascertained, and actually recent studies indicate that androgens may have anti-atherogenic and vasodilatory actions at the vascular level [27–29]. It is consequently difficult to deduct that progestins with androgenic properties will have deleterious effects on the vessels, while non-androgenic progestins will act neutrally, as it has been proposed in the past [3,30]; the impact of the single molecules should instead be verified in adequate clinical trials.

In addition to the regulation of vascular tone, sex steroids have been shown to be tightly involved in the regulation of the atherosclerotic process [4,15]. Indeed, animal studies with ovariectomized *Cynomolgus* monkeys consistently indicate that ovarian ablation is associated with accelerated atherosclerosis, and that administration of hormonal replacement prevents this process, particularly if started in healthy monkeys early after surgical menopause [31,32]. The data published using the monkey model indicates that progesterone does not impair the positive effect of estrogens, but there are no data regarding the actions of other progestins [33,34].

6. Progesterone and progestins signaling in vascular cells

The molecular basis of the effects of progesterone and progestins on the blood vessels has been investigated in the last few years, and much data have been provided as to the possible mechanisms through which these steroids may modulate the function of vascular cells via the interaction with progesterone receptors.

Progesterone has been shown to regulate the proliferation of vascular endothelial cells (EC) [35], causing an arrest in the G1 phase of the cell cycle. This effect involves a reduction in cyclin-dependent kinase activity, and the altered expression of cyclin E and A in accordance with G1 arrest. Progesterone also regulates smooth muscle cell (SMC) growth induced by serum or endothelin-1 (ET-1) [36]. The mitogenic effect of ET-1 and serum depends on mitogen-activated protein kinase (MAP-K) and MAP-kinase activities, and these were significantly inhibited by

P, that also inhibited mitogen-stimulated c-fos and c-myc, downstream targets for MAP-K action [36]. While natural progesterone seems to have some antiproliferative effects on vascular smooth muscle cells (VSMC) (that would turn into an anti-atherogenic action), MPA instead does not inhibit the expression of mitogenic genes such as PDGF-A, IL-1, IL-6 and c-myc in VSMC, suggesting that this antiproliferative effect may not be shared by this synthetic progestin. Although the antiproliferative effect of progesterone is well established *in vitro*, opposite effects were observed *in vivo*; there is evidence of a worsening of carotid response-to-injury in wild type mice exposed to progesterone, but not in PR-knock-out mice [37], thus suggesting a more complex role than previously postulated for progesterone on VSMC.

A relevant complication of sex steroid use is the increased risk of thromboembolic events. Although sex steroids have pro-coagulant effects mainly through the liver, they also promote hemostasis indirectly by increasing the pro-coagulant activity of blood vessels. Indeed, treatment of VSMC with different progestins (progesterone, 3-keto-desogestrel, gestodene, and MPA) up-regulates proteolytically activatable thrombin receptor (PAR-1) expression, resulting in a potentiated thrombin-induced tissue factor expression and surface pro-coagulant activity [38]. In contrast, the progestins levonorgestrel, norethisterone, and norgestimate do not share such effects [38]. In addition, long-term administration of progesterone, 3-keto-desogestrel, or MPA to ovariectomized rats increased PAR-1 protein level in the arterial wall, resulting in an increased responsiveness of isolated aortic rings to thrombin [38]. Therefore, there is evidence that different progestins may have a different impact on the haemostatic function of the vessels.

Progesterone control of VSMC gene expression has also functional relevance for the pathophysiological processes leading to hypertension. Indeed, while estradiol reduces VSMC expression of AT(1) receptor, on the contrary, progesterone causes AT(1) receptor overexpression via PI3-kinase activation [39]. Because AT(1) receptor regulation plays a central role in the pathogenesis of hypertension and atherosclerosis, the detrimental effects of progesterone on the expression of this gene may be important for cardiovascular disease.

The regulatory effects of progesterone on endothelial and smooth muscle cells may also turn into anti-atherogenic actions. For instance, the expression of endothelial-leukocyte adhesion molecules induced by pro-atherogenic cytokines is one of the earliest detectable events during atherogenesis [15]. Estrogens and SERMs are known to have potent anti-inflammatory actions at this level, inhibiting the inflammation-driven expression of these molecules [40,41], and, at least for estrogen, this has been shown to be mediated by an interference with the activation of the transcription factor NF- κ B [41]. An inhibitory action on adhesion molecules has also been shown for the mixed estrogenic and androgenic/progestogenic molecule tibolone [42]. Recently, our unpublished observations (Simoncini and Genazzani,

unpublished), as well as other groups [43], have shown that progesterone is also able to decrease the expression of endothelial-leukocyte adhesion molecules, although this feature is not shared by MAP.

7. Progesterone and vascular tone regulation: non-transcriptional effects

The actions described before are consistent with the classical concept of progesterone receptor (PR) being a transcription factor regulating the expression of target genes [44]. Indeed, binding of progesterone to the specific PR ligand binding domain (LBD) induces a conformational modification of the receptor, followed by the separation of the receptor from cytoplasmic chaperone proteins, such as heat shock protein 90 (HSP90) and by the exposure of nuclear localization sequences (NLS). This allows nuclear translocation and binding to progesterone response elements (PRE—i.e. nucleotide sequences specifically recognized by PRs) on the promoter regions of the target genes, thus regulating gene expression by interfering with the transcription machinery [44].

However, in addition to the effects on EC and VSMC gene expression, progesterone and progestins may also act via rapid mechanisms, which are independent of protein synthesis. Different terms have been used to distinguish these non-conventional signaling mechanisms, the more popular of which is “nongenomic”. As practical rules, non-transcriptional effects can be indicated as: (a) actions that are too rapid to be compatible with RNA and protein synthesis; (b) actions that steroid hormones induce in cells with highly compacted chromatin, in which RNA and protein synthesis are absent (such as spermatozoa); (c) actions that can be reproduced in the presence of inhibitors of RNA or protein synthesis; (d) actions that can be reproduced by using steroid hormones coupled to cell-membrane-impermeable molecules. A full understanding of the real nature of these mechanisms is still far from being accomplished, and up to now only a patchwork of descriptive data on different non-transcriptional effects of steroid hormones have been cumulated. However, there is growing evidence that some of these effects could be linked to the presence of functional and yet unidentified cell membrane-steroid hormone receptors [45]. Cell membrane progesterone receptors also have been suggested, and they have been found to be of particular relevance in sperm cells, where they are responsible for the rapid, nongenomic activation of Ca^{2+} and Cl^{-} channels, leading to the acrosomal reaction [46].

Compatible with the presence of nongenomic effects of progesterone is the evidence of the rapidly ensuing vasodilatation upon exposure of either intact or isolated vessels. Indeed, in Sprague–Dawley rats, bolus intravenous injections of P significantly decrease pressure responses to norepinephrine [47]. At the same time, progesterone induces a significant relaxation of vascular strips isolated from tail artery or aorta [47], and these effects may be dependent on

the blunting effect on L-type calcium channel currents in isolated VSMC [47]. These suggestive laboratory data are confirmed by the evidence of acute, possibly nongenomic, vasodilatory actions of progesterone in primate coronary arteries [48].

A prototypical “nongenomic” action of estrogen receptors is represented by vasodilatation induced by estrogen, which occurs in a matter of seconds to minutes [49]. This acute effect is the result of a regulation of ionic fluxes as well as of vasoactive molecules release on endothelial and smooth muscle cells. Regulation of nitric oxide (NO) synthesis and release is a major target of estrogen at the endothelial level, and estrogen has been shown to regulate NO release by several means. The principal mechanism is probably represented by the rapid activation of the endothelial isoform of nitric oxide synthase (eNOS) [50]. Estrogen receptor α is involved in the genesis of this phenomenon, which is independent from gene transcription and has been proposed to be in part due to the activation of MAP or tyrosine kinases-dependent pathways. Unpublished data from our laboratory confirm this hypothesis, indicating that estradiol treatment of human endothelial cells results in an ultra-rapid activation of the Raf-1/MEK 1-2/ERK 1-2 MAP kinase module (Simoncini and Genazzani, unpublished). In addition to these important non-transcriptional actions of estrogens, we have recently characterized a novel, nongenomic mechanism of ER α signaling in endothelial cells, explaining a major part of estrogen’s rapid effects on nitric oxide synthesis [50]. Indeed, we have shown that upon binding with estradiol, ER α physically and functionally couples with the regulatory subunit of the lipid kinase phosphatidylinositol 3-OH kinase (PI3K), thus triggering an activation of the catalytic subunit and increasing intracellular production of phosphoinositides [50]. One of the principal targets of this cascade is the serine-threonine protein kinase Akt/protein kinase B. The activation of Akt mediates many of the downstream cellular effects of PI3K, including rapid eNOS activation, accomplished through eNOS phosphorylation by Akt.

Additional evidence shows that interaction with PI3K may be a feature shared by other steroid hormone receptors, such as glucocorticoid receptors. Indeed, GR interacts with PI3K upon binding with different synthetic glucocorticoids, resulting in nitric oxide-dependent anti-inflammatory effects on blood vessels, but also in dramatic anti-ischemic effects in mice hearts [51].

Since PR has also been shown to activate PI3K [39], and is known to have acute vasodilatory effects, it may be possible that part of these actions are mediated by activation of the PI3K-Akt-eNOS pathway.

8. Conclusions

In summary, progesterone is considered a cardiovascular-active sex steroid, which is able to regulate the structure and function of the blood vessels both in physiological as well

as in pathological conditions. Natural progesterone and the different synthetic progestins often have substantially differing effects and, based on the current data available, it is difficult to draw conclusions on the possible superiority of a single molecule versus the others. Understanding the molecular mechanisms through which these actions are exerted represents an important frontier, which should ultimately allow for the engineering of newer progestins with optimal cardiovascular profiles for the treatment of women.

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