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THE EFFECT OF ESTROGEN ON MICROVASCULAR SMOOTH MUSCLE CELLS: IMPLICATIONS FOR PERIPHERAL VASCULAR DISEASE

by
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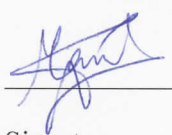

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AN ABSTRACT OF THE DISSERTATION OF

Manal Muin Fardon for Doctor of Philosophy
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Title: The Effect of Estrogen on Microvascular Smooth Muscle Cells: Implications for Peripheral Vascular disease

Cardiovascular disease (CVD) remains the leading cause of morbidity and mortality worldwide. Although the risk factors of this disease are common to males and females, the prevalence of CVD is gender dependent. This suggests the implication of the female hormone, estrogen or 17βestradiol, in CVD. How estrogen affects various CVD has long been a dazzling question. Substantial amount of research has been undertaken to assess the effect of estrogen on various CVDs. To date the mechanisms by which estrogen affects CVD remain incompletely understood, particularly estrogen-evoked signaling in arteriolar inflammation, Raynaud's phenomenon (RP), and hypertension. In this study, we mechanistically assessed the effect of estrogen on microvasculture, at which the aforementioned vascular pathologies are manifested.

First, we showed that estrogen attenuated inflammation of human arteriolar smooth muscle cells. This anti-inflammatory effect of estrogen is achieved by inhibiting major events of phenotypic switch, namely proliferation and migration. This was mirrored by decreased activation of mitogenic extracellular signal–regulated kinase (ERK1/2) and focal adhesion kinase (FAK), involved in cell proliferation and migration, respectively. Further investigations showed that estrogen induced vascular smooth muscle cell (VSMC) senescence via the regulation of tumor suppressor genes, p53 and pRb. In addition, estrogen increased the expression of contractile differentiation markers: calponin and caldesmon. Therefore, due to the potential of estrogen to favorably modulate VSMC phenotype, our results may explain the lower incidence of CVDs in estrogen-replete premenopausal females.

Then, we evaluated the effect a novel homoisoflavonoid, 7-O-methylpunctatin (MP), on arteriolar inflammation. We showed that MP decreased VSMC proliferation, migration, invasion, and adhesion. MP also attenuated adhesion of THP-1 monocytes to microVSMCs, abolished FBS-induced expression of MMP-2, MMP-9, and NF-κB, as well as reduced activation of ERK1/2 and FAK. Furthermore, MP-treated VSMCs showed an increase in differentiation markers and a decrease in osteopontin, a protein highly expressed in synthetic VSMCs. Taken together, these results corroborate an anti-inflammatory action of MP on human microVSMCs. Therefore, by inhibiting the synthetic phenotype of

microVSMCs, MP may be a promising modulator for inflammation-induced arteriolar pathophysiology.

Exaggerated cold induced vasoconstriction leads to RP, which is much more prevalent in females. We have previously established that estrogen increases the expression of alpha 2C-adrenergic receptors (α_{2C}-Ars), the sole mediators of cold-induced vasoconstriction.We showed that estrogen upregulates α_{2C}-AR expression via an EPAC-mediated JNK/AP-1-dependent mechanism. These results provide an insight into the mechanism by which exaggerated cold-induced vasoconstriction (RP) occurs in estrogen-replete females and identify Epac and JNK as potential targets for the treatment of this condition.

The prevalence of hypertension in premenopausal women is lower compared to age-matched men, suggesting a vasorelaxant effect of estrogen. However, recent evidence showed that estrogen-based hormone replacement therapy (HRT) may lead to an increase in blood pressure (BP) in postmenopausal females, arguing against the hypotensive effect of estrogen. In fact, estrogen was reported to mediate vascular smooth muscle cell (VSMCs) contraction by stimulating myosin light chain (MLC) phosphorylation. However, the mechanism by which estrogen induces VSMC contraction is yet to be investigated. In chapter VI, we showed that estrogen induces VSMC contraction by phosphorylating MLC via ROS/Rho/p38 pathway. This pathway presents a novel insight to the mechanism underlying estrogen-induced hypertension, and provides therapeutic targets for the treatment of the disease.

Taken together, our results show the implication of estrogen in several conditions at the level of microvasculature. Whether estrogen's role is beneficial or not, the answer is it depends.

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CHAPTER I

LITERATURE REVIEW

Cardiovascular disease (CVD) is the leading cause of morbidity and mortality worldwide [1,2]. According to WHO, 17.9 million people died from CVD annually, accounting to one-third of global deaths [2]. More than 75% of these deaths occur in low- and middle- income countries [2], which are witnessing epidemiological transition to non-communicable diseases (NCDs) [3,4]. Indeed, deaths from CVD have been decreasing in high income countries, while increasing in middle and low income countries [5]. Knowing that CVD-related deaths could be highly prevented [6,7], this trend may be due to limited access to health care services in low- and middle-income countries [8].

Studies have shown that 50 % of CVD-caused deaths occur with no prior symptoms [3,9]. However, there exist risk prediction charts by WHO ISH (International Society of Hypertension) risk prediction charts and other risk prediction tools that can assess the risk of developing CVDs [10]. According to WHO, the global behavioral risk factors of CVDs include:

1- Tobacco smoking:

Smoking is a totally avoidable risk factors. Smoking causes 10% of total CVD [11]. In addition, it is the second global leading CVD risk factor, in terms of attributable deaths [11]. Prevalence of smokers varies among the world, with the highest in Europe, and the lowest Africa [12].

2- Physical inactivity:

Physically inactive people are at 20-30 % higher risk of all-cause mortality [13]. In addition, physical inactivity has been associated with increased risk of CVD [14,15], ranking fourth in

mortality risk factors [11]. The highest prevalence of physical inactivity was reported in America and Eastern Mediterranean [16].

3- Unhealthy diets:

Unhealthy diet (high-fat diet, low fruit and vegetable intake) is a risk factor of CVD [7]. When combined with physical inactivity, it leads to obesity, which is associated with many CVD, mainly atherosclerosis [5]. In addition, high-salt diet increases the blood pressure of hypertensive and normotensive individuals [17]. Consequently, lower intake of dietary salt would ameliorate blood pressure and CVD [18].

4- Excessive use of alcohol:

CVD risk is directly related to level of alcohol consumption [19,20]. In fact, more than 50% of alcohol-attributable deaths are due to 3 diseases, including CVD [11].

CVD is largely preventable, as the aforementioned risk factors can be avoided. However, long-exposure to these behavioral risk factors sets stage for pathophysiological conditions, which are themselves major CVD risk factors [5]. Hypertension, diabetes, and dyslipidemia are on top of the list.

1- Hypertension:

Hypertension is the leading CVD risk factor in terms of attributable deaths, accounting for 13% of CVD-related deaths [11]. Hypertension is positively and continuously related to CVD [5]. It has been reported that early detection and treatment of hypertension greatly reduces risk to CVDs [7]. The prevalence of hypertension is highest in Africa, and least in America [13].

2- Diabetes:

Diabetes is a major CVD risk factor. Notably, CVD risk in diabetic individuals is 2-3 times higher, and 60% of diabetic patients die from CVDs [21-23].

3- Dyslipidemia:

Raised serum cholesterol and triglyceride is associated to CVD risk [7]. Globally, high cholesterol accounts for one third of ischaemic heart disease incidences [11,24]. It has been reported that treatment of dyslipidemia results in reduction in heart diseases [5].

Although these risk factors are common between males and females, substantial amount of evidence reported a gender/sex difference in the prevalence of CVD [25]. In fact, males are at a higher risk of CVD [26], and the total CVD-related morbidity is approximately 2 folds higher in men [27]. Conversely, premenopausal females are at a lower CVD risk compared to men [27]. For instance, at the age of 40, the risk of developing CVD is 49% in men and 32 % women [28]. As such, gender is considered a key determinant in CVD; however, the effect of this sex gap decreases with age due to increased prevalence of CVD in postmenauposal females [28]. This observation led to speculation on the effect of the female hormone, estrogen, on CVD [29]. The effect of estrogen on three cardiovascular pathologies, namely vascular inflammation, Raynaud's phenomenon, and hypertension, are discussed respectively.

A. Estrogen In Vascular Smooth Muscle Cells: A Friend Or A Foe?
Authors: Fardoun M, Dehaini H, Abou-Saleh H, El-Yazbi A, Eid AA, Eid AH.

Cardiovascular disease (CVD) continues to be the leading cause of death worldwide. The effect of estrogen on these diseases has been assessed in in vitro and in vivo models, as well as in

observational studies. Collectively, these studies alluded to a cardiovasculo-protective effect of estrogen. However, comprehensive clinical investigation failed to produce concrete proof of a cardiovascular protective effect for hormone replacement therapy (HRT), let alone rule out potential harm. These seemingly paradoxical effects of estrogen were explained by the 'theory of timing and opportunity'. This theory states that the effect of estrogen, whether cardiovasculo-protective or pathological, significantly depends on the age of the individual when estrogen administration takes place. Here, we review the conflicting effects of estrogen on vascular smooth muscle cells, mainly proliferation and migration as two cellular capacities intimately related to physiology and pathophysiology of the cardiovascular system. Furthermore, we critically discuss the major parameters and signaling pathways that may account for the aforementioned paradoxical observations, as well as the key molecular players involved.

1. Introduction

Estrogens represent a class of hormones that is mainly composed of 17-β-estradiol (E_2), in addition to estrone (E_1) and estriol (E_3), with 17-β-estradiol being the most potent [30,31]. They are synthesized by the ovaries, adrenal cortices and liver, as well as by the placenta during pregnancy [30]. Estrogens are responsible for the development of primary and secondary female sex characteristics. Furthermore, they take part in the induction of female growth spurts by stimulating bone growth, increasing body metabolism and enhancing fat and protein deposition [30]. Two nuclear receptors, namely estrogen receptor alpha (ERα) and beta (ERβ), in addition to the G protein-coupled estrogen receptor (GPER) mediate the actions of estrogens [32-35].

It has been suggested that estrogens impart a beneficial effect on the cardiovascular system. This was primarily based on multiple cohort studies which showed a negative correlation

between the use of estrogen supplements and coronary heart disease (CHD), as well as other fatal cardiovascular diseases among postmenopausal women [36,37]. The Danish Osteoporosis Prevention Study's randomized controlled trial (RCT) provided some support for this protective effect [38]. This study showed that postmenopausal women who received hormone replacement therapy (HRT) with a cyclical protocol including synthetic 17-β-estradiol and a combination of 17-β-estradiol and norethisterone (for patients with intact uterus) or 17-β-estradiol alone (for patients with a hysterectomy) were at lower risk of myocardial infarction (MI) [38].

Yet, not all studies support a cardio-vasculoprotective effect of estrogen. Indeed, a RCT conducted by Women's Health Initiative revealed that postmenopausal women on HRT experienced higher rates of CHD, stroke, venous thromboembolism (VTE), deep vein thrombosis (DVT) and pulmonary embolism (PE) [39]. However, it is noteworthy that patients in this study received conjugated equine estrogens with extensive first pass metabolism and variable *in vivo* potency[40]. Additionally, the difference in mean subject age between the above mentioned trials could potentially underlie this discrepancy, with an older patient cohort in the Women's health Initiative Study. In fact, some authors suggest that the vast difference in the dosage of estrogenic component between oral and other routes of administration, including transdermal application, that is driven by the first pass metabolism, triggers unfavorable cardiovascular effects caused by disproportionate hepatic exposure to estrogens in oral preparations [41]. Detailed discussion of these effects is beyond the scope of this review.

The unfavorable effects of estrogens become further evident in yet another vascular disease, namely Raynaud's phenomenon (RP). RP is an exaggerated, cold-induced constriction of peripheral cutaneous arterioles [42]. Although patients with RP exhibited signs of improvement upon the short-term administration of estrogen [43], a 10-year literature review alluded to a positive

association between increased risk of RP and estrogen therapy [44]. This is not surprising given that nearly 70% of RP patients are females [45,46], and the prevalence of this disease in premenopausal females is nine times higher than in age-matched men [47-49]. Furthermore, RP is more predominant in premenopausal women compared to postmenopausal ones [42]. Interestingly, postmenopausal women receiving HRT have a significantly higher incidence rate of RP than the control group [50]. In this study, 114 women received estrogen replacement therapy, the majority of who received conjugated equine estrogens while only 12 patients reported using the transdermal patch. Clearly, these findings support the notion that estrogen plays a major contributing role to the onset or pathogenesis of RP [51].

The Flavahan group elegantly showed that the entirety of cold-induced vasoconstriction was shown to be mediated by vascular alpha 2C-adrenoceptors (α_{2C}-ARs) [51,52]. The same group further showed that α_{2C}-AR, once dubbed a "vestigial" receptor, assumes a rather unique biology. Indeed, while this receptor remains intracellularly trapped in the endoplasmic reticulum and Golgi apparatus at 37 °C, it is spatially and functionally rescued to the plasma membrane upon moderate cold exposure [53-55]. Once at the membrane, this receptor can readily bind to epinephrine, its natural agonist, and thus elicit vasoconstriction. It was later showed that estrogen leads to heightened cold-induced vasoconstriction by virtue of its ability to increase the expression and function of α_{2C}-ARs in human arteriolar smooth muscle cells [51].

VSMCs are major structural and functional components of the vessel wall. They carry out several functions, such as regulation of vasotone and blood flow, both of which are critical for tissue perfusion, metabolic demand and homeostasis. In addition to their role in physiology, VSMCs are also important players in the pathophysiology of several diseases including atherosclerosis [56-58]. Atherosclerosis is the most common cause of ischemic heart disease as well

as stroke, both of which are major contributors to CVD-associated morbidity and mortality [59,60]. Indeed, VSMCs contribute to the formation of the atheroma within the tunica intima either by proliferating and migrating from tunica media [61,62], or by arising from proliferating multipotent vascular stem cells (MVSCs) that reside in the vessel wall [63]. Additionally, VSMCs are involved in restenosis, the re-narrowing of arterial lumen that occurs post-angioplasty [64]. Restenosis results from excessive migration and proliferation of VSMCs which eventually culminate in the formation of neointimal hyperplasia [64,65].

Due to the controversy that has arisen in regards to the use of estrogens in HRT, our aim in this review is to dissect and critically discuss the effects of estrogens on proliferation, migration and apoptosis of VSMCs. These parameters represent major hallmarks of VSMC phenotype, a crucial player in cardiovascular physiology and pathophysiology.

2. Modulation of VSMC proliferation and migration by estrogen

The literature contains contradictory reports on the effect of estrogen on VSMC proliferation and migration. These discrepancies are likely due to several factors related to VSMCs, such as the vascular bed from where cells were isolated, age of the animal, cell population number, cell phenotype in culture and whether cells are stimulated or not (figure 1). The chemical type of estrogen used, as well as its concentration, are also among the factors that contribute to the variable effects of estrogen on VSMCs' proliferation and migration (figure 1).

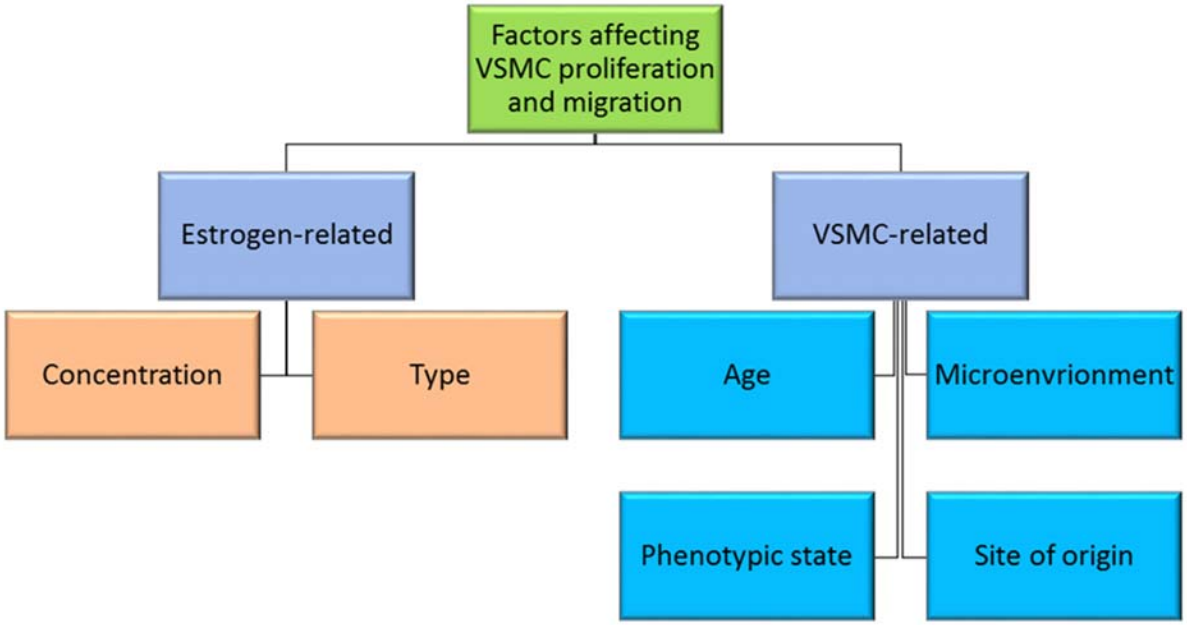

Figure 1. Schematic representation of the effect of estrogen on VSMCs.

The concentration/dose as well as the type of estrogen used are two critical factors inherent to estrogen's effects. The age of the subject/animal and the vascular bed from where VSMCs are isolated contribute to phenotypic behavior of these cells in the presence of estrogen. Microenvironmental cues, such as the nature of the extracellular matrix proteins available, are also contributing factors to VSMC phenotype.

Overwhelming evidence clearly shows that the effect of estrogen on VSMCs is largely affected by the site from where they were isolated. For instance, 17β-estradiol inhibited neointimal hyperplasia, characterized by increased proliferation and migration of VSMCs, in the femoral artery of rats and mice [66,67], the aorta and iliac artery of rabbits [68], as well as the carotid artery of mice, rats and pigs [69-73]. Using estrogen receptor knockout mice, Pare *et al.* attributed these effects to ERα receptor [73]. This was reflected by findings showing that the protective effect of *in vivo* 17β-estradiol treatment against neointimal hyperplasia were abrogated with conditional ERα knockout [67]. On the other hand, 17β-estradiol promoted the proliferation of

pulmonary VSMCs obtained from rats and canines in a concentration-dependent manner in the same concentration range reported to inhibit VSMC proliferation in the previous studies [74]. This apparent paradox could potentially be explained by differential regulation of ERα transcriptional activity in VSMCs from different sources. Huang *et al.* showed that while 17β-estradiol inhibited the proliferation and migration of VSMCs extracted from human saphenous and umbilical veins , the same concentration seemed to increase proliferation of VSMCs from varicose veins of the same patients. This potentiation appears to be due increased expression of IQ-domain GTPase-activating protein 1 (IQGAP1), which is a scaffold protein that aids in the activation of the mitogen-activated protein kinases/extracellular signal-regulated kinases (MAPK/ERK) pathway, and thus promotes ERα transcriptional activity as discussed in section 3 [75]. Whether this is a universal mechanism or other factors contribute to the difference in response to 17β-estradiol observed in VSMCs from different vascular beds remains to be determined. Nevertheless, these results clearly show that VSMCs' response to estrogen is affected by the vascular bed from which they are collected, as well as the initial state of VSMCs prior to estrogen treatment. Accordingly, one may argue that HRT can be either beneficial or harmful depending on the site of vascular injury.

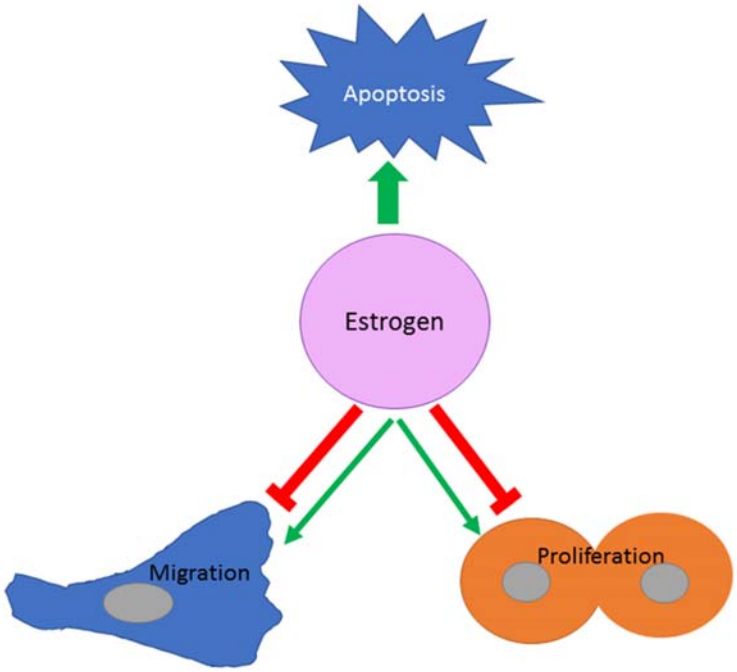

Figure 2. Modulation of VSMC proliferation, migration and apoptosis by estrogen.

While estrogen-induced apoptosis is a consistent finding, modulation of proliferation and migration by estrogen appears to be affected by several other factors. While estrogen may promote proliferation and migration, it can also inhibit them if the physiological or pathophysiological cues change

The microenvironment of VSMCs is another factor that determines their response to estrogen. Indeed, 17β-estradiol inhibited the proliferation and migration of VSMCs that were cultured in a medium containing mitogenic factors, such as basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), platelet-derived growth factor (PDGF) and serum [76-79]. Ueda *et al.* showed that this effect is mediated by non-nuclear ERα signaling leading to Akt and Erk dephosphorylation [78]. The same effect of 17β-estradiol was observed for VSMCs grown in conditions that mimic physiologic or pathophysiologic stress such as hyperlipidemia [80], hypoxia [81], or oxidative stress [82]. Interestingly, 17β-estradiol had no effect on unstimulated VSMCs [76].

Therefore, it can be proposed that the microenvironment of the VSMCs alters their phenotypic state, and this affects their response to estrogen.

The phenotypic state of VSMCs can either be contractile under physiologic conditions or synthetic under inflammatory and stimulatory conditions [83]. Synthetic VSMCs are highly proliferative and migratory, and produce a high amount of extracellular matrix (ECM) proteins [84]. Contractile VSMCs, on the other hand, exhibit low proliferative and synthetic states, and they express contractile proteins such as myosin heavy chain (MHC) and elastin [84]. Under the influence of 17β-estradiol, contractile VSMCs had a longer G_0 phase before entering the cell cycle and proliferating extensively [83]. Synthetic VSMCs, however, responded to estrogen by increasing their cell division rate [83]. This finding suggests that the inhibitory effect of estrogen on proliferation and migration of VSMC can be a retardation of the switch from contractile to synthetic phenotype.

The age of the animal from which VSMCs are isolated may greatly dictate estrogen's effect on cellular proliferation and migration. In an experiment conducted on old and young mice, estrogen inhibited neointimal hyperplasia in the carotid arteries of young mice, but instigated neointimal hyperplasia in the carotids of old mice [85]. Interestingly, this study implicated both ERβ and the novel G protein-coupled estrogen receptor (GPR30) in the protective effect observed in young rats. Hence, it can be speculated that with time, VSMCs change their expression of proteins that respond to estrogen from anti-inflammatory molecules to pro-inflammatory ones. Interestingly, barring the differences in route of administration and estrogen/progestin component used, women who benefited from HRT in the Danish Osteoporosis Prevention Study were younger than those who had increased cardiovascular events in the WHI's RCT [38,39]. Indeed, a sub-group analysis of younger patients in the Women's

Health Initiative study indicated a potential protective effect [86]. Consequently, all these findings support the "windows of opportunity and timing" hypothesis, which states that an early onset of HRT in postmenopausal women helps reduce the risk of cardiovascular diseases, while a later initiation has no beneficial effect and may actually be harmful [87].

With regards to the age-dependent estrogen effects, it is important to consider the variability in serum estrogen levels in different ages, since opposite effects of estrogen on VSMCs can be also attributed to the concentration/dose used. Serum 17β-estradiol levels were reported to be around 0.2 nM, up to 1.6 nM, and up to 0.15 nM in pre-pubertal, adult, and post-menopausal females, respectively [88]. At concentration levels matching those found in prepubertal and postmenopausal women (0.3 nM), 17β-estradiol promotes the proliferation of human umbilical VSMCs [88]. However, at high concentrations that match those found during the ovulatory phase of premenopausal women (3 nM), 17β-estradiol inhibits VSMC proliferation [88]. Similar results were observed in rat VSMCs, where physiological concentrations of 17β-estradiol (1 nM) stimulated proliferation, but higher concentrations (>10 nM) inhibited it [89]. Interestingly, women of WHI's RCT received 0.625 mg/day of conjugated equine estrogen and suffered increased risk of cardiovascular diseases [39], while those from the Danish Osteoporosis Prevention Study received 2 mg/day and were at lesser risk of cardiovascular diseases [38]. All these results support the bimodal dose dependency hypothesis, which states that estrogen increases or decreases VSMC proliferation at low or high concentrations respectively [88]. One way to partly explain the bimodal dose dependency hypothesis is by observing 2- and 4-hydroxyestradiols, both of which are estrogen metabolites [89]. As the concentration of estrogen increased, these metabolites accumulated and caused an intracellular increase in reactive oxygen species (ROS), which damaged DNA and resulted in VSMC senescence [89].

Another determinant of estrogen's effect on VSMC is the type of estrogen used in experiments. In an attempt to differentiate among the clinically used estrogens, it was found that an inhibitory effect on VSMCs is noted upon the use of estradiol valerate, estradiol cypionate, and estradiol benzoate [90]. Estrone, estrone sulfate, estriol, and 17α-estradiol, on the other hand, failed to display any inhibitory effect [90]. This result may provide another explanation for the opposing conclusions met in the aforementioned RCTs, since the Danish Osteoporosis Prevention Study used synthetic 17β-estradiol [38], while WHI used conjugated equine estrogens[39].

The role of individual estrogen receptors, especially ERα and ERβ, in mediating estrogen's role in vascular injury is also important. Estrogen inhibited vascular injury in both ERα- and ER-β knock out mice [91,92]. Interestingly, in double gene knock out mice, 17β-estradiol failed to suppress intimal thickening after vascular injury yet still inhibited proliferation of VSMC [73,93]. These studies alluded to a potential contribution from an ERα splice variant, although the possibility of a yet unidentified estrogen-responsive receptor cannot be eliminated.

Despite the aforementioned varying effects on proliferation and migration of VSMCs, a consistent finding was that estrogen induced apoptosis of VSMCs in all the reviewed studies (figure2). This finding is consistently observed in VSMCs obtained from human or murine aortas [94-96]. This suggests that the anti-proliferative effect of E_2 on VSMCs can be due to E_2's induction of apoptosis, which occurred at the G_2-to-M phase, despite the fact that VSMCs had already entered the cell cycle [96].

3. Signaling pathways implicated in estrogen's effects on VSMC proliferation and migration

The contentious observations regarding the effect of estrogen on VSMCs are reflected in the experiments that studied the signaling pathways implicated in VSMC proliferation,

migration and apoptosis. One of the most extensively studied pathways is the MAPK/ERK pathway. ERK1/2 translocate to the nucleus and phosphorylate transcription factors required for proliferation [97]. Their mode of action is biphasic: the initial step is a transient increase in ERK1/2 for up to 10 minutes, followed by a lower, but sustained peak for the duration of G_1 phase [98]. These two steps are necessary to drive the G_1-to-S phase transition into completion [97]. This may appear to suggest that both rapid non-genomic and longer-lasting genomic effects are implicated. Importantly, the notion that both these pathways are based on phosphorylation makes it more likely that the effects are independent on gene expression. However, this remains to be investigated.

Using rat VSMCs, 17β-estradiol induced proliferation by promoting ERK1/2 phosphorylation [95]. However, it inhibited ERK1/2 phosphorylation and the consequent proliferation and migration in murine, human and porcine VSMCs [99-101], inducing differentiation instead particularly when GPER agonist, G-1, was used [100]. Interestingly, other reports suggest that 17β-estradiol -induced phosphorylation of ERK1/2 may cause inhibition of murine aortic VSMC proliferation and migration [78,102]. This effect appears to be due to estrogen-induced upregulation of striatin [78,102], a calmodulin-dependent scaffolding protein [103].

While these results appear conflicting, a closer look at the underlying molecular mechanisms may help resolve this paradox. Indeed, in human aortic VSMCs, activation of ERα inhibited proliferation by suppressing prolonged ERK phosphorylation via the upregulation of manganese superoxide dismutase (MnSOD) [104,105]. Henceforth, it can be concluded that estrogen induced ERK1/2 phosphorylation momentarily, causing VSMCs to enter the cell cycle and striatin to be expressed, before inhibiting the sustained ERK1/2 phosphorylation of the biphasic model. A limitation to this experiment, however, is that the cells were treated with high glucose

(HG) [104], which is known to induce expression of inflammatory genes in VSMCs [106]. Thus, these findings need to be replicated under other conditions before our argument can be cemented.

Another signaling molecule that plays an integral role in cellular proliferation is the retinoblastoma protein (pRb). pRb is a tumor suppressor protein, which binds to and inhibits E2F transcription factors [107]. When phosphorylated, pRb is inactivated, allowing E2F transcription factors to translocate to the nucleus and drive the G_1-to-S phase transition [107]. In an experiment conducted on rat aortic VSMCs, 17β-estradiol inhibited pRb phosphorylation, resulting in inhibition of proliferation at the G_1 phase [108] (figure 3). This study showed that this effect was elicited through ERα, since stimulating A10, a rat aortic smooth muscle cell line that expresses ERβ but not ERα, with PDGF was not inhibited by 17β-estradiol or raloxifene [108]. However, when these cells were transfected with ERα, estrogen potently inhibited PDGF-induced cyclin D1 expression [108]. This is in marked contrast to findings showing that inhibition of a different related receptor named estrogen related receptor-α, ERRα, induced hyperphosphorylation of pRb, inhibited proliferation and migration of rat aortic smooth muscle cells, as well as attenuated neointima in rat artery subjected to balloon injury [109], with both studies reporting the involvement of the cyclin-dependent kinase inhibitor (CDK), $p27^{Kip1}$. Importantly, by suppressing the Ras-pRb pathway, 17β-estradiol inhibits premature senescence of VSMCs isolated from young (2 months) female rats [110]. Contrarily, this is reversed to a senescence-promoting effect in cells isolated from old (18 months) female rats. Non-selective ER blockade abolished the protective effect but promoted the senescent effect that appeared likely to be due to estrogen metabolites since it was blocked upon treatment with a cytochrome P450 inhibitor [110]. Together, these findings provide insight into the “time window theory” as well as provides

insight into how HRT could be vasculoprotective in younger versus being a risk factor in older postmenopausal women.

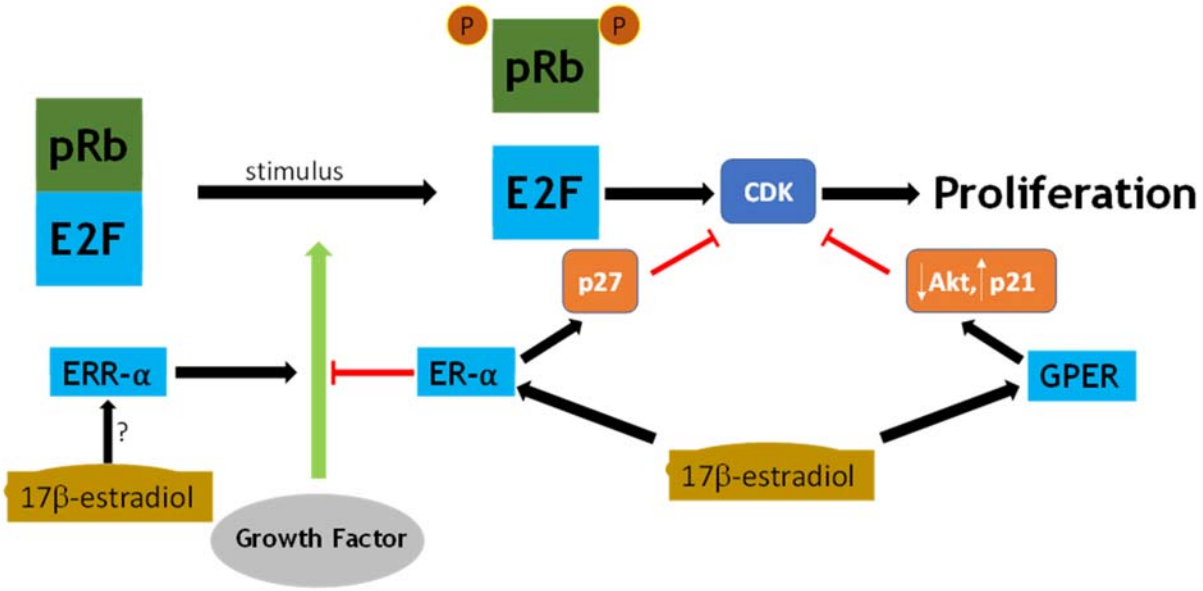

Figure 3. Estrogen modulation of growth factor-stimulated, pRb-E2F-mediated proliferation of VSMCs.

17β-estradiol modulates proliferation via different pathways. Its inhibitory actions are elicited by virtue of its ability to increase levels of p21 and p27, which then inhibits CDK and reduces proliferation. It can also act through ERα to inhibit growth-factor-induced stimulation of pRb phosphorylation. However, when it activates ERRα, it may induce the opposite effect by promoting dissociation of pRb from E2F, thereby upregulating CDK levels and promoting proliferation.

The role of Akt in proliferation is extensively established in various cell types including VSMCs. In particular, Akt is also an important mediator of estrogen-induced inhibition of VSMC proliferation. Indeed, in VSMCs isolated from the carotid artery of human and porcine origin, stimulation of G-protein coupled estrogen receptor 1 (GPER) inhibited proliferation by decreasing Akt phosphorylation and inducing p21 expression [100]. p21 is a CDK inhibitor that induces G_1/S cell cycle block. Recently, it was also shown that 17β-estradiol, acting via Akt, reduced proliferation and migration of rat aortic smooth muscle cells [111]. These effects occur

mainly by virtue of estrogen's ability to decrease sirutin 1 (SIRT1) [111]. Importantly, because SIRT1 levels are decreased with aging [112], estrogen may fail to impart its potent anti-proliferative effect on VSMCs. Together, these findings may partly explain the dichotomy of estrogen's effects depending on the age of the cells stimulated with estrogen.

4. Signaling pathways implicated in estrogen's effects on VSMC apoptosis

The notion that estrogen induces apoptosis of VSMCs is well-accepted. Indeed, when treated with 17β-estradiol, rat synthetic VSMCs exhibited a dramatic increase in the number of apoptotic cells, marked by an increase in Bax/Bcl-2 ratio at the G_2-to-M phase [96]. Interestingly, this pro-apoptotic effect of estrogen occurs together with increased expression of cyclin D_1 and CDK4 concomitant with increased G1 to S transition [96]. Therefore, estrogen established its anti-proliferative effect by inducing apoptosis in the very cells that it accelerated their entry into cell cycle. In human aortic VSMCs, 17β-estradiol also increased apoptosis by promoting rapid and temporary phosphorylation of p38 [94], a MAPK that is usually activated by stress stimuli [113]. Interestingly, ERK1/2 pathway, which caused proliferation, and the p38 pathway, which resulted in apoptosis, were both activated in VSMCs treated with 17β-estradiol [95]. Hence, the ultimate effect of estrogen may result from the balance between these two antagonistic pathways, especially that the inhibition of one of them stimulated the other [95].

One of the key signaling molecules that has been shown to be involved in estrogen's induction of apoptosis is Protein kinase A (PKA). While 17β-estradiol activated the MAPK/ERK pathway, it concomitantly inhibited PKA, resulting in apoptosis [114]. This effect was observed in freshly isolated rat aortic VSMCs [114]. In cultured VSMCs, however, ERK was dephosphorylated and PKA was activated; this resulted in the loss of estrogen-induced apoptosis [114]. A possible

explanation to this discrepancy is the expression difference of GPR30, a G-protein coupled receptor of estrogen. Indeed, while GPR30 expression is high in freshly isolated VSMCs, its expression in cultured cells is barely detectable [114]. Importantly, estrogen-GPR30 complex was shown to be responsible for the inhibition of PKA and the induction of apoptosis [114]. It appears that a balance between ER-activated PKA and GRP30-inhibited PKA is critical for defining estrogen's ultimate effect on apoptosis of VSMCs. Consequentially, this indicates that *in vitro* experiments that study estrogen's effect on VSMCs but undermine the role of GPR30 in estrogen's pro-apoptotic effect may need to be revisited.

5. The Effect of Estrogen on VSMC Differentiation

Under normal physiological conditions, differentiated VSMCs assume a contractile phenotype [115]. They are characterized by the expression of smooth muscle cell (SMC) differentiation markers such as calponin and smooth muscle α-actin [115]. The expression of these markers is downregulated in response to vascular insults that ultimately drive VSMCs to switch to a synthetic phenotype [115]. The effect of estrogen on differentiation of VSMCs and consequently the expression of differentiation markers is not well defined. It may be postulated that estrogen would increase the differentiation markers by virtue of inhibiting VSMC proliferation and migration. However, pathways that increase the expression of contractile molecules and increase the migratory phenotype could occur simultaneously [116-118]. Furthermore, Motague *et al.* showed that the activation of ERα reduces SMC differentiation markers in aortic SMCs [119]. On the other hand, Huang *et al.* suggested that the inhibition of ERα, ERβ, and/or GPR30 reduced the SMC marker, calponin, expression [120]. Furthermore, Myocardin, cardiac and smooth muscle cell specific protein, activates many VSMC differentiation markers [121].

Interestingly, it was shown that this protein interacts with ER co-activator, SRC3, to up-regulate the SMC differentiation markers [121].

6. Epigenetic regulation of ERs in VSMCs:

Since estrogen elicits its effects through binding to its receptors, the epigenetics of these receptors may affect the cellular response to estrogen. Indeed, ER-α promoter does not appear to be methylated in normal aorta (in situ), but exhibits higher methylation status in proliferating VSMCs isolated from human aortae [122]. This hypermethylation is an important contributor to VSMC phenotype [122]. Other studies have shown that hypermethylation of ER-α is correlated with atherosclerosis and ischemic stroke, in a manner proportional to the disease severity [123,124]. This relation is further confirmed in a study showing that insulin potentiated the expression of DNA methyltransferases, leading to increased ER methylation [125]. This in turn caused a loss of ER-α- inhibited proliferation of VSMCs and thus resulted in atherosclerosis [125]. ER-β hyper-methylation was also observed in atherosclerotic tissues [126]. Interestingly, unlike ER-α, the correlation of ER-β expression with atherosclerosis is independent of age [127].

7. Conclusion

It is now evident that the effect of estrogen on cardiovascular diseases depends on age as well as many other factors. Physiological/pathological states, VSMC microenvironment, the particular estrogen derivative used, the predominant estrogen receptor expressed, and the epigenetic status of the estrogen receptor promotor all dictate the outcome of VSMC exposure to estrogen. These factors must be taken into consideration when studying the cardiovascular role of estrogen. Concerted and coordinated efforts among basic science researchers and clinicians are needed to optimize a suitable model to assess the role of estrogen in the vasculature.

B. Raynaud's Phenomenon: a Brief Review of the Underlying Mechanisms

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Raynaud's phenomenon (RP) is characterized by exaggerated cold-induced vasoconstriction. This augmented vasoconstriction occurs by virtue of a reflex response to cooling via the sympathetic nervous system as well as by local activation of α_{2C} adrenoceptors (α_{2C}-AR). In a cold-initiated, mitochondrion-mediated mechanism involving reactive oxygen species and the Rho/ROCK pathway, cytoskeletal rearrangement in vascular smooth muscle cells (VSMCs) orchestrates the translocation of α_{2C}-AR to cell membrane, where this receptor readily interacts with its ligand. Different parameters are involved in this spatial and functional rescue of α_{2C}-AR. Of notable relevance is the female hormone, 17β-estradiol, or estrogen. This explains the high prevalence of RP in pre-menopausal women compared to age-matched males. In addition to dissecting the role of the aforementioned players in this review, we further discuss the role of pollution as well as genetic background in the onset and prevalence of RP. We finish by discussing the different therapeutic approaches employed as treatment modalities for this disease. The lack of an appropriate animal model for RP mandates that more efforts be undertaken in order to better understand and eventually treat this disease. Hence, it is important to note that precaution remains the best way to avoid Raynaud's disease, as there is no approved therapy for the disease.

1. Introduction

Cold-induced vasoconstriction of cutaneous arterioles is a normal physiological process that redirects blood from the superficial circulation to internal organs in order to protect the body

from excessive heat loss [128]. This constriction is mediated by reflex sympathetic release of norepinephrine [128] as well as increased sensitization of the vasculature [129]. When this cold-induced constriction is exaggerated, it leads to a pathological condition known as Raynaud's phenomenon (RP) [130]. This disease can be clinically classified as primary or secondary [131] (Fig. 1). Primary RP is idiopathic, and it is the most common form of the disease [132]. On the other hand, Secondary RP could be due to myriad of underlying health conditions such as autoimmune diseases or cancer, as well as lifestyle conditions such as smoking or certain medications [133] (Fig. 1). Indeed, 95% of patients suffering from Scleroderma are diagnosed with RP [134].

Raynaud's disease affects up to 10% of the general population [45]. Affected individuals suffer from cold-provoked vasospastic attacks [135] which are associated with the classic triple-color change (pallor, cyanosis, and erythema) [136], in addition to puffiness and ulcerations mainly at the level of fingers [137]. Other distal body organs such as the nose, toes, and nipples are reported to be affected [131,138]. While there are different manifestations that can be used to diagnose RP, changes in some parameters may also be helpful . For example, serological tests of Raynaud's patients show rather increased levels of endothelin-1 [139], tumor necrosis factor-α (TNF-α) [140], fibrinogen [141], platelet factor (PF-4), and von Willebrand's factor (vWF) [140]. Magnesium ions and S-nitrosothiols levels appear to decrease in Raynaud's patients compared to unaffected individuals [142,143]. Furthermore, anti-centromere and anti-centriole antibodies are detected in patients' sera [144,145].

Many hypotheses have been proposed to dissect and explain the underlying mechanisms implicated in the pathogenesis of RP. Recent evidence appears to lend a strong support for the mosaic theory of this disease [146]. This theory consolidates the multi-etiology of the disease, involving local, neuronal, and hormonal mediators [147]. Impaired function of any of these

mediators may contribute to an exaggerated constriction of cutaneous arteries in response to noradrenaline [148]. Noradrenaline elicits its effects through binding to adrenergic receptors located on the surface of vascular smooth muscle cells (VSMCs) [149]. Typically, VSMCs have three types of adrenergic receptors (ARs): α_1, α_2, and β_2. β_2 adrenoceptors are involved solely in vasodilation [150], whereas α_1 and α_2-ARs are responsible for vasoconstriction (figure 4A). While α_1-ARs have a wide expression pattern across the vascular tree, α_2-ARs are predominantly present in smaller blood vessels or arterioles [151]. At one point, these receptors were surprisingly found to be present in the protein extract of minced aortas [152]. However, further histochemical analysis showed that these receptors were rather expressed in the *vasa vasorum* of the aorta [152].

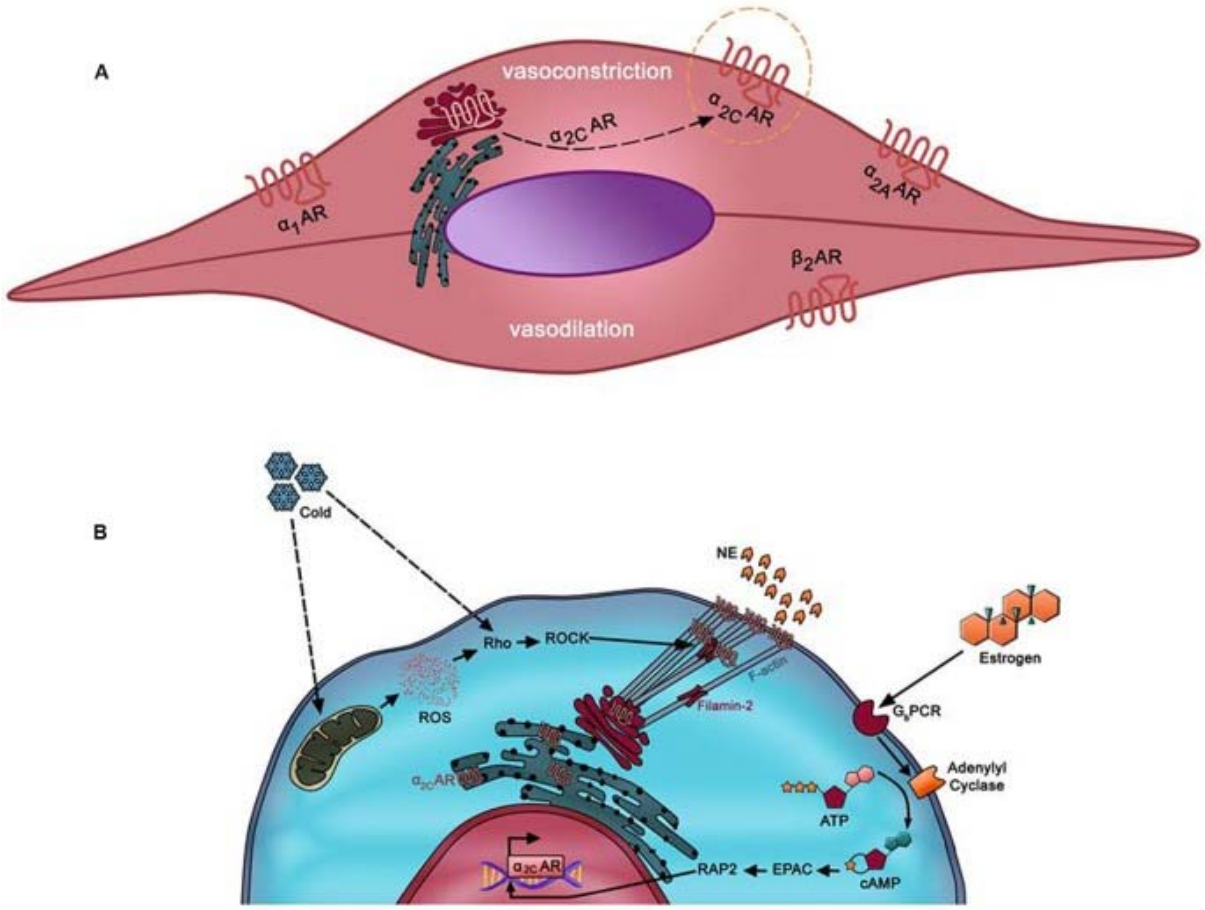

Figure 4. The α_{2C}-ARexpression and translocation in VSMCs

(A) Predominant adrenergic receptors in arteriolar vascular smooth muscle cells (VSMC). β2AR mediates mediates vasodilation of small microvessels. Vasoconstriction of these vessels occurs via α1-AR, α_{2A}-AR, and α_{2C}-AR. Whereas α1-, α_{2A}-, and β_2-ARs in these cells are localized at the cell surface, α_{2C}-AR (in dotted orange circle) is uniquely trapped intracellularly (mostly *trans*-Golgi). However, it can be mobilized to the membrane by various stimuli such as cold temperatures. α_{2C}-AR mediates cold-induced vasoconstriction, which when exacerbated may lead to Raynaud's phenomenon (RP). **(B)** Mechanism of cold-induced mobilization of α_{2C}-AR. In cutaneous arteriolar SMCs, a decrease in temperature is sensed by the mitochondria, which then releases reactive oxygen species (ROS). ROS, in turn, activates the Rho/ROCK pathway. Subsequent cytoskeletal rearrangements involving F-actin and filamin-2 promote mobilization of α_{2C}-AR from the endoplasmic reticulum/Golgi to the cell surface.

Early evidence clearly pointed to the prominent role of α_2-ARs in local cooling-induced constriction of cutaneous arteries. It is important to note that local cooling causes vasodilation [153] as well as inhibits α_1-AR-mediated vasoconstriction [154]. Paradoxically, this very cooling also causes vasoconstriction by virtue of its potential to selectively amplify α_2-AR-mediated constrictive effects [53,155] . Because they play the key role in the sympathetic constriction of cutaneous vessels, selective potentiation of α_2-ARs allows their cold-induced constrictive effects to overcome the vasodilatory effects. Accordingly, α_2-AR antagonists were, at one point, used to potentially treat peripheral cold-induced vasoconstriction [156].

Molecular, genetic and pharmacologic studies show that α_2-ARs actually comprise three subtypes: α_{2A}, α_{2B}, and α_{2C} [157]. These subtypes have their corresponding genes on three different chromosomes, and they are all coupled to inhibitory hetero-trimeric G protein [157]. The search for the particular subtype responsible for α_2-AR-mediated cold-induced vasoconstriction remained unclear for some time. α_{2A}-ARs did not seem to play any role in cold-induced constriction [52] . Some reports pointed to the potential use of α_{2B}-AR antagonists as a treatment option for vasospasms in Raynaud's Disease [157]. Much to our surprise, we could not find a strong evidence that supports this assertion about α_{2B}-AR.

Of the α_2-AR subtypes, α_{2C}-AR was thought to be a vestigial receptor for two main reasons. The first is that α_{2C}-ARs are sequestered in an intracellular compartment [158], and thus their function was not easily detected by immunohistochemistry assays [157]. The second is that neither the α_{2C}-AR knockout nor the transgenic mice showed major changes; both remained viable, fertile, and almost normal [159]. On the other hand, other evidence emerged to argue against the apparent vestigiality of α_{2C}-AR. First, α_{2C}- ARs exhibit highly conserved domains present in other adrenoceptors [160]. Second, the apparently normal phenotype may be due to compensation by other α_2-ARs, and third, α_{2C}-ARs are differentially expressed in cells of different tissues [157].

One interesting and rather unique feature of its biology is that upon certain physiologic and pathophysiologic stimuli, α_{2C}-AR can translocate from the endoplasmic reticulum (ER) and Golgi apparatus to the cell membrane. This spatial rescue renders the receptor available for its ligand, whose binding then awakens the receptor function [52,53,152]. Upon moderate physiological cooling (i.e. 28 °C), α_{2C}-AR is mobilized from the ER/Golgi to the cell surface [55]. The now membrane-localized receptors can readily interact with their agonists, become activated and evoke cutaneous vasoconstriction in response to norepinephrine [53]. Indeed, it is now evident that the entirety of cold-induced constriction of cutaneous arteries is due to an increased activity of α_{2C}-ARs [55,155]. As such, α_{2C}-ARs appear to play an important role in the augmented vasoconstriction observed in RP [55].

The mechanism by which α_{2C}-AR translocation takes place involves different players such as reactive oxygen species (ROS), Rho/Rho kinase, and the actin cytoskeleton (figure 4). Bailey *et al.* reported that the Rho/Rho kinase pathway becomes activated as early as few minutes after cells get exposed to cold temperatures [55]. The now active Rho evokes the mobilization of α_{2C}-AR to the membrane, and consequently precipitates cold-induced

vasoconstriction [55]. In this sense, it seems that Rho, rather than α_{2C}-AR, is the "thermosensor" [55]. However, additional and rather elegant investigations from the Flavahan group further showed that the mitochondrion is the "thermo-sensitive" organelle in VSMCs [161]. Indeed, upon cold stress, it is the mitochondria that initiate the process by releasing ROS, which in turn triggers a redox signal that activates the Rho/Rho kinase pathway leading to a spatial and functional rescue of α_{2C}-ARs [161]. This cooling-induced Rho activation may then act through calcium sensitization or via modulation of cytoskeletal architecture [53,162,163].

2. RP and the Actin Cytoskeleton

The Cytoskeleton plays a major role in fundamental cellular processes like cell division, migration, cell-cell communication and protein trafficking [164]. The translocation of α_{2C}-ARs, a main player in RP, from the endoplasmic reticulum/ Golgi to the cell membrane of VSMCs is critical for their activation. This translocation involves many cytoskeletal components such as F-actin and actin/myosin filaments. It is through modulation of the actomysoin filaments that VSMC contraction and ultimately vasoconstriction occur.

Cold-induced, Rho-mediated architectural change occurs by virtue of a rearrangement of the actin superstructure evident by an increase in F-actin, a downstream effector of Rho kinase signaling [54]. Interestingly, immunocytochemical analysis show that α_{2C}-AR and F-actin appear to be co-localized [165]. In a rather elegant and orchestrated series of events, α_{2C}-ARs then get in close proximity and associate with actin filaments, readying themselves for the trafficking process [54]. This intimate association appears to be mediated by a direct interaction between α_{2C}-ARs and filamin-2, a cross-linker of actin filaments [166]. Indeed, further *in silico* protein-protein docking examinations confirmed that the interaction between α_{2C}-AR and F-actin occurs via the direct binding of α_{2C}-AR to filamin, the actin binding protein [167]. Interestingly, this interaction

has evolved only in warm blooded animals among all other animals [167]. Therefore, elucidation of similar protein-protein interactions can help establish more efficient therapies for exaggerated vasoconstriction. One scenario would include approaches that seek to disrupt the interaction between α_{2C}-AR and the cytoskeletal component, F-actin.

3. Raynaud's Disease and Estrogen:

Evidence from epidemiological studies reveals a rather interesting finding regarding the prevalence of RP. There is a significantly higher incidence of this disease in females versus age-matched males [45,46]. Indeed, 70% of all American patients suffering from Raynaud's disease are females [46]. Among patients affected with RP, the ratio of pre-menopausal females compared to age-matched males is close to 9:1 [47]. This clearly illustrates a gender-based element in the prevalence of the disease, and thus hints to a potential role of sex hormones in its onset or pathology [46]. Although it is reported that cardiovascular diseases in general are more prevalent in men and post-menopausal women [168], being a female is among the risk factors of RP [45]. This conclusion is partly based on a meta-analysis study asserting the much higher prevalence in females compared to males [45]. In particular, the incidence is higher in pre-menopausal versus post-menopausal women, with an interesting association between the menstrual cycle and cold-modulated digital blood flow [146]. Further analysis revealed that post-menopausal females receiving unopposed estrogen replacement therapy (ERT) are more likely to suffer from the disease than post-menopausal women that are not receiving ERT [169]. Together, these findings clearly demonstrate that estrogen may explain the higher incidence in premenopausal women (Table 1). Interestingly, in post-menopausal women receiving opposed estrogen therapy (estrogen and progesterone together), the incidence of RP was not significantly higher than that

in premenopausal women [50]. This may suggest that progesterone negates estrogen's effect in this context, but this remains to be established.

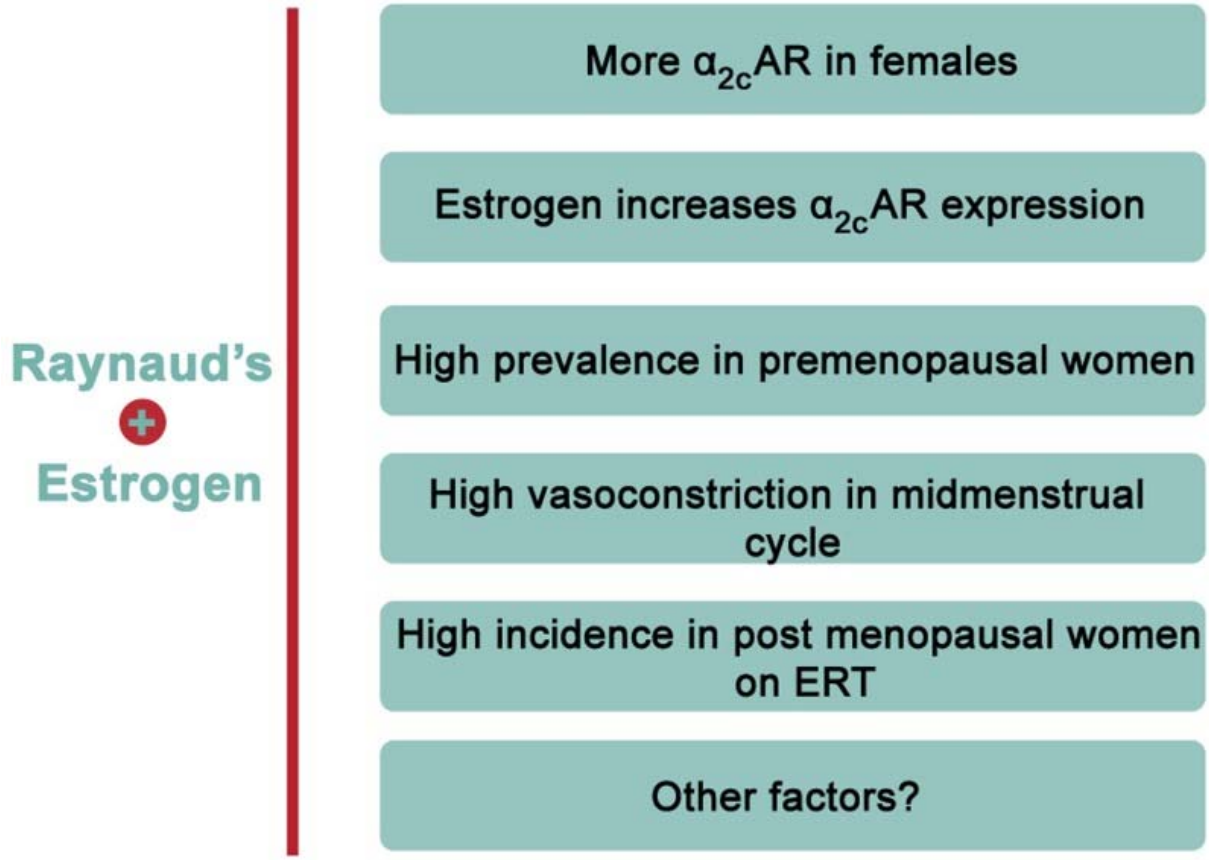

Table 2. Evidence of positive association between estrogen and RP.

Accumulating evidence points to an overwhelming association between estrogen and RP. For instance, estrogen increases α_{2C}-AR but not α_{2A}-AR in human arteriolar smooth muscle cells. Moreover, females have higher expression of α_{2C}-AR than males. Epidemiologically, RP is reported to have remarkably high incidence in premenopausal females or post-menopausal females on estrogen replacement therapy (ERT)

It is worth mentioning that in premenopausal females, noradrenaline-mediated vasoconstriction is higher at the mid-menstrual cycle, characterized by relatively high estrogen level, than during the early stage of the cycle [170]. Moreover, human and rat females of reproductive age exhibit higher vascular responsiveness than males [171]. Interestingly, male

vascular responsiveness is potentiated when 17β-estradiol is externally supplemented [171]. This implies that estrogen has a direct effect on vasoreactivity, though the mechanisms for such potentiation may be far from clear.

The fundamental role of estrogen in regulating body temperature has been defined [172]. Although estrogen has a vasodilatory effect, it may in many instances decrease body temperature [172]. Since RP can be considered a vascular thermoregulatory control disorder [173], the implication of estrogen in the disease becomes obvious especially in light of the exaggerated response to cold in premenopausal women as well as the higher prevalence of RP in younger females. This is further supported by the findings of English *et al.* that there is a gender difference in vasomotor activities in response to estrogen, and that this difference may be a critical contributor to the etiology of vasospastic diseases [174], such as RP.

Evidence indicates that estrogen increases α_{2C}-AR expression in vascular smooth muscle cells and that α_{2C}-AR-mediates cold-induced vasoconstriction in rat tail arteries [51]. A notable finding is that among the α_2-ARs, only the α_{2C}-AR subtype is differentially expressed in rat tail arteries, with a remarkably greater expression in females [175]. We had also reported that in human VSMCs, estrogen does not modulate the expression of α_{2A}-AR [51]. The Flavahan group had also established that α_{2C}-AR mediates the entirety of cold-induced vasoconstriction. We then hypothesized and later confirmed that estrogen indeed increases the expression and function of α_{2C}-AR [51]. This estrogen-induced activity of α_{2C}-AR was followed by a potentiated cold-induced vasoconstrictive response in mouse tail arteries [51]. Collectively, these pieces of evidence highlight a positive association between estrogen and RP.

4. Raynaud's and Genetics:

As mentioned earlier, RP is either idiopathic, or secondary to another disease like scleroderma. There have been some speculations that genetic predisposition may be a contributor to the onset of this disease [176] (figure 5). However, sequencing results showed no mutations in candidate genes that are suspected to play a role in the etiology of the disease [177]. These candidate genes are the beta subunit of the muscle acetylcholine receptor and the serotonin 1B and 1E receptors [177]. Nonetheless, others continued to suggest that there is a genetic factor contributing to the prevalence of this disease [178]. This assertion is supported by familial studies and twin analysis [178]. Recently, there was a reported case of a one-month male baby diagnosed with RP [179]. In light of this case, it was speculated that there could be a genetic basis of the disease. However, much evidence remains lacking before a strong causative link between genetics and RP can be affirmed.

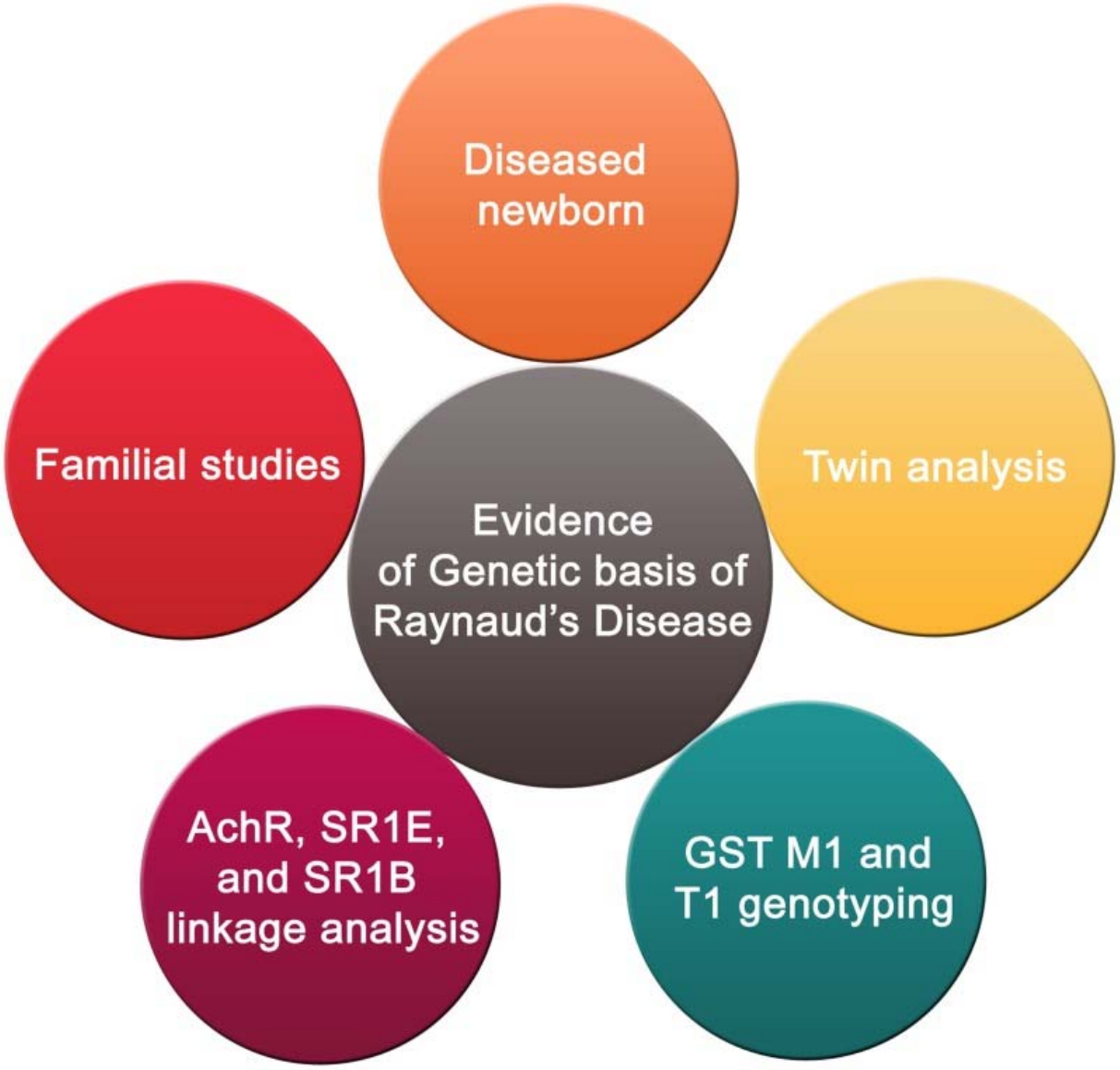

Figure 5. Genetic basis of RP.

The genetic basis of RP is supported by familial studies and twin analysis in addition to a reported case of a 1-month male baby diagnosed with the disease. Furthermore, a combination of positive genotypes for both genes encoding glutathione *S*-transferase M1 and T1 subtypes may have a role in susceptibility to RP. Linkage analysis pinpointed five areas corresponding to three candidate genes (β-subunit of muscle acetylcholine receptor, 1E and 1B serotonin receptors) which could be associated to RP.

Interestingly, studies of RP patients that were exposed to vinyl chloride monomer

(VCM) suggest that the interaction between a certain genetic background and environmental

conditions may play a role in increasing the onset of RP in VCM-exposed individuals [180]. In 2006, Fontana *et al* investigated whether there is an association between polymorphisms in glutathione S transferase M1 and T1 genes and RP patients exposed to VCM [180]. The results showed that the combination of positive genotypes for both genes may increase susceptibility to RP [180]. In another study, using 298 microsatellite markers, a 2-stage whole genome screen of six extended families having at least 3 RP patients in each family was undertaken [177]. Linkage analysis identified five chromosomal areas of possible linkage. These were mapped to three candidate genes (β-subunit of muscle acetylcholine receptor, 1E and 1B serotonin receptors) which could be associated with RP [177]. This provides evidence of a genetic basis for RP susceptibility. The fact that five possible linkages were highlighted indicates that RP may be an oligogenic rather than monogenic condition. However, more research is needed to ascertain this suggestion, since some of the findings reported may be false positives [177]. It would, therefore, be interesting to screen in a large pool of RP patients, for mutations or SNPs in these candidate genes.

5. Raynaud's and Toxicology:

Many of the heightened vasoreactivity responses observed in RP are due to either sympathetic or local causes. Stressors such as cold temperatures or emotional anxieties fall under the sympathetic category, since they cause vasoconstriction via noradrenaline. On the other hand, mechanical and chemical stresses fall under the "local" category since they directly affect a body organ that will show symptoms of the disease. A prominent body area that could be affected by these "local" insults would be the digits. Prolonged exposure to vibration at the level of the hand and arm is an example of mechanical stress. Also known as vibration-induced white finger, this hand-arm vibration syndrome is indeed one form of secondary RP that is due to occupational

hazards [181]. Continuous insults of the hand and arm by vibrating machines can prime these organs for increased vasospastic attacks upon a thermal or emotional stress. With the progression of this condition, such vibration can cause increased digital vasospasm even at room temperature [181]. Therefore, it is not surprising that vascular symptoms are highly prevalent among workers who manipulate vibrating tools [181].

One of the prominent examples of chemical stressors in Raynaud's phenomenon is vinyl chloride monomer. This monomer is a colorless gas used in the manufacturing of plastic, particularly poly vinyl chloride (PVC). Interestingly, almost one third of workers exposed to PVC suffer from RP [182]. Angiography of these patients' hands showed vascular tone changes and vascular lesions such as narrowing of the digital arteries [183]. This is not surprising since angiographic and cappilarsocopic examinations have shown that exposure to VCM is toxic for the endothelium [183,184]. Furthermore, exposure to VCM was shown to significantly contribute to acroosteolysis of distal phalanges of hands, which was recurrently associated with symptoms of RP [185]. Indeed, and as mentioned earlier, a higher prevalence of RP among French workers exposed to PVC was reported [180]. Taken together, these observations support the notion that a persistent toxic effect of polyvinyl chloride can contribute to the onset and pathogenesis of RP.

There are other chemical compounds or even medicinal drugs that are linked to the onset of RP. Some examples include Arsenic, nicotine and the drug Gemcitabine. Indeed, a positive correlation seems to exist between Arsenic and RP. A study in Chile shows that increased prevalence of signs and symptoms of peripheral vascular disease, including RP, are associated with Arsenic-contaminated drinking water [186]. Contextually, Arsenic-exposed smelter workers exhibit heightened vasospastic reactivity in the fingers, reminiscent of RP [187-189]. Together, these findings provide some evidence of Arsenic being a player in the etiology of RP.

Smoking has been long found to positively associate with RP [45]. Our ongoing studies further support the implication of cigarette smoke in cold-induced vasoconstriction (unpublished observations). Moreover, nicotine, one main constituent in tobacco, is known to significantly decrease blood flow and increase vascular resistance [190]. It is thus not surprising that nicotine can exacerbate symptoms of RP [191,192], so much so that avoiding nicotine has been suggested as one element in the treatment of RP of the nipple [138]. For example, Gemcitabine, a nucleoside analogue used in chemotherapy [193], was reported in some patients to evoke symptoms reminiscent of Raynaud's disease [144]. Indeed, when orally administered, it appears to cause pain, swelling, and whitening of the digits, all of which are typical of RP [193].

6. Treatment of Raynaud's Disease:

Significant efforts have been undertaken to better understand and treat RP [194,195]. However, no definite therapy for this disease has yet been approved by the U.S. Food and Drug Administration (FDA) [196]. One of the limiting factors in the war against this disease in the incomplete understanding of its pathophysiology [196], which is further compounded by the lack of appropriate animal models for RP. Despite that, some medications or treatment options that are thought to alleviate symptoms of the disease are being employed in the clinic. These options can be collectively classified into traditional pharmacological, ethno-pharmacological, non-traditional treatments, and most recently surgical intervention (Table 3).

	Treatment	Effectiveness	Reference
Traditional treatment	Calcium channel blockers	Effective; first-line of treatment	Halawa, 2001; Thompson and Pope, 2005
	PTK inhibitors	Efficient	Furspan et al., 2004, 2005
	PDE5 inhibitors	Inefficient	Lee et al., 2014
	Beta-blockers	Controversial	Marshall et al., 1976 Koltringer et al., 1991
	Statins	Emerging/powerful	Abou-Raya et al., 2008
	Prostacyclins	Efficient	Rademaker et al., 1987
	ACE inhibitors	Variable effect	Henness and Wigley, 2007
	Endothelin receptor antagonists	Variable effect	Poredos and Poredos, 2016
	Serotonin receptor antagonists	Effective	Coleiro et al., 2001
Surgery	Botulinum toxin type A	Efficient	Neumeister et al., 2014
	Chinese herb	Ineffective	Wu et al., 2008
	Ginkgo biloba	Ineffective	Muir et al., 2002
	Acupuncture	Efficient	Appiah et al., 1997
	Laser therapy	Efficient	Hirschl et al., 2004
Surgery	Thoracic sympathectomy	Effective	Coveliers et al., 2011
	Hand stripping	Effective	Balogh et al., 2002
	Nerve stimulation	Effective	Kaada, 1982
	Fat grafting	Encouraging results	Bank et al., 2014

Table 4. Various lines of treatment of Raynaud's disease.

Traditional pharmacological drugs alleviate RP symptoms by reducing vasoconstriction, inducing vasodilatory effect, or by a yet unclear mechanism. Drugs used for a vasodilatory effect include calcium channel blockers, cyclic guanosine monophosphate (cGMP)-specific phosphodiesterase type-5 (PDE5) inhibitors, prostacyclins, prostaglandin analogs, and alpha-1 blockers. Calcium-channel blockers are the most common first-line treatment [197,198]. Cyclic guanosine monophosphate (cGMP)-specific phosphodiesterase type-5 (PDE5) inhibitors have been used as well [199]. PDE5 inhibitors decrease vasospastic attacks and improve digital blood flow [194].

Vasodilation-inducing prostacyclins are reported to decrease the number of cutaneous lesions in RP patients [200]. Moreover, ACE (Angiotensin Converting Enzyme) inhibitors, PTK (protein tyrosine kinase) inhibitors and endothelin-receptor antagonists are utilized in the treatment of RP owing to their ability to reduce vasoconstriction. The therapeutic benefit of using ACE inhibitors in the management of RP seems to be variable [201]. Some studies have reported that they may have minor benefits albeit to a lesser extent than traditional therapies [202]. The increased phosphorylation of PTK is associated with the α_{2C}-AR-mediated vasoconstriction, thus PTK inhibitors are used to reverse the contractile response to cooling [203,204] . When endothelin receptor antagonists were employed, not all patients responded positively; nonetheless, these antagonists were able to at least alleviate the severity and frequency of vasospastic attacks [195].

Whether beta blockers have a therapeutic value remains controversial. While some argue that they disturb the microcirculation causing Raynaud's disease as a side effect [205], or that their use is associated with higher incidence of RP [206], others report that beta blockers could be beneficial particularly because of their ability to reduce blood viscosity [207]. Interestingly, a recent study showed that a combination treatment of beta blockers with calcium channel inhibitors is very effective in reducing Raynaud's disease symptoms [208].

There are other drugs that appear to have a potential for use in the management of RP. These include statins [209] and serotonin receptor antagonists [210] . Although their mechanism of action is not fully clear, they appear to retard vascular injury, lessen severity and reduce pain associated with RP.

A recent report discussed the potential benefit of using a rather non-traditional approach for the treatment of RP. Botulinum toxin type A can be locally injected to improve ulcerated

digits and alleviate the associated pain [211]. This improvement may be due to better perfusion and improved vascularity; however, the exact mechanism remains unknown. Notably, studies have shown that the use of botulinum toxin type A is safe and could be somewhat efficient [211].

It is worth mentioning that in some patients, the aforementioned pharmacological drugs may cause several side effects such as headaches and dizziness. As such, many patients resort to alternative therapies in the hope of avoiding such undesired side effects. Herbal therapies are one common approach. Of particular interest in the management of RP is Ginkgo biloba plant extracts [212] or a combination of two Chinese herbal medications, Duhuo-Tisheng Tang and Danggui- Sini Tang [213] . However, contradictory reports suggest that that digital vascular response of Raynaud's patients receiving this therapy was not changed in patients consuming the above herbal combination [213] . It has also been suggested that acupuncture and laser therapy can be employed in the treatment of RP, as they were shown to decrease the frequency and severity of digital vasospastic attacks [214,215].

It is important to note that surgical therapies may be considered as an option of treatment [196]. These therapies include thoracic sympathectomy, hand stripping, and nerve stimulation [216-218]. Although invasive, these are considered to be successful in pain reduction and ulcer healing [196]. Finally, fat grafting in the patient's hands is a new and rather unconventional surgical therapy for RP patients [219]. This novel treatment originated from clinical improvements observed after fat grafting in hands suffering from burns and radiation dermatitis [220] . When it was later “tested” on a group of RP patients, the results were encouraging and included alleviation of pain, decrease of ulcers, and decline in cold attacks [219]. Although the mechanism by which fat grafting caused these effects is largely unclear, it is hypothesized that pathways involving neoangiogenesis and stem cells are likely implicated [219] .

The variability of the treatments and their altered efficacies calls for urgent and concerted efforts to better understand the molecular mechanisms underlying the disease, as well as to develop more targeted and efficient drugs. These drugs may include blockers of α_{2C}-ARs as well as inhibitors of protein tyrosine kinases and Rho-kinase [221].

7. Conclusions and Perspectives:

Despite the exponentially growing research and biomedical advances, a treatment for RP still poses a real and elusive challenge. The molecular mechanism underlying RP is still not fully elucidated due to the multifactorial etiology (hormonal, neuronal and endothelial) of the disease. Another challenge is the absence of an appropriate animal model of the disease. The fact that α_{2C}-AR is expressed in many brain regions such as the olfactory bulb and the cerebral cortex further complicates the hunt for an RP-specific drug. This is especially challenging because α_{2C}-ARs are also implicated in presynaptic regulation of the heart. Thus, targeting α_{2C}-ARs in an attempt to treat RP would not be most suitable, since it will affect the heart and brain as well. However, it is tempting to speculate that applying topical creams containing α_{2C}-ARs blockers to affected body parts could be beneficial, and likely with fewer side effects. However, rigorous basic research and clinical trials are needed to support this suggestion. So far, precaution remains the best option to avoid some of the complications of RP, especially that there is no approved therapy for the disease.

C. The Hypertensive Potential of Estrogen: An Untold Story

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Cardiovascular disease (CVD) is the major cause of morbidity and mortality worldwide. The implication of estrogen in this disease has been extensively studied. While the vast majority of published research argue for a cardioprotective role of estrogen in vascular inflammation such as in atherosclerosis, the role of estrogen in hypertension remains far from being resolved. The vasorelaxant effect of estrogen has already been well-established. However, emerging evidence supports a vasoconstrictive potential of this hormone. It has been proposed that the microenvironment dictates the effect of estrogen-induced nitric oxide synthase-1 (nNOS) on vasotone. Indeed, depending on nNOS product, nitric oxide or superoxide, estrogen can induce vasodilation or vasoconstriction, respectively. In this review, we discuss the evidence supporting the vasorelaxant effects of estrogen, and the molecular players involved. Furthermore, we shed light on recent reports revealing a vasoconstrictive role of estrogen, and speculate on the underlying signaling pathways. In addition, we identify certain factors that can account for the discrepant estrogenic effects. This review emphasizes a yin-yang role of estrogen in regulating blood pressure.

1. Introduction

Cardiovascular disease (CVD) continues to be the world's leading cause of debility and mortality [222]. According to the World Health Organization (WHO), CVD accounts for 31% of annual global deaths [223]. Common risk factors for CVDs include smoking, unhealthy diet, diabetes mellitus, hyperlipidemia and hypertension [224]. The latter is considered a major contributor to CVD-associated morbidity and mortality [225].

The American College of Cardiology and American Heart Association (ACC/AHA) define hypertension as systolic blood pressure (SBP) above 130 mm Hg or diastolic blood pressure (DBP) above 80 mm Hg [226]. It affects 1.13 billion people and accounts for 9.4 million deaths per year [223], with an expected increase in prevalence by 30 percent by 2025 [227]. Hypertension is more common in low- and middle-income countries [223], where access to healthcare is limited and lifestyle facilitates its development [228].

Hypertension is mostly asymptomatic [229], but it sets the stage for several debilitating diseases. These include CVDs, cerebrovascular accidents (CVAs), retinal vascular disorders and hypertensive renal disease [230], most of which are associated with high mortality and morbidity [231]. Thus, it is no surprise that hypertension is referred to as the 'silent killer' [229]. Hypertension induces arterial remodeling, where small vessels undergo wall thickening and lose their elasticity in a process called arteriosclerosis, leading to the so termed 'target organ damage' [232]. In brain arteries, arteriosclerosis narrows the lumen and hardens the vessel wall, resulting in ischemic or hemorrhagic stroke [233]. It also damages coronary vessels, which can progress to a myocardial infarction (MI) [234]. Renally, arteriosclerosis stiffens the nephron, also called nephrosclerosis [235], impeding filtration, which in turn leads to electrolyte imbalances and volume overload [236]. Hypertensive retinopathy is another manifestation of arteriosclerosis, in which retinal vessels are damaged, causing blurry vision and even blindness [237]. Moreover, these vessels can leak and allow fluid to build up behind the retina, resulting in retinal detachment and acute blindness [238].

17β-estradiol (E_2), estrone (E_1) and estriol (E_3) constitute an endogenous group of sex steroid hormones called estrogens [239]. E_2 is the predominant and most biologically active form [239]. It executes its physiologic effects via two members of the nuclear receptor superfamily,

estrogen receptor-alpha (ER-α) and -beta (ER-β) [240]. Both receptors are structurally similar but differ in their DNA-binding and ligand-binding domains, leading to different transcriptional activation programs [241]. E_2 binding to and signaling through ER homo- or heterodimers elicit a genomic effect. Additionally, they activate several signal transduction pathways, such as extracellular signal-regulated kinases 1 and 2 (ERK1/2), p38 mitogen-activated protein kinase (p38 MAPK) and phosphoinositide 3-kinase-serine/threonine-specific kinase B (PI3K/AKT) [242]. E_2 can also rapidly mediate non-genomic actions by activating plasma membrane bound estrogen receptors, called G protein-coupled estrogen receptor (GPER, formerly known as GPR30)[51,243]. GPER can activate multiple downstream signaling cascades, like PI3K/AKT and MAPK [244,245]. Historically, E_2 has been described as a vasorelaxant [246-248], but recent observations argue for a vasoconstrictive effect of the hormone. In this review, we highlight the emerging role of estrogen as a vasoconstrictive agent. We also speculate on the molecular players that may be involved in the underlying mechanism.

2. Vasorelaxant Effects of Estrogen

The vasoprotective role of estrogen has been extensively documented. As a result, prior to menopause, females have less incidence of CVDs than age-matched men [249]. The cardioprotective sexual dimorphism is evident in many cardiovascular disorders. Indeed, when compared to men, premenopausal women show better endurance to ischemia and reperfusion during open-heart surgery [250,251]. In addition, females show significantly more adaptive cardiac remodeling in response to aortic stenosis, while males show upregulated inflammatory and fibrotic markers and genes [252].

With regards to blood pressure (BP), a lower incidence of hypertension is observed in premenopausal women [253,254]. With aging, BP increases in both sexes, but postmenopausal women have a more abrupt increase [255], encompassing 75 percent of women above 60 [256]. Accordingly, older women tend to have a higher BP than men of similar age group [255]. This discrepancy between the BP of pre- and postmenopausal women prompted scientists to focus on the potential benefits of estrogen in the context of hypertension. In the context of hypertension, estrogen's hypotensive role has been classically attributed to the following observations (figure 6):

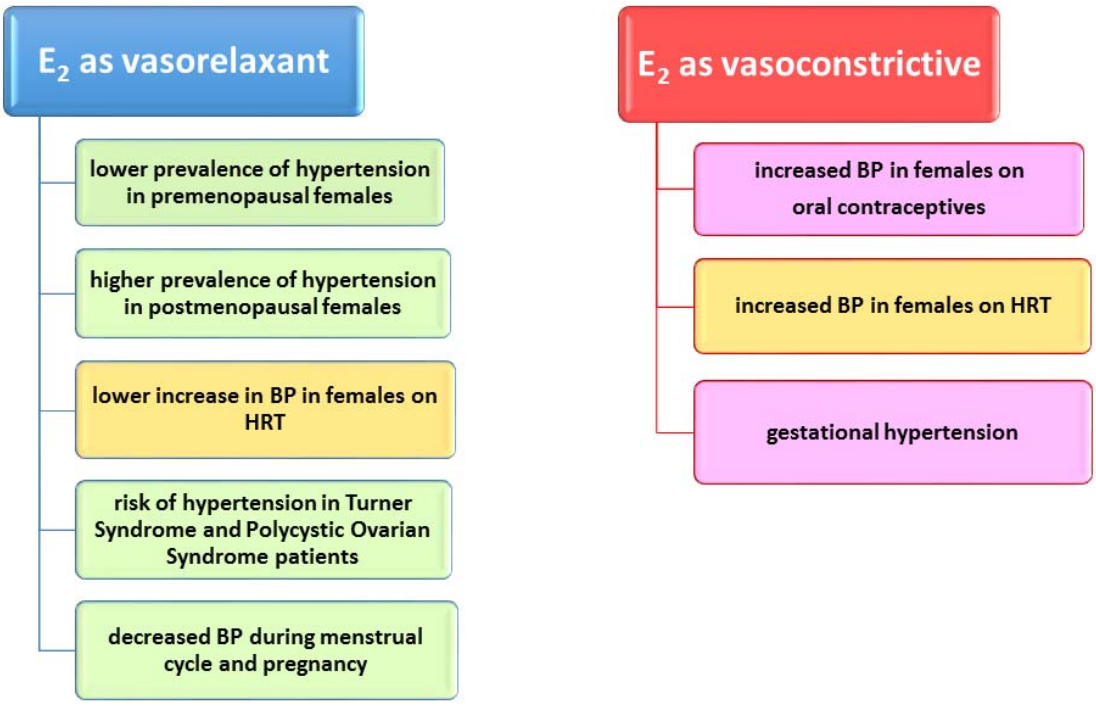


Figure 6. Various clinical observations supporting either the vasorelaxant or the vasoconstrictive effect of estrogen.
Several observations support the well-established role of estrogen. These include lower prevalence of hypertension in premenopausal females and higher prevalence of the disease in postmenopausal females. Decreased blood pressure in during menstrual cycle and during the first two trimesters of pregnancy further support a vasorelaxant role of the hormone. In addition, Turner Syndrome and Polycystic Ovarian Syndrome patients, characterized by low estrogen level, are at higher risk of hypertension. Elevated blood pressure in females on oral

contraceptives and pregnancy-induced hypertension support a vasoconstrictive role of estrogen. Blood pressure in post-menopausal females on ERT is still a matter of controversy.

a. Hypertension in females as compared to males

The prevalence of hypertension is generally lower in premenopausal women compared to age-matched men [253]. It was reported that the average SBP and DBP in males under 60 is higher than females by 6-7 and 3-5 mm Hg respectively [257-259]. This gender-related difference was traditionally correlated to the difference in blood estrogen concentration between the two groups [255,258]. In this respect, estrogen was labeled as hypotensive.

Interestingly, sexual dimorphism in BP is observed in animals as well. For instance, male rats, including Dahl salt-sensitive rats [260,261], deoxycorticosterone acetate-salt hypertensive rats [262], spontaneously hypertensive rats and New Zealand genetically hypertensive rats have higher BP as compared to their corresponding female rats [263-266].

On the other hand, the incidence of CVDs is higher in postmenopausal women when compared to age-matched men [253,267]. While this may appear contradictory to the notion that women are generally at lower risk of dying from hypertension-related CVDs [268], it is noteworthy to mention that these "women" included both pre- and post-menopausal groups, i.e. estrogen-replete and deplete subjects, respectively. Indeed, 41% of postmenopausal women are at risk of being hypertensive [269], with a similar DBP to age-matched men, but higher SBP [270].

b. Hypertension in premenopausal versus postmenopausal females

Conventional and ambulatory evaluations of BP in women have shown that postmenopausal women have an SBP higher by 4-5 mm Hg compared to premenopausal and perimenopausal women [271-274]. However, a recent study reported that the values of SBP and DBP

are significantly higher in postmenopausal females as compared to premenopausal ones [275]. Cross-sectional studies have also shown that menopause, including surgical menopause [276], increases the risk of hypertension by 2-fold even after adjustment for age and body mass index (BMI) [277,278]. These observations confirm a correlation between serum estrogen level and hypertension in females. This is in addition to reports showing that postmenopausal women show a non-dipping pattern of BP. Normally, a 10% circadian decrease in BP is observed between day and night, a decrease referred to as BP dipping. Postmenopausal women, however, tend to show less than 10% decline in their nocturnal BP [279].

c. Hypertension in postmenopausal females on hormone replacement therapy (HRT)

The effect of HRT on CVDs remain controversial, as this effect mainly depends on the HRT preparation and HRT initiation time since menopause [280]. However, many observational studies established that postmenopausal women on HRT have a lower rate of CVDs than those who are not [281]. This can be partially explained by the smaller increase in SBP in females on HRT compared to their counterpart [282,283] . It was also reported that postmenopausal women administered with E_2 have lower daytime and nocturnal SBP and DBP when compared to placebo-treated women [284]. A recent meta-review has shown that transdermal estrogen has a beneficial effect on BP of postmenopausal females [285].

d. Hypertension in females with Turner syndrome and polycystic ovarian syndrome (PCOS)

In addition to menopause, there are other cases where a decrease in estrogen levels may be associated with hypertension. Females with Turner syndrome or PCOS, both of which are characterized by low estrogen levels [286,287], are at higher risk of developing hypertension [286-289].

Moreover, a study has shown that there is a negative correlation between estrogen and DBP in females with Turner Syndrome [290]. This implies a beneficial effect of estrogen on BP in these patients.

e. Blood pressure during menstrual cycle and pregnancy

The BP-lowering potential of estrogen can also be inferred from its effect during the menstrual cycle and pregnancy. In the menstrual cycle, BP is lower during the luteal phase, when estrogen levels peak, than in the follicular phase when these levels drop [291,292]. In pregnancy, BP decreases during the first and the second trimesters [293], in concomitance with an increase in estrogen [294].

3. Estrogen's Vasorelaxant Direct Effect on Arterial Wall: Signaling Molecules

Estrogen grants protective effects to the cardiovascular system, particularly to the vasculature. Whereas the anti-inflammatory role of estrogen in atherosclerosis has been well established [295,296], the role of estrogen in hypertension is beginning to be cemented. Emerging evidence from experimental and clinical research points to both vasodilatory and vasoconstrictive effects of estrogen.

E_2, the most active endogenous estrogen in premenopausal women, has been documented to induce vasodilation through several molecular pathways [297]. While estrogen's hypotensive effect is mainly through the release of vasodilators from the endothelium [298], it can also lead to vasculature relaxation in an endothelium-independent manner [299]. A myriad of agents are responsible for estrogen's vasodilatory effects. Below we discuss the most important ones (figure 7).

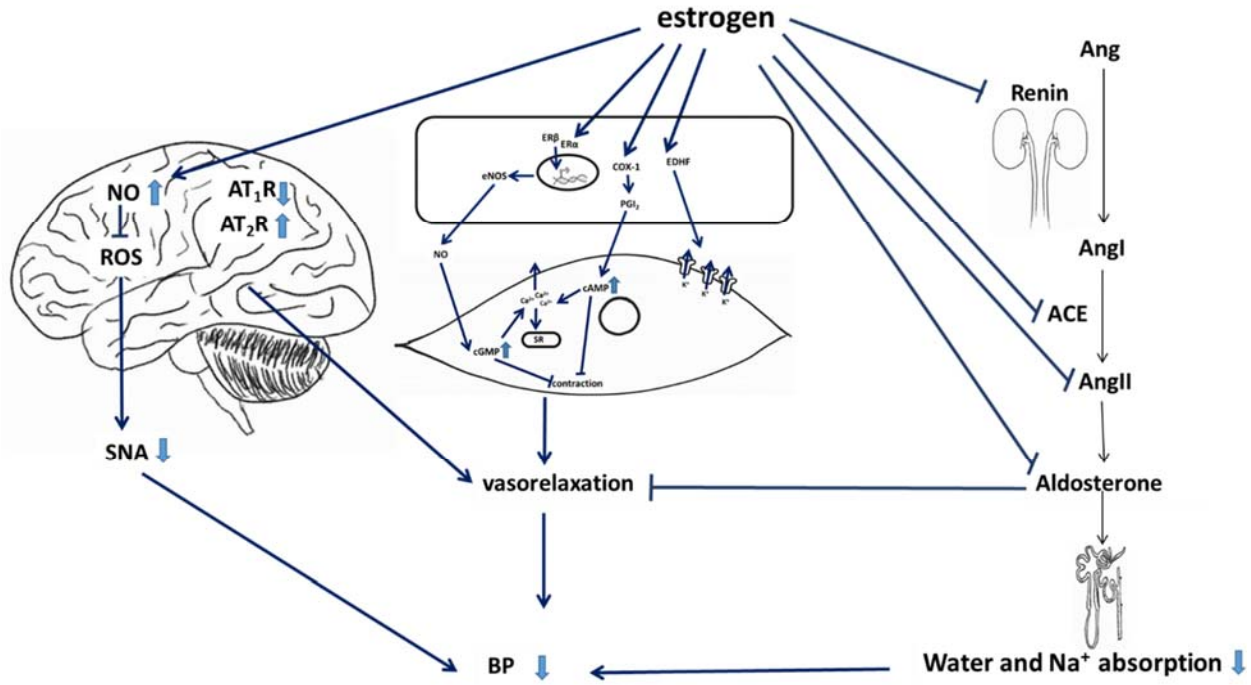


Figure 7. Signaling pathways mediating estrogen-induced vasorelaxation.

Estrogen works on three different lines to elicit its hypotensive effect. First, it decreases the sympathetic nervous action (SNA) by increasing NO production and AT_2R expression. In addition, estrogen attenuates AT_1R expression and ROS production. At the level of the RAAS, estrogen Renin and ACE activities, leading to decreased AngII and aldosterone levels. Estrogen also attenuates water and sodium absorption in the kidneys. At the level of blood vessels, estrogen binds to its receptors on endothelial cell, leading to eNOS transcription, and thus NO release. In addition, estrogen stimulates COX-1-mediated PGI_2 release, which decreases intracellular Ca^{2+}. In addition, estrogen induces EDHF release, causing K^+ through their corresponding channels. These events lead to membrane hyperpolarization and consequently vasorelaxation.

a. Nitric Oxide

Nitric oxide (NO) is one of the most well-studied and canonical estrogen-induced vasodilators [300]. Indeed, it has been demonstrated that inhibiting nitric oxide synthase (NOS) attenuates the vasodilatory effect of E_1 in phenylephrine-contracted Wistar rat aortic ring [301].

Similarly, another study shows that E_2 induces vasorelaxation in porcine coronary arteries through NO [302]. E_2 was reported to increase endothelial nitric oxide synthase (eNOS) activity in ovariectomized diabetic rat model [298]. It also upregulates eNOS expression via cytosolic ER-mediated genomic effect or membranous ER signaling and the consequential activation of the PI3K/cyclic adenosine monophosphate (cAMP) pathway [295,300,303]. In addition to being a vasodilator, this cAMP exerts many biological effects on arteriolar smooth muscle cells [54,155,166,304,305].

Further evidence, however, shows that estrogen's vasodilatory effect can be elicited in the absence of the endothelium as well, supporting a direct effect of estrogen on vascular smooth muscle cells (VSMCs). Indeed, estrogen can cause production of NO in VSMCs themselves [306]. This is attained mainly via lowering VSMCs' intracellular levels of calcium (Ca^{2+}), which can be the result of diminishing Ca^{2+} influx or stimulating Ca^{2+} efflux [306]. The VSMC-produced NO, in turn, stimulates potassium (K^+) channels, whose opening further leads to decreased intracellular Ca^{2+}, further propagating vascular relaxation [307]. Darkow *et al*. show that estrogen can induce the relaxation of endothelium-denuded porcine coronary arteries through local production of cyclic guanosine monophosphate (cGMP) and NO, which then open large-conductance Ca^{2+} and voltage-activated K^+ channels (BKCa) [308].

However, NO appears to be dispensable for estrogen-mediated vasodilation in several cases. For example, a study shows that NO inhibition does not affect estrogen-induced relaxation of resistance sized mesenteric arteries in spontaneously hypertensive rats (SHRs) [309]. Additionally, estrogen was able to induce vasorelaxation of de-endothelialized serotonin-precontracted male rat aortic strips via the inhibition of serotonin-induced Src kinase, which is NO-independent [299].

b. Carbon Monoxide

In addition to NO, carbon monoxide (CO) is considered a potent endothelial-derived vasorelaxant. The role of CO in estrogen-induced vasorelaxation was reported by Pósa *et al* [310]. This group showed that heme oxygenase-generated CO contribute to estrogen-induced vasodilation of ovariectomized rat aorta. They also demonstrate that estrogen induce vasorelaxation in a hydroxide (HO^-)–CO–NO signaling pathway [310].

c. Cyclic Nucleotides

Inhibition of cAMP and cGMP production has no effect on E_2-induced vasorelaxation of mesenteric arteries of SHRs [311]. However, in response to estrogen, cGMP plays a key role in the relaxation of porcine coronary artery [308], including endothelial-denuded ones [312]. Moreover, in endothelin-1 (ET-1)-stimulated aortic rings of Wistar rats, estrogen causes relaxation via a mechanisms that depends on guanylate cyclase (GC) [301]. Additionally, cAMP bridges GPER-mediated relaxation [313], especially in porcine coronary artery [314]. It is proposed that cAMP activates both protein kinase A (PKA) and exchange protein activated by cAMP (EPAC) in a synergistic manner to dephosphorylate myosin light chain (MLC) and induce vascular relaxation [314]. However, further experimentation is warranted to better elucidate the interplay between EPAC and PKA in modulating estrogen's cardiovascular effects.

d. Mitogen-activated Protein Kinases

MAPKs are key signaling molecules involved in a plethora of cellular processes. However, their role in estrogen-induced vasorelaxation is still not fully elucidated. Two recent

studies show discrepant results in this respect. The vasorelaxant effect of E_1 on rat aortic rings was not affected by ERK1/2 or p38 inhibition [301]. This indicates that MAPKs do not play a role in estrogen-induced vasorelaxation. It is reported, however, that inhibiting ERK1/2 potentiates ER agonist-induced relaxation of ET-1-constricted porcine coronary artery [314]. This suggests that estrogen inhibits ERK1/2 to achieve part of its vasodilatory effect. On the other hand, a study has shown that ERK1/2 mediate the rapid, non-genomic vasorelaxation of E_2 and ER-α agonist [315].

e. Phosphoinositide 3-Kinase-serine/threonine-specific Kinase B

The role of PI3K in vasorelaxation is well-established. In estrogen-induced relaxation, De Oliveira *et al*. report that E_1 induces relaxation of phenylephrine-contracted rat aortic rings in PI3K/Akt-dependent manner [301]. Particularly, PI3K/Akt activate eNOS, leading to the release of endothelial NO, which in turn increases vascular cGMP and leads to vasorelaxation [301]. This pathway has been shown in action in response to an estrogen-receptor agonist in phenylephrine-contracted resistant mesenteric arteries too [316].

f. Protein Kinase G

The role of protein kinase G (PKG) in mediating the effects of estrogen remains controversial. PKG is activated by the second messenger cGMP, which is produced by soluble GC [317]. The interaction between E_2 and PKG has been investigated for years. Earlier studies showed that E_2-induced vasorelaxation of mesenteric arteries in SHRs was not affected by cGMP or PKG inhibition [311]. However, these findings are yet to be conclusive, especially that PKG was reported to be a key mediator of estrogen-induced arterial vasodilation in ApoE-/- mice [318]. In fact, it has been demonstrated that estrogen activates PKG, which in turn stimulates the

production of hydrogen sulfide (H_2S), a potent vasodilator that decreases BP [311,319]. Further studies are thus warranted to conclusively determine the interplay between estrogen and PKG in regulating vasotone.

4. Estrogen's Vasorelaxant Effect: Roles of the Renin-Angiotensin-Aldosterone System (RAAS) and the central nervous system (CNS)

a. Effect on RAAS

Vasoconstriction, and by extension BP, are regulated by more than just local metabolites. A vital component of the recipe for BP maintenance is the RAAS. RAAS involves renin, an enzyme released by the renal juxtaglomerular apparatus, and angiotensinogen (AGT), a protein synthesized in the liver. Renin proteolyzes AGT to angiotensin I (Ang I), which in turn is activated by angiotensin converting enzyme (ACE), that is found in lungs and kidneys, to angiotensin II (Ang II) [320]. Ang II binds to Ang II type 1 receptor (AT1R), resulting in a cacophony of physiologic changes that eventually lead to elevation in BP [320]. These physiologic changes include vasoconstriction, sympathetic nervous system (SNS) stimulation, increased sodium reabsorption at the nephron and the release of aldosterone and anti-diuretic hormone (ADH) from adrenal and pituitary glands respectively [320-322]. Moreover, prolonged activation of AT1R is associated with pathologic processes, like arteriosclerosis and atherosclerosis [323].

The interaction between estrogen and RAAS is intricate. An initial glance at the literature suggests a RAAS-activating role of estrogen. This is inferred from postmenopausal women who have a less responsive RAAS to orthostatic stress than premenopausal females [324], and the decreased expression of AT1R and ACE in ovariectomized animals [325,326]. Additionally, estrogen has been shown to increase plasma renin activity, AGT, Ang I and Ang II [324,325,327,328].

Nevertheless, estrogen seems to have a hypotensive role through RAAS. Indeed, evidence shows that despite increasing RAAS substrates, E_2 downregulates AT1R and stimulates the expression of Ang II type 2 receptor (AT2R) instead [325], which has vasorelaxant and natriuretic effects [323,325], both of which drop BP. E_2 also induces renal and cerebral expression of angiotensin converting enzyme 2 (ACE2) [325,329], which catalyzes the conversion of Ang II to angiotensin 1-7 (Ang 1-7) [330]. Ang 1-7 is a peptide that causes vasodilation and natriuresis renally [323], and attenuates SNS centrally [329], reducing BP. On the other hand, hypoestrogenism in menopause causes increased AT1R to AT2R ratio [323,325], hence the observed postmenopausal hypertension.

b. Effect on CNS

Activation of the SNS increases CO and TPR and acutely raises BP; however, the role of the SNS in the long-term control remains under investigation [331]. Joyner *et al.* investigated the relationship between SNS and chronic BP maintenance. Their work showed a proportional relationship between SNS and TPR of young men, tapered by an inversely proportional relationship with CO and vascular adrenergic insensitivity. They also demonstrated that with age, men's BP increases alongside an increase in sympathetic neuronal activity (SNA). Interestingly, premenopausal women showed no relationship between their BP and SNA due to the notion that β_2-adrenergic receptor (β_2-AR)-mediated vasodilation makes this relation; this trait is lost after menopause [332]. Consequentially, postmenopausal women ended up with both an increased SNA and a stronger association between elevated BP and SNS than in men [332]. This result goes in concordance with Chobanian *et al.*'s work, in which postmenopausal females had higher SBP than age-matched men [270]. Furthermore, the loss of vasodilation by β_2-AR activation post menopause indicates a substantial role for estrogen in this mechanism. This is confirmed by

other studies showing that low-dose E_2 reduced sympathetic effect on vessels and by extension BP in rats with postmenopausal hypertension [333]. This is also in accordance with other reports indicating that 17β-estradiol-evoked vasodilation in renal and mesenteric arteries is diminished and may be masked by β_2-AR-mediated vasodilation [334].

The extent of estrogen's influence on SNS goes beyond regulating its effect on vascular tone. Centrally, SNA is controlled by several cerebral regions, like the subfornical organ (SFO), paraventricular nucleus (PVN) and the rostral ventral lateral medulla (RVLM), all of which express ERs [335]. Intracerebroventricular infusion of propyl-pyrazole-triol, a selective ER-α agonist, ordiarylpropionitrile, a selective ER-β agonist, alleviated hypertension in ovariectomized rats [336]. In addition, silencing these receptors in the cerebroventricular region augmented hypertension in intact female rats. These results confirm a central hypotensive role of estrogen mediated by both ER subtypes the brain regions that control BP. Subsequent studies have localized ER-β to PVN and RVLM and ER-α to SFO [336,337], but there is a need for further investigation into the pathways each ER subtype indwells.

5. Vasoconstrictive Effect of Estrogen

Emerging evidence supports a new and rather surprising effect of estrogen, a hypertensive effect. This estrogenic action was principally inferred from the rise of BP in premenopausal women on contraceptives and postmenopausal women on HRT [338-342]. The positive association between hypertension and the third trimester of pregnancy, an estrogen-rich phase, further underpinned the hypertensive role of estrogen [343-345].

Several subsequent reports further validated this effect in animal models. Long-term estrogen exposure lead to increased BP in mice [346]. Similarly, long-term administration of Envoid, an oral contraceptive, significantly increased the BP of ovariectomized female rats [347]. This was the first *in vivo* evidence showing the effect of contraceptives on hypertension. Another study by Fowler *et al*., confirmed that it is estrogen in contraceptives that is responsible for BP elevation [348]. Later, Byrne *et al*., showed that administration of ethynylestradiol, a synthetic derivative of E_2, to female Sprague-Dawley rats induced an increase in BP [349]. In addition, Lew *et al*., showed that estrogen administration to rats increased their BP by 4 percent [350]. *In vitro* experiments further supported the hypertensive estrogen effect. Estrogen (10–100 nmol/L) was reported to induce contraction of rat aortic VSMCs [351,352]. Also, estrogen increased contractility of VSMCs from Ephrin-B3 knock-out mice [353]. This was confirmed *in vivo*, where ovariectomy lead to normal BP of EFNB3 knock-out female mice [353]. These studies mostly showed a slight but significant increase in BP (1.5- 2 mm Hg). However, this slight increase is dangerous since it is associated with the development of serious cardiovascular events [354,355], including coronary atherosclerosis [356].

The emerging vasoconstrictive potential of estrogen stems from the following observations (figure 6):

a. Hypertension in women on oral contraceptives

Women on oral contraceptives are at a higher risk of developing hypertension [357,358]. In fact, these women showed an increase in both SBP and DBP [348,359-361]. Notably, progesterone in contraceptive pills does not contribute to BP elevation [362-364], implying that it is estrogen in oral contraceptives that exerts their hypertensive effect. This hypertensive effect appears to be

estrogen dose-dependent [365,366], since newer preparations of oral contraceptives that have lower estrogen content are associated with lower elevations in BP [258,339]. Conversely, women using preparations containing more than 50 μg of estrogen have a BP greater than 140/90 mm Hg [367]. It is worth mentioning that upon cessation of estrogenic contraceptives, women returned to normal BP [368-370].

b. Hypertension in women on hormone replacement therapy

Postmenopausal women resort to HRT to compensate for the lost estrogen beneficial effects. Indeed, estrogen is assumed to be responsible for reduced cardiovascular events such as aortic stenosis, fibrosis, and ischemia/reperfusion (I/R) injury [300]. However, several studies showed that HRT can lead to an increase in BP in postmenopausal females [341,354], even after adjusting other factors [268]. Specifically, increased SBP was observed in postmenopausal females receiving estrogen plus progestin and estrogen-only hormonal preparations [342,371].

It is worth mentioning that the endothelial integrity of small vessels is compromised in postmenopausal woman [372], which may affect estrogen-induced vasodilation. In addition, menopause-related arterial stiffness favors hypertension [373]. These factors, in addition to the route of HRT, whether oral or transdermal [374], hormonal preparation and method of study may account for the discrepant results of reports assessing the effect of HRT on BP [375].

Observations on the effect of HRT on BP remain controversial. While we earlier discussed the BP-lowering or rising effect of HRT, other significant studies show that HRT does not really have a profound effect on BP, regardless of its formulation. The Women's Health Initiative (WHI) is the largest (16,000 women) randomized, placebo-controlled trial that has evaluated the effect of postmenopausal HRT on cardiovascular outcomes [341]. It reported that at

5.2 years, HRT produced only a small mean increase (1.5 mmHg) in systolic pressure compared with placebo [341]. In the Postmenopausal Estrogen/Progestin Interventions (PEPI) trial, use of ERT with or without progestins did not affect BP at three years among 875 healthy postmenopausal women [376]. In the Kronos Early Estrogen Prevention Study (KEEPS), neither of the two different formulations of HRT evaluated produced any significant change in BP or endothelial function among recently postmenopausal women without high BP at baseline [377].

c. Hypertension in pregnant women

Pregnancy-induced hypertension is detected after 20 weeks of gestation and resolves within 12 weeks of delivery [378]. It includes gestational hypertension, preeclampsia (gestational hypertension plus proteinuria) and eclampsia (seizure during or within 24 hours of delivery in preeclamptic woman) [378,379]. Studies show that preeclampsia affects 3 to 7 percent of pregnant women [380], while gestational hypertension takes in almost 6 percent of pregnancies [381]. Since pregnancy is characterized by high estrogen levels, especially towards the last trimester [294,382,383], it may be postulated that increased estrogen levels are associated with gestational hypertension. In fact, estrogen levels increase to their maximum in the last trimester [294], during which the ambulatory blood pressure (ABP) reaches its highest level throughout pregnancy [293].

6. Mechanisms of Estrogen-Induced Vasoconstriction

The mechanism by which estrogen induces vasoconstriction is not fully elucidated yet. However, several research groups proposed possible pathways by which estrogen can induce high BP. These pathways include central actions of estrogen directly on certain brain regions,

estrogen modulation of the RAAS system, modulation by calcium channels, or phosphorylation of myosin light chain (MLC) (figure 8).

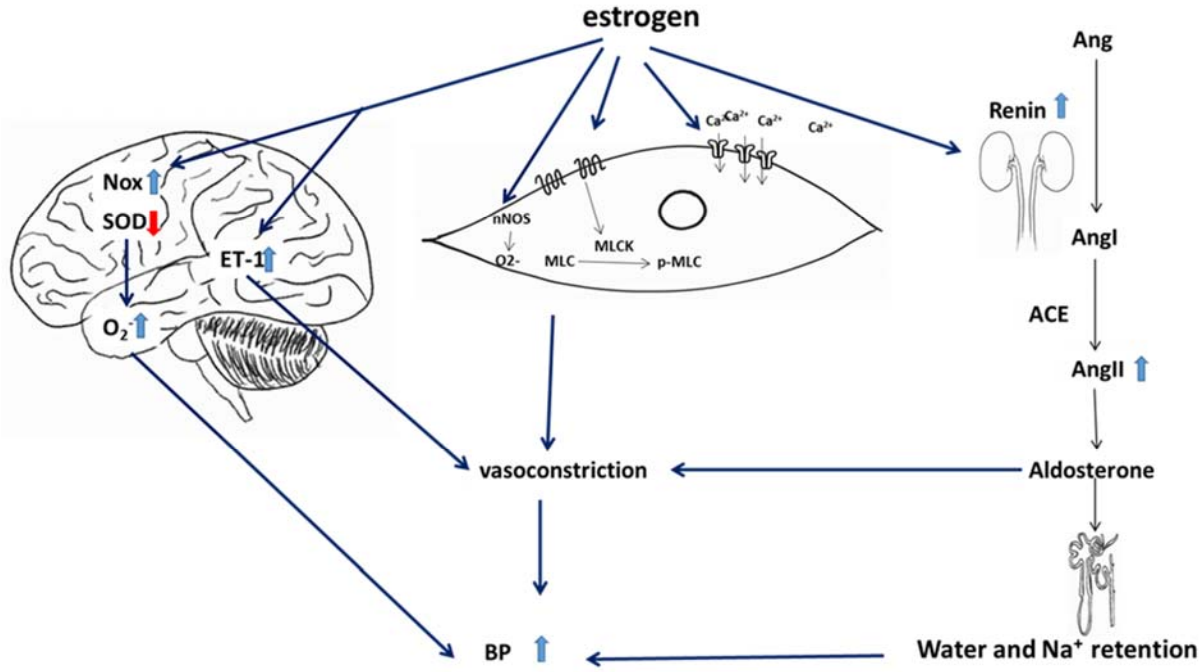


Figure 8. Putative signaling pathways mediating estrogen-induced vasoconstriction.

Estrogen mainly affects three levels, the brain, the RAAS, and the VSMC itself, to induce vasoconstriction. In RVLM, estrogen increases ET-1 leading to vasoconstriction. In addition, estrogen increases Nox or decreases SOD, leading to O_2^- pileup, and consequently increased blood pressure. At the level of the RAAS, estrogen increases the level and the activity of renin, and subsequently level of AgII and aldosterone, leading to increased blood pressure. In the VSMC, estrogen increases cell sensitization to Ca^{2+}, activates MLC, and induces nNOS-released O_2^-. These pathways are known to induce VSMC contraction, and thus vasoconstriction.

a. Brain sites involved in BP regulation

It is well established that the central nervous system regulates BP via sympathetic nervous activity [335]. This activity involves several brain regions, whose neurons express ERs. Thus, the effect of estrogen on BP may be elicited via these neurons [384]. It was suggested that

chronic exposure to E_2 lead to a concomitant increase in BP and superoxide species in the rostral ventrolateral medulla (RVLM) of Sprague Dawley female rats [346]. Interestingly, this brain area expresses both ER-α and ER-β and is involved in BP regulation [385]. Moreover, administration of anti-oxidants that lowered superoxide species also reduced BP [346]. Thus, it was postulated that estrogen leads to accumulation of superoxide anions in the RVLM, thereby leading to BP elevation [346]. The same group later showed that chronic estrogen exposure activated the expression of different sets of genes in RVLM depending on the age of the rat [386]. While chronic estrogen exposure increased the expression of the vasoconstrictor ET-1 in both young and old mice, it upregulated the expression of NAPDH oxidases (NOX-1 and NOX-2) in young mice only, while downregulating the expression of superoxide dismutase in old rats [386]. Thus, estrogen exposure may cause hypertension by activating various molecular signaling pathways in the rostral ventrolateral medulla, with ET-1 and O_2^- being common mediators [386], albeit to different extents in young versus aging animals.

b. RAAS Activation

The RAAS plays a critical role in fluid homeostasis and BP regulation [322,387-389]. Contextually, estrogen and estrogen-containing contraceptives increased the plasma levels of the renin substrate, angiotensinogen, in studies involving human subjects [389-392]. This increase is often associated with elevation in plasma renin activity and plasma levels of Ag II [322,390,393]. It is this activation of RAAS components that is known to increase BP [394,395]. Moreover, *in vivo* studies showed that estrogen (ethynylestradiol) increased SBP of rats. This increase was concomitant with an increase in the activity and levels of renin and Ag II [396]. This indicates a positive relationship between hypertension and RAAS with estrogen [349]. Additionally, inhibition

of the RAAS with enalapril, an ACE inhibitor, decreases BP in both males and females of SHRs [264]. More importantly, this enalapril partially removes the sex difference-related BP variances [264]. Similarly, suppression of this system prevented contraceptive-induced BP elevation in rats [397].

It is worth mentioning that RAAS inhibition could not completely normalize BP between males and females of SHRs, suggesting that other mechanisms contribute to the sex-related hypertension differences [264]. These results were confirmed in another study where ACE inhibitors failed to normalize BP in male SHRs, also demonstrating that pathways different from Ang II contribute to hypertension in these animals [349].

c. Calcium Channels

The role of calcium channels in arterial vasoreactivity is extensively documented [398-400]. In the context of estrogen-modulated vasoreactivity, a study reports that contraceptive-induced hypertension in rats is mediated by calcium channels [401]. Administration of nifedipine, a L-type calcium channel blocker, significantly inhibited the contraceptive-induced hypertension [401]. This result was further confirmed by another study which showed that calcium channels mediate estrogen-induced vasoconstriction [402]. In fact, it was speculated that estrogen elicited its vasoconstrictive effect via a signaling pathway that increases the sensitivity of Ca^{2+} influx in VSMCs [353].

d. Myosin Light Chain Phosphorylation

Phosphorylation of MLC is a key event in VSMC contraction, and consequently vasoconstriction [403,404]. It has been reported that estrogen induces VSMC contraction via phosphorylation of MLC in a time- and concentration-dependent manner [351,352]. Furthermore,

MLC played a role in estrogen-induced vasoconstriction and BP elevation, which was attenuated by ovariectomy [353]. It was proposed that estrogen acts via GPER to reduce phosphorylation of myosin light chain kinase (MLCK), leading to MLC phosphorylation and eventually vasoconstriction [353]. This may be supported by the fact that G_1, a GPER agonist, induces while G_{15}, a GPER antagonist, inhibits MLC phosphorylation in aortic ring segments [405] .

7. Factors Affecting Estrogen Vasoreactivity

An antihypertensive effect of estrogen has been well-established. This was mainly supported by the lower BP in premenopausal women and higher BP in postmenopausal females, as compared to age-matched men [406] [253,256,404]. However, this vasoprotective role of estrogen has been questioned, especially after the women's health initiative (WHI) trials initiated by the National Institutes of Health (NIH), which showed that HRT may increase the risk of CVDs [341,405] [407] . Additionally, the Heart and Estrogen/progestin Replacement Study I (HERS I) and HERS II studies showed that HRT failed to provide any protection against primary or secondary cardiovascular events [408]. In the context of hypertension, the inconsistency in the reports assessing the vasoreactive role of estrogen calls for an urgent inclusion of other factors in future studies so as to arrive at a better understanding of their respective effects in estrogen's vascular effects. Below we discuss some gender-and non-gender-related factors that may contribute to effect of estrogen on BP (figure 9).

Figure 9. Factors contributing to estrogen's effect on vasculature.

Several factors affect the overall estrogen-induced vasoreactivity. These include estrogen dose and treatment duration, the studied model and its age, endothelial integrity, sex chromosome content and genetic variations in ERs.

a. Effect of sex chromosome content

In addition to estrogen, gender-based effects in hypertension are also function of sex chromosome genes [409]. Studies revealed that sex-linked genes have a direct effect on hypertension [410]. For example, females with Turner Syndrome, caused by the absence of one X chromosome, are at a higher risk of hypertension [289]. This suggests the presence of pathways coded by sex chromosomal genes that control hypertension in the general population. Therefore,

gender-based differences in hypertension may be the result of an intricate relationship between sex hormones, particularly estrogen, and sex chromosomes. To differentiate between the contribution of sex steroid hormones or sex chromosome genes to the sex-related differences such as hypertension, Four Core Genotype mouse models have been developed by Arnols *et al*. These mice are either Sry-deficient or have a Sry transgene autosomally expressed, thus making mice that are XX females, XY males, XY females or XX males [411]. When steroid hormones were abrogated by castration or ovariectomy, contribution of sex steroids to sex-related differences in these animals can be separated from the contribution made by sex chromosomes genes [411,412]. Using the Four Core Genotype mouse models showed that, so far, there is no clear evidence suggesting that sex chromosomes alone affect CVDs, hypertension included [412].

b. Genetic variations in estrogen receptors

Estrogen elicits its vasomotor effects via ER-α, ER-β or GPER, all of which are receptors expressed in the vasculature [413,414]. It has been reported that polymorphisms in ESR1 and ESR2 genes, that encode ER-α and ER-β respectively, contribute to BP regulation, as certain ER genetic variants are associated with hypertension [415,416]. In addition, different ERs show sex-related differential gene expression. The potential differential expression and cellular and intracellular localization ER-α, ER-β or GPER, may, at least partially, explain some of the sex differences in estrogen-induced responses [412]. One more consideration is that ER expression shows age-related changes and could be a source of estrogen response variation between pre- and postmenopausal women [412].

c. Vascular bed type and endothelial integrity

One important factor that affects the response to estrogen is the type of vessels used in *ex vivo* experiments, and the type of vascular bed from which VSMCs are extracted for *in vitro* experiments. On the other hand, the endothelium also plays a major role in mediating vascular estrogenic effects. Indeed, endothelial integrity is a major determinant of the hormonal-induced vasoconstrictive or vasorelaxant effect. Wang *et al*., attribute these discrepancies to the extent of endothelial cell removal in a given experiment [353].

d. Estrogen's dose, exposure duration and experimental model used

The dose of estrogen administered to animal models, and the duration of administration significantly affect vasoreactivity in response to estrogen. One study proposes that the reason behind the previously established vasorelaxant effect of estrogen and the recently reported vasoconstrictive effects is due to higher dose of estrogen used in earlier studies [417]. Subramanian *et al.* clearly dissects the effect of estrogen dose in estrogen-treated mouse models [346]. The reason behind the discrepancy in their results in cardiovascular parameters, including BP, and results from previous studies is the used estrogen dose. While they used a 20 ng estrogen preparation, previous studies used a 20 fold higher concentration (0.2 to 10 µg of various estrogenic preparations) [418,419]. Indeed, higher estrogen dose leads to higher serum concentration (190 ± 20 pg/ml), which is far from physiological level of estrogen in female rats (5-140 pg/mL). In this context, it is worth mentioning that pharmacological concentration of estrogen (10–100 nmol/L) used in *in vitro* experiments are far from physiological levels of the hormone (30-1500 pmol/L) [351,352]. In addition, estrogen exposure time adds another level of complexity to vasoreactivity in response to estrogen [346].

One additional contributor to estrogen vasoreactivity may be the animal model used i.e. whether Wistar rats, Sprague-Dawley rats, SHRs, genetic hypertensive rats, or C57BL/6J mice are used. For instance, genetically hypertensive rats needed higher doses of estrogen to increase their BP [350]. At the cellular level, the expression of GPER, which mediates estrogen-induced MLC phosphorylation and thus vasoconstriction, is reduced in cultured VSMCs compared to freshly isolated ones [351]. Thus, the time spent in culture may affect the response of the VSMCs to estrogen.

e. Age of the subject

The age of the experimental subject plays a major role in responsiveness to estrogen. The estrogenic response is largely affected by age, both in animal models and in humans [420,421]. For instance, 3-month old normotensive rats exhibited higher BP increase after estrogen treatment than 6-months old rats [422]. Similar pattern of BP change in response to estrogen was observed in genetically hypertensive rats [422]. This indicates that responsiveness to estrogen is more prominent at younger age [350]. It appears that age influences eNOS-dependent estrogen-induced vasodilation [420]. Postmenopausal women receiving acute or chronic estrogen treatment showed an abrogation in the estrogen-induced vasodilatory response; this abrogation was more significant in women of older age or in women who stayed longer without receiving estrogen treatment [420,423]. In addition, estrogen's direct action on the vasculature may be more important in older women because their vascular endothelium becomes increasingly dysfunctional with advanced age [421,423-425]. However, clinically, it is postulated that the postmenopausal females who were on HRT at a late stage in their lives are at a higher risk of CVDs [426]. This is known as

the “windows of opportunity and timing” hypothesis, in which the age of starting HRT affects its risk [243].

f. nNOS microenvironment

Recent evidence suggests that estrogen may elicit vasodilation or vasoconstriction of denuded porcine coronary arteries via its novel target, type 1 neuronal NOS (nNOS). nNOS is expressed in VSMCs and is characterized by reductase and oxygenase domains and binding sites for a variety of cofactors [427,428]. Thus, nNOS can lead to NO production or O_2^- production, depending on its microenvironment [402]. Accordingly, estrogen may employ nNOS-derived NO or nNOS-derived O_2^- to induce vasodilation or vasoconstriction, respectively (figure 10) [402]. It was later speculated that estrogen-induced vasodilation takes place via ER-α/PI3K/Akt/nNOS/NO signaling pathway[429]. This same pathway may lead to vasoconstriction if nNOS microenvironment was more favorable for O_2^- production, as in the case of diabetes mellitus [429].

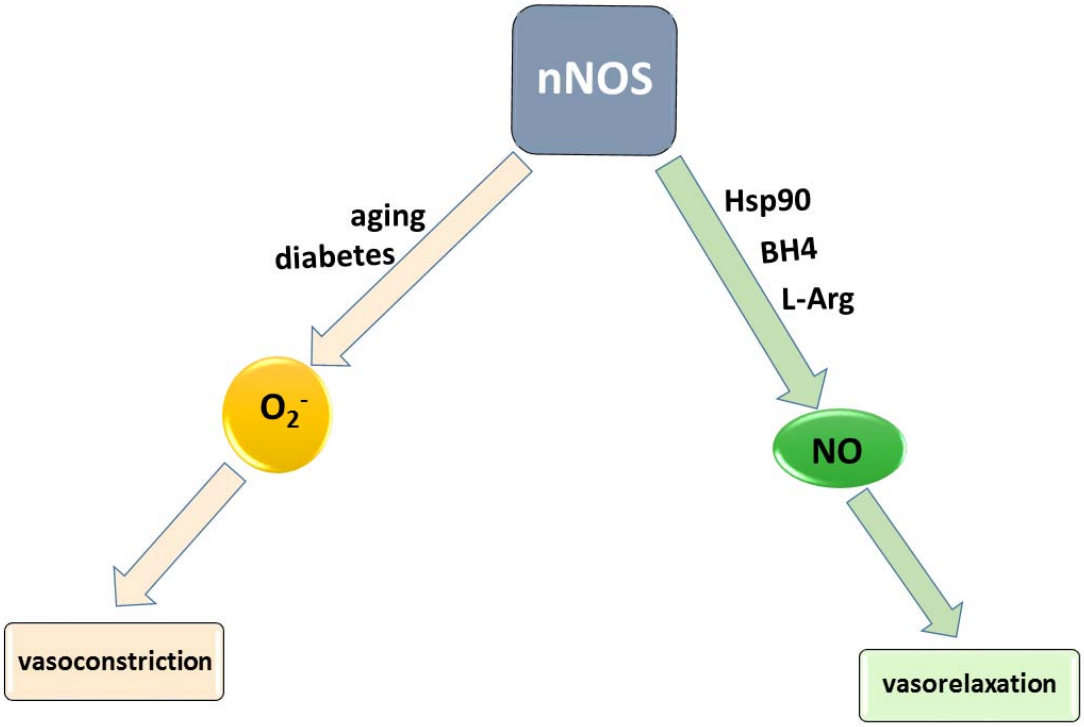


Figure 10. The nNOS microenvironment in VSMC dictates estrogen vasoreactivity.

Owing to its several domains and cofactor-binding sites, nNOS may lead to NO or O_2^- production, depending on its microenvironment. Aging and diabetes favor O_2^- release, leading to vasoconstriction. Cofactors such as Hsp90, BH4, and L-Arg activate the reductase function of nNOS, leading to NOS production, and thus vasorelaxation.

Estrogen-induced coronary artery relaxation may also be regulated via another nongenomic pathway involving the chaperone Hsp90 [430]. Interestingly, Hsp90 level was shown to decline with age [429]. This may explain the less potent vasorelaxant effect of estrogen in aged females. In addition, aging leads to decreased levels of L-arginine and tetrahydrobiopterin (BH4), cofactors needed to maintain nNOS in the NO-producing state [431]. Accordingly, aging will aid in nNOS switch to O_2^- release in response to estrogen [402] . This may explain the vasoconstrictive effect of estrogen in HRT [402] (figure 10).

8. Conclusion

Taken together, the effect of estrogen on VSMC contractility and ultimately BP is still unresolved. The most explicit example would be the discrepant effect of estrogen on BP during pregnancy. Further studies are needed to clearly identify novel factors affecting vasoreactivity and how they elicit their effects. Similar future studies are needed to better characterize the net vascular effect of estrogen and the factors that determine estrogenic responses. Knowing that awareness and control of hypertension continues to be suboptimal in women [432], these studies aid in a better delineation and guidelines concerning sex and gender in relation to hypertension.

CHAPTER II

MODEL

CVD may affect either the heart or blood vessels. Cardiomyopathy, heart failure, and hypertensive heart disease are examples of CVDs affecting the heart. On the other hand, coronary artery disease, renal artery stenosis, and aortic aneurysm are among CVDs affecting blood vessels. Whereas these vascular diseases involve large vessels, other vascular pathologies take place at the level of micrvasculature. These include arteriolar inflammation, Raynaud's phenomenon (RP), and hypertension. These vascular pathologies are manifested at the level of arterioles. Thus, VSMCs extracted from human arterioles were used for *in vitro* experiments, while mouse tail mesenteric arteries were employed in *ex-vivo* experiments to evaluate vasoreactivity.

Arterioles constitute the tree-type network of smaller and more numerous branches of the arterial system [433]. Arterioles are uniquely characterized by multiple layers of VSMCs, leading to the highest wall to lumen ratio among all blood vessels[434]. In addition, arterioles are highly enervated and highly responsive to external chemical and physical stimuli [435,436]. This responsiveness is further orchestrated through the arteriolar wall due to the presence of gap junctions in VSMCs and endothelial cells [437,438]. By virtue these characteristics, arterioles play two main functions: nutrient and oxygen delivery, and maintenance of blood pressure [433]. Owing to large changes in their diameter, arterioles determine the blood pressure of an individual. For instance, arterioles may dilate up to 50% of their normal diameter [433]. Conversely, arterioles were reported to cause to a 50–60% decrease in blood pressure [439]. During maximal stimulation,

arterioles are capable of complete closure [436]. In addition to their key role in transport and blood flow regulation, arterioles provide a line of defense during inflammation [440]. This immunologic role is achieved by increased arteriolar permeability allowing leukocyte infiltration. Anatomically, arterioles are made up of three layers: tunica intima, tunica media, and adventia [434].

1- Adventia: the outermost layer made up of collagen and fibroblasts [434].
2- Media: composed of several layers of VSMCs arranged in a circular manner. The mean thickness of this layer is around five μm. This layer is enervated with sympathetic nerve fibers and enclosed by connective tissue [434].
3- Intima: made up of endothelial cells surrounded by the internal elastic lamina. In fact, two basement membranes exist between arteriolar smooth muscle cells and endothelial cells [434]. One adheres to the basal surface of endothelim, and the other adheres to the surface of the inner most layer of VSMCs [434].

Among arteriolar components, VSMCs are of critical importance. This is due to their phenotypic plasticity and their role as vasotone effectors. Notably, these two features are regulated by estrogen, the female hormone.

VSMCs were extracted from foreskin of a newborn using the non-enzymatic sprouting method, and were grown in complete medium, Dulbeco Modified Eagle Medium (DMEM: F12), supplemented with 5% FBS and 1% Pen/Strep. This medium was used to propagate the cells. However, not only is this medium far from the natural niche of VSMCs, but also induces their inflammation leading to their phenotypic switch. To reduce this effect, cells were starved in serum-free, phenol red- free medium for 48 hours prior to any experiment. The rationale behind starvation was to synchronize the cells and bring them to G0/G1 phase. In addition, starvation

allows turnover of cellular proteome. Knowing that phenol red has estrogenic structure, starvation was done using phenol-red free medium to avoid the background noise resulting from phenol red.

In our experiments, we used VSMCs and tail arteries of male origin to test the effect of estrogen. This is called a cross-over approach. Knowing that hormones have a relatively long half-life, the aim of this approach is to decrease the carryover of the tested hormone from the donors, in an attempt to decrease the background noise [441]. However, a drawback of this approach is that under physiological conditions, cells originating from a subject of a given sex are not exposed to the hormone of the opposite sex, at experimental concentrations. Notably, the estrogen level in female mice varies during estrous cycle. Knowing that estrogen increases α_{2C}-AR expression in VSMCs [442], using female mice will affect our results, as the exact phase of the estrous cycle cannot be maintained during all experiments.

CHAPTER III

ESTROGEN ATTENUATES PHENOTYPIC SWITCH OF SERUM-INDUCED HUMAN ARTERIOLAR VASCULAR SMOOTH MUSCLE CELL

Authors: Manal Fardoun, Hassan Dehaini, Elias Baydoun, Ali H. Eid

Cardiovascular diseases (CVD) remains the leading cause of mortality worldwide. Vascular inflammation is a major contributor to the onset and the pathogenesis of this disease. In response to inflammatory stimuli, vascular smooth muscle cells (VSMCs) switch from a contractile to a dedifferentiated synthetic phenotype, characterized by decreased expression of differentiation markers and increased proliferative and migratory abilities. By virtue of its ability to attenuate these processes, estrogen (E_2) is known to play a vasculoprotective role. While the vast majority of studies investigating the role of estrogen on phenotypic modulation were conducted on VSMCs isolated from large vessels, the effect of estrogen on VSMCs extracted from microvessels (microVSMCs) remains largely obscure. Thus, we sought to assess the effect of estrogen on phenotypic switch of microVSMCs, and characterize the underlying molecular mechanism.

We were able to extract microVSMCs from human dermal arterioles by enzymatic sprouting method. The purity of the cells was identified by their morphology and by immunostaining of two VSMC markers: smooth muscle alpha-actin and calponin**.** In addition, we previously assessed the concentration-dependent effect of estrogen on the VSMC response, with 10^{-10} M being the optimal physiologic concentration. Results showed that E_2 (10^{-10} M) inhibited FBS-induced cell proliferation and migration. This estrogen-induced inhibition was

accompanied by decreased activation of mitogenic extracellular signal–regulated kinase (ERK1/2) and focal adhesion kinase (FAK), involved in cell proliferation and migration, respectively. Furthermore, E_2 (10^{-10} M) increased 5' AMP-activated protein kinase (AMPK) phosphorylation. In addition, E_2 blocked the cells in G0/G1 phase of cell cycle. These estrogenic effects were not inhibited by ICI 280,780 (5 μM, estrogen receptor (ERα/β) antagonist). Surprisingly, ICI 280,780 mimicked estrogen, acting as an ER agonist. Furthermore, estrogen-induced cell cycle arrest was concomitant with increased expression of p53 and decreased phosphorylation of retinoblastoma protein (pRb). The regulation of the aforementioned tumor suppressor proteins, p53 and pRb, was not mirrored by apoptosis evident by the absence of E_2-induced caspase-3 cleavage. Alternatively, SA-β-gal staining showed that E_2 induced senescence of VSMC. Moreover, E_2 increased the expression of contractile differentiation markers: calponin and caldesmon.

Taken together, this is the first study to report the effect of estrogen on phenotypic switch of VSMCs extracted from human arterioles. Our results indicate that estrogen attenuated VSMC proliferation by inducing VSMC senescence, via an ERα/β-independent p53/pRb-mediated mechanism. These results potentially implicate estrogen membrane receptor, GPR30, as the mediator of rapid estrogenic response in microVSMCs. In addition, due to the potential of estrogen to favorably modulate VSMC phenotype, our results may explain the lower incidence of CVDs in estrogen-replete premenopausal females.

A. Introduction:

Vascular smooth muscle cells (VSMCs) are characterized by phenotypic plasticity[443]. This property allows VSMCs to switch between differentiated contractile phenotype and synthetic de-differentiated phenotype[443]. The contractile phenotype is adopted under normal physiological conditions, where VSMCs exhibit undermined proliferative and migratory abilities[444]. In addition, contractile VSMCs are characterized by the expression of contractile markers such as calponin, caldesmon, and α-SM actin[445]. This phenotype allows VSMCs to maintain vascular homeostasis[115] by granting blood vessels contractility and ability to accommodate to blood pressure[444]. However, under pathological conditions, differentiated contractile VSMCs switch to de-differentiated synthetic phenotype[446]. The latter phenotype is characterized by increased VSMC proliferation and migration[446,447], and by decreased expression of the afore-mentioned contractile VSMC makers[446]. Instances whereby VSMC phenotypic switch take place include Atherosclerosis and vessel injury [445], where VSMCs proliferate and migrate to the intima of the blood vessel[448].

Estrogen (17β-estradiol or E_2) is believed to confer protection against cardiovascular diseases[449]. It is suggested that estrogen plays this vasoprotective role through is anti-inflammatory effect on vasculature[450]. By inhibiting VSMC proliferation and migration, estrogen halts the main processes of cardiovascular inflammation[78,102]. Estrogen binds to cytoplasmic receptors, ERα and ERβ, to initiate a genomic-response[451]. Some reports show that estrogen protective effects are due to ERα binding[452], others show that ERβ is the mediator of these effects[453]. In addition, the membrane GPR30, is emerging as an important estrogen-receptor responsible for rapid non-genomic estrogen response in VSMCs[114,454-456].

The efficiency of estrogen in cardiovascular protection along with its mechanism of action are dependent on many factors[243,341]. These factors can be estrogen-related such as estrogen form[90], concentration[83,89], and treatment duration[38]. Other factors depend on the VSMCs, such as VSMC age[457], condition[75], phenotype[83] and origin[66,458] of VSMCs and the gender[459] of the organism from which VSMCs where extracted. In addition, the estrogenic anti-inflammatory signaling pathway depends on stimulus used to induce VSMC inflammation[70,80,105] . Importantly, the molecular mechanism by which estrogen affects VSMCs is largely dependent on the vascular bed from which VSMCs were extracted[460]. While most studies used VSMCs extracted from large vessels, the effect of estrogen on micro VSMCs has not been reported. Indeed, there are many cases where the small arteries get affected/re-modelled in many cases [461,462], an example of which is small vessel disease[463]. Interestingly, one of the risk factors of this disease is low estrogen level[1].

In this study, we assessed the effect of estrogen on quiescent and FBS-stimulated arteriolar SMCs. We reported the estrogen increased the expression of contractile VSMC markers: calponin and caldesmon, in quiescent VSMCs, but it had no effect on their proliferative and the migratory abilities. On the other hand, estrogen attenuated proliferation and migration of FBS-induced VSMCs. Interestingly. ICI 182,780, a pure estrogen antagonist, did not negate the effect of estrogen on these cells, indicating a role for the GPR30. In addition, estrogen increased the proportion of VSMCs in G0/G1 phase of cell cycle. The FBS-induced activation of ERK1/2 was attenuated by estrogen. While estrogen did not activate caspase-3, it regulated pRb and p53, leading to cell senescence.

[1] American Heart Association

B. Materials and Methods:

1. Reagents:

Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham DMEM: F12 (BE12-719F), Penicillin/Streptomycin (17-602E), trypsin (BE02-007E), and DMSO (0231) were purchased from Lonza (Basel, Switzerland). Fetal Bovine Serum FBS (F9665), Phosphorous Buffer Saline PBS (D1408), L-glutamine (G7513), and propidium iodide (P4170) were obtained from Sigma Aldrich (Schnelldorf, Germany). 17-β-estradiol (ab120657), tetrazolium (ab146345), anti-Calponin antibody (ab46794), anti- Caldesmon antibody (ab32330), anti-caspase-3 antibody (ab13847), anti-βactin antibody (ab119716), anti-p38 antibody (phospho: ab7952, total: ab38238), anti-c-JNK antibody (phospho: ab124956, total: ab179461), anti-AMPK antibody (phospho: ab133448, total: ab8003), and DAPI (ab 90229) were purchased from Abcam®. Anti-Rb antibody (phospho: 8516S, total: 9309S) and anti-p53 antibody (phosphor: 9284, total: 48818S) were purchased from Cell Signaling TECHNOLOGY®. Insulin-Transferrin-Selenium ITS (41400045) and charcoal stripped FBS (S-FBS-SA-0450) were obtained from Thermofischer Scientific. DC™ Protein Assay kit and Clarity™ Western ECL Substrate kit were obtained from Biorad (CA, USA), TRiTc-Phalloidin (CS-207796) from Mito science, and ICI 182,780 (sc-203435) from Santa Cruz.

2. Cell culture:

VSMCs were extracted from human dermal arterioles isolated from post-circumcision skin of newborn healthy human, using nonenzymatic sprouting method. The cells were identified by their morphology and by the immunostaining of two VSMC markers: smooth muscle alpha-actin and calponin. Only cells of passages 9-12 were used. Cells were grown in Ham's Growth

medium (DMEM: F12, 50:50; supplemented with 10% FBS, and 1% penicillin/streptomycin) in a humidified incubator at 37°C with 5% CO_2 atmosphere. Before estrogen treatment, cells were starved in serum-free phenol red-free medium (DEMEM, 50:50, supplemented with L-Glutamine, insulin-transferrin-selenium mix, and 1% penicillin/streptomycin).

3. MTT assay:

Cell proliferation was assessed using MTT assay. Cells were grown in 96-well plate in complete medium until 30-40% confluency, then starved for 48 hours. Following treatment, 20 µl of 5mg/ml MTT solution was added to each well and then incubated (37°C, 5% CO_2) for 1hr. The media was then removed, and 200 µl of DMSO was added to each well. The plate was put on shaker for 20 minutes to allow DMSO to dissolve formazan. Using an ELISA Multiscan EX Reader (Thermo), the optical density (O.D.) of the plate was read at 550 nm. O.D. is directly correlated with number of viable cells.

4. Hypertrophy assay*:*

Cells were grown in 100 mm^2culture dishes to a confluency of 10%. Cells were then starved for 48 hours. Following treatment, five random fields were photographed at 5X magnification using a phase-contrast microscope (Zeiss). The cross-sectional area of 5 cells in each field was measured using ZEN software (ZEN 2011). The average area of a cell was compared to that of vehicle-treated cells.

5. Cell protein content:

Cells were grown in 100 mm^2 culture dishes to a confluency of 30%, then starved for 48 hours. Following treatment, cells were trypsinized and an aliquot was used for counting by trypan blue. Cells were then centrifuged at 300g and the pellet was re-suspended in 20 µl lysis buffer (2% SDS and 60 mM Tris (pH 6.8)). Proteins were quantified using BCA assay. To get the protein content per cell, total protein quantity was divided over the cell number of each plate.

6. Wound healing assay:

30.10^3 VSMC per well were grown in 12-well tissue culture dishes until 90-95 % confluence then starved for 48 hours. Using a sterile plastic pipette tip (1-10 µl), a scrape was made through the confluent monolayer. The media was then aspirated, wells were washed with PBS to remove cellular debris and new medium containing the treatment. . Using Axio Vert microscope at 5X magnification, the width of the wound was measured at an arbitrary place marked on the outside of each well. The difference between the distance at time zero and at different time points (1, 2, 4, 6, 8, 12, and 24 hrs) reflects wound closure with time. Photomicrographs were taken and ZEN imaging software from Zeiss used to measure the width of the scratch.

7. Western Blot:

Cells were washed twice with PBS, then scraped into lysis buffer (2% SDS and 60 mM Tris (pH 6.8)[155]. Protein concentration was determined by DC™ Protein Assay kit. Equal protein quantities (25-30 µg) were resolved onto 10% SDS-PAGE, and then transferred to PVDF membranes. Prior to their incubation (overnight, 4º C) with the primary antibody, PVDF

membranes were blocked with 5% fat-free dry milk dissolved in TBS-T (TBS and 0.05% Tween 20). The suitable HRP-conjugated secondary antibody conjugated to horseradish peroxidase was used. Bands were detected by ECL chemiluminescent substrate and quantified using Image Lab program.

8. PI staining:

Cells were seeded in 100 mm culture dishes. At 70-80% confluency, cells were starved for 48 hours then treated. After treatment, cells were trypsinized and pelleted by centrifugation at 4°C. The cells were then washed twice with ice-cold PBS and resuspended in 500 µl PBS. Equal volume of 100% ethanol was added and incubated for 15 mins at -20°C for fixation and permeabilization. Cells were then pelleted, washed twice with PBS, and resuspended in 1mg/ml propidium iodide in PBS. PI fluorescence was read using Guava EasyCyte8 Flow Cytometer (Luminex, USA). Cell cycle analysis was done using Guava Soft 2.7 software.

9. Actin staining:

Cells were seeded on coverslips in 12-well plate. At 80% confluency, cells were starved for 48 hours. After treatment, cells were then washed twice with warm PBS. Fixation was achieved by incubating the cells for 10 minutes in 4% formaldehyde. Cells were then washed and permeabilized by incubating them for 5 minutes in 0.5% Triton-100. For actin staining, cells were washed and incubated for 90 minutes in Rhodamine phalloidin stain. Nuclei were stained by DAPI for 20 minutes at room temperature. Cells were mounted on an anti-fading agent and visualized using Zeiss Axio fluorescent microscope. Images of different fields were captured.

10. Statistical Analysis

Results were reported as means ± S.E.M. of the number of experiments. A Student's *t*-test was used for either paired or unpaired observations. For multiple comparisons, ANOVA was used: either one-way ANOVA (with Dunnett's post hoc test) or two-way ANOVA (with Tukey-Kramer's post hoc test). A p value of <0.05 was considered statistically significant.

C. Results:

1. Estrogen attenuates FBS-induced VSMC Proliferation

VSMC proliferation is a key step in vascular remodeling. The effect of E_2 on VSMC proliferation was assessed using MTT assay. E_2 (10^{-10} M) showed no significant effect on the proliferation of non-stimulated cell (figure 11.A). Then, we sought to check the effect of E_2 on the proliferation of challenged-VSMCs. 5% charcoal stripped-FBS was used as mild inflammatory factor to induce cell proliferation. As shown in figure 11.B, FBS induced cell proliferation in a time-dependent manner, reaching a 1.7±0.06 fold increase at day 9 ($p<0.05$). This FBS-induced cell proliferation was inhibited by pre-treatment with E_2 (10^{-10} M). Therefore, estrogen attenuates FBS-stimulated but not basal cell proliferation.

To further assess the inhibitory effect of estrogen on FBS-stimulated cells, ICI 182,780, estrogen receptor antagonist, was used. Surprisingly, ICI 182,780 (3 μM) did not antagonize the inhibitory effect of estrogen on FBS-stimulated VSMC proliferation (figure 11.C). In fact, ICI 182,780 inhibited FBS-induced VSMC proliferation in a manner comparable to that of estrogen inhibition (figure 11.C). This suggests that the ICI 182,780 may play the role of ER agonist.

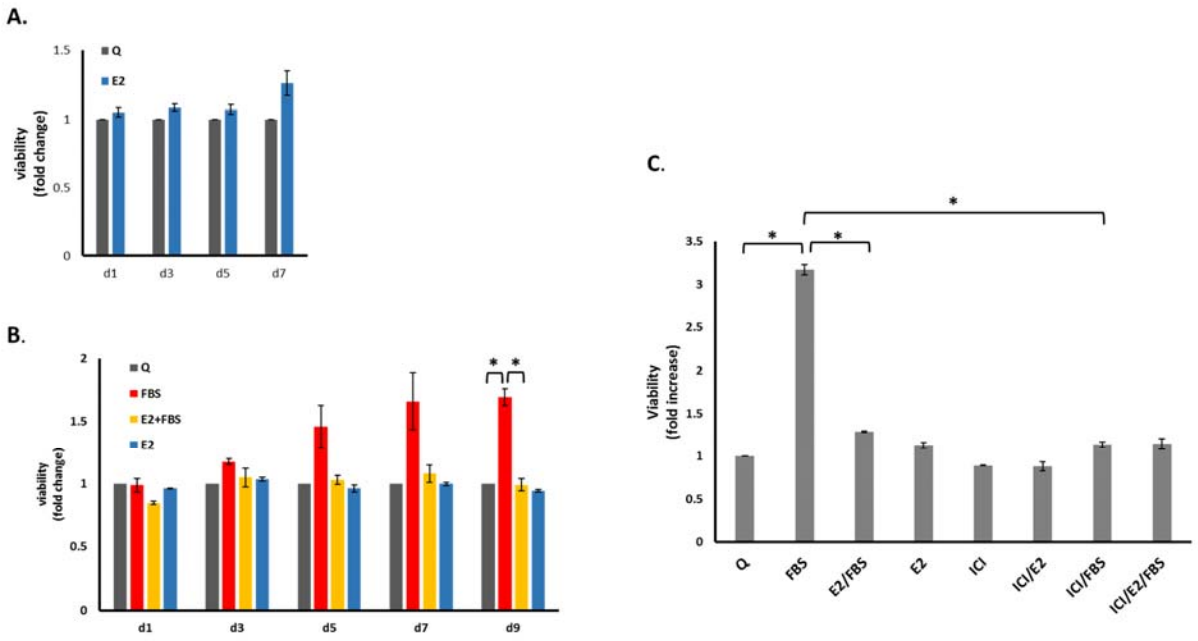


Figure 11. Effect of estrogen on VSMC proliferation

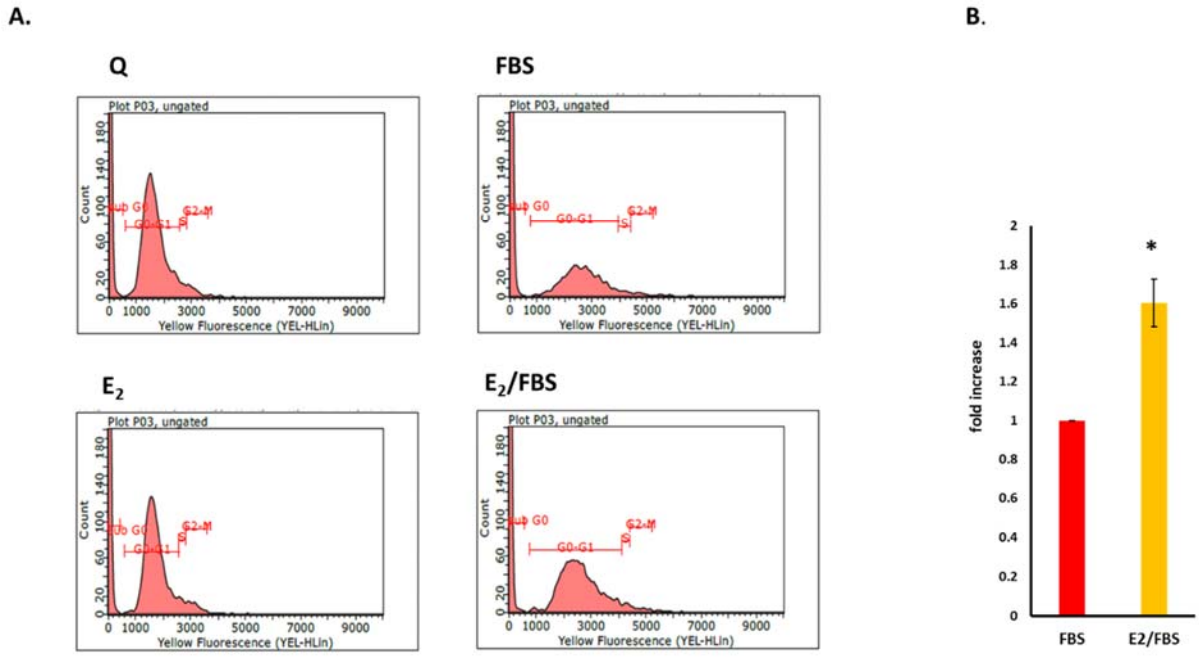
A. Quiescent cells were treated with E_2 (10^{-10} M) for 1, 3, 5, and 7 days. B. Cells were treated with 10% FBS in the presence or absence of E_2 (10^{-10} M) for 1,3,5,7, and 9 days. E_2 was added 30 minutes before and during treatment with FBS. C. cells were treated with 10% FBS in the presence or absence of E_2 (10^{-10} M), ICI 280,780 (5 μM), or both. E_2 was added 30 minutes before and during treatment with FBS, and ICI 280,780 was added 30 minutes before and during treatment with E_2 or FBS. Cell viability was assessed by the overall metabolic activity measured by MTT. Values are calculated as % of the corresponding vehicle control value and represented as mean ± SEM of three different experiments, each run in triplicates. *p<0.05, **p<0.01 and ***p<0.005

2. Estrogen attenuates cell cycle of FBS-induced VSMCs

Having established that E_2 attenuates FBS-induced cell proliferation, we next sought to assess the effect of E_2 on cell cycle progression. Flow cytometry analysis showed that quiescent and estrogen-treated cells had a similar cell cycle profile, with almost all cells blocked in the G0/G1 phase (figure 12.A). This is in accordance with MTT assay results. As expected, FBS induced cell cycle progression. This FBS-induced cell cycle progression was attenuated by E_2

(10^{-10} M) (figure12.A), significantly increasing the proportion of cells in the G0/G1 phase (1.6 ±0.12, $p<0.05$) (figure12.B).

Interestingly, pretreatment of FBS-stimulated cells with either E_2 (10^{-10} M) or ICI 182,780 (3 µM) showed the same cell cycle profile (figure12.C). In addition, the ICI 182,780 did not negate the inhibitory effect of estrogen on FBS-treated cells (figure12.C). These results show that E_2 attenuated the proliferation of FBS-induced cells by blocking the cells in the G0/G1 phase. Similar effect was noted for ICI 182,780. This indicates that estrogen elicits its effect in an ERα/β-independent manner. In addition, ICI 182,780 may act as an estrogen agonist.



C.

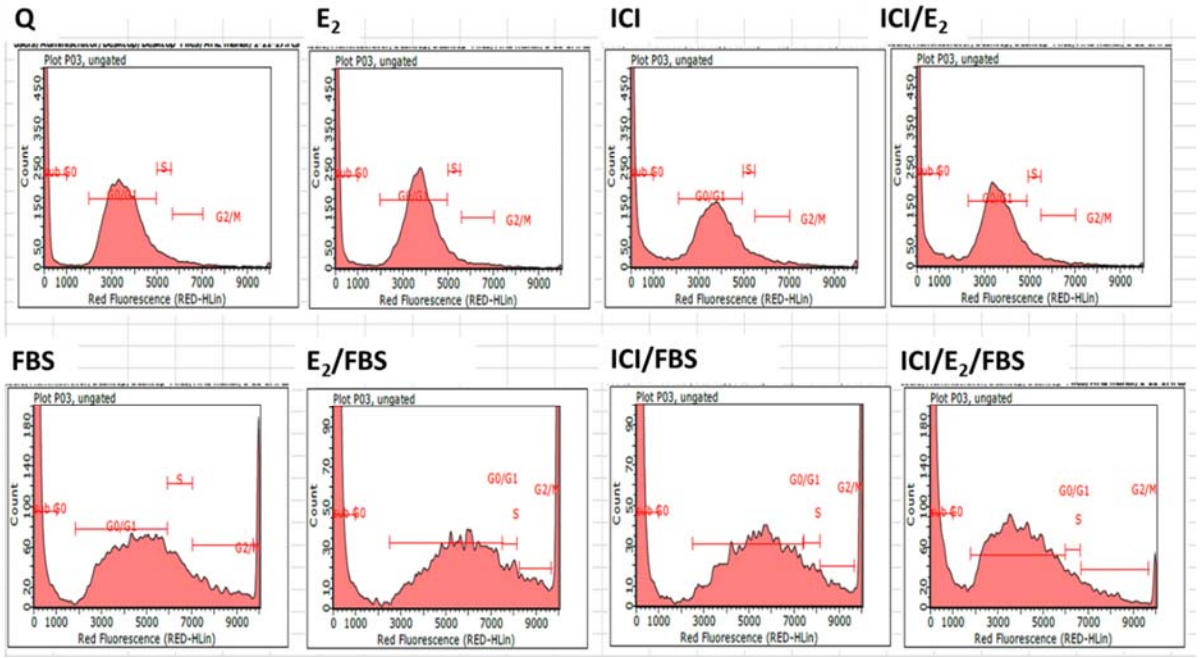


Figure 12. Effect of estrogen on cell cycle of VSMCs

A. E_2 (10^{-10} M) induced G0/G1 cell-cycle arrest. VSMCs were treated with 10% FBS, with or without prior administration of E_2 (10^{-10} M), for 5 days. Cells were then stained with PI and sorted by flow cytometer. Guavasoft 2.7 was used to analyze the results. B. The percentage of cells in G0/G1 phase in the VSMC treated with FBS+E_2 relative to that in the VSMC treated with FBS alone. C. Pre-treatment with ICI 182,780 (5 μM) showed similar cell cycle profile as E2 (10-10 M) treatment.

3. Estrogen induces VSMC senescence by regulating pRb and p53

The tumor suppressor proteins, p53 and pRb, are involved in G1 to S phase transition of cell cycle. The accumulation and the phosphorylation of p53 lead to cell cycle arrest. However, the phosphorylation of pRb leads to its inactivation, and thus cell cycle progression. We wanted to examine the estrogen effect on p53 and pRb . As expected, FBS increased the expression of pRb but decreased p53 expression (figure 13.A). These FBS-induced effects were attenuated by E_2 (10^{-10} M) (figure 13.A). Furthermore, E_2 (10^{-10} M) attenuated FBS-induced phosphorylation of

pRb but increased p53 activation (figure 13.B). These results indicate that estrogen attenuates cell cycle progression by regulating p53 and pRb.

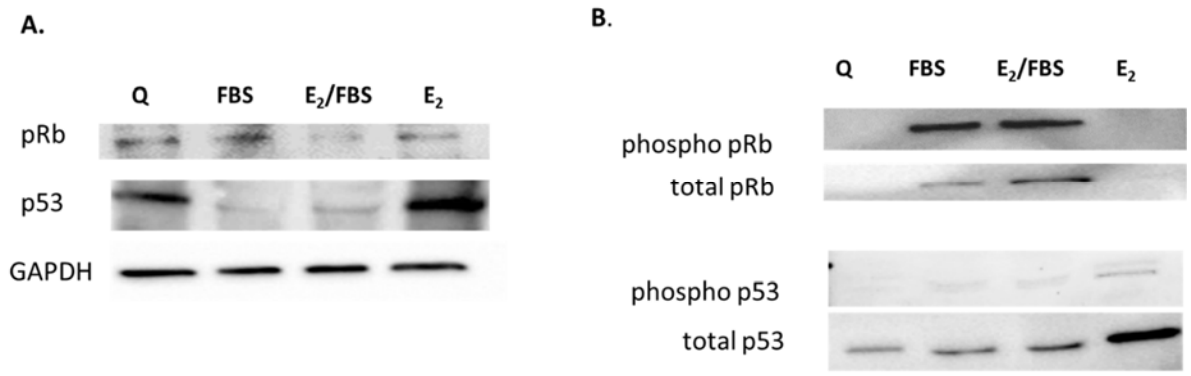


Figure 13. Effect of estrogen on pRb and p53 in serum-induced VSMCs.

Cells were treated with FBS in the presence or absence of E_2 (10^{-10} M). E_2 was added 30 minutes before and during FBS treatment. The expression (A) and the activation (B) of pRb and p53 was determined by Western blotting.

Since the afore-mentioned tumor suppressor proteins are involved in apoptosis and cell senescence, we sought to assess the effect of estrogen on apoptosis and cellular senescence. We first assessed the effect of estrogen on caspase-3, a protein involved in intrinsic and extrinsic apoptotic pathways. Expectedly, FBS decreased the expression of caspase-3 (figure 14). This inhibitory effect of FBS was attenuated by E_2 pretreatment (10^{-10} M) (0.51 ±0.09 vs 1.08 ±0.19, *p<0.05, **p<0.01) (figure 14). However, E_2 (10^{-10} M) did not induce caspase-3 cleavage. These results indicate that estrogen does not activate caspase-3, and therefore does not induce apoptosis.

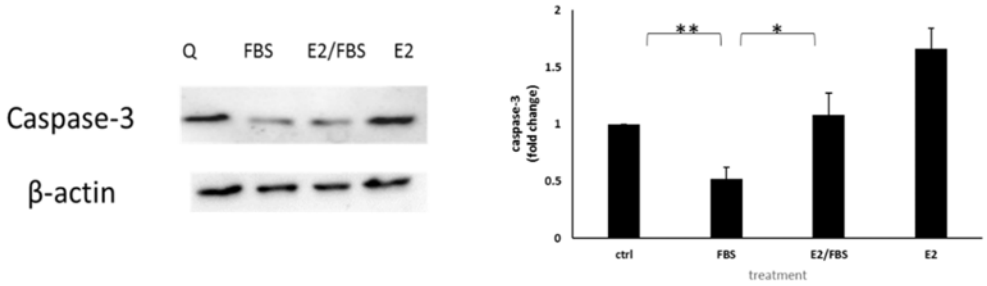


Figure 14. Effect of estrogen on caspase-3 in VSMCs.

Cells were treated with FBS in the presence or absence of E_2 (10^{-10} M). E_2 was added 30 minutes before and during treatment with FBS. The expression of caspase-3 was determined by Western blotting. The results are expressed as fold increase from the control and are represented as mean±SEM. *$p<0.05$

We next employed SA β-gal to stain senescent cells. E_2 (10^{-10} M) induced senescence of quiescent and FBS-stimulated VSMCs (blue dots) (figure 15). Similarly, ICI 182,780 (3 μM) induced senescence of these cells. This indicates that estrogen, as well as ICI 182,780, attenuates cell proliferation by inducing senescence.

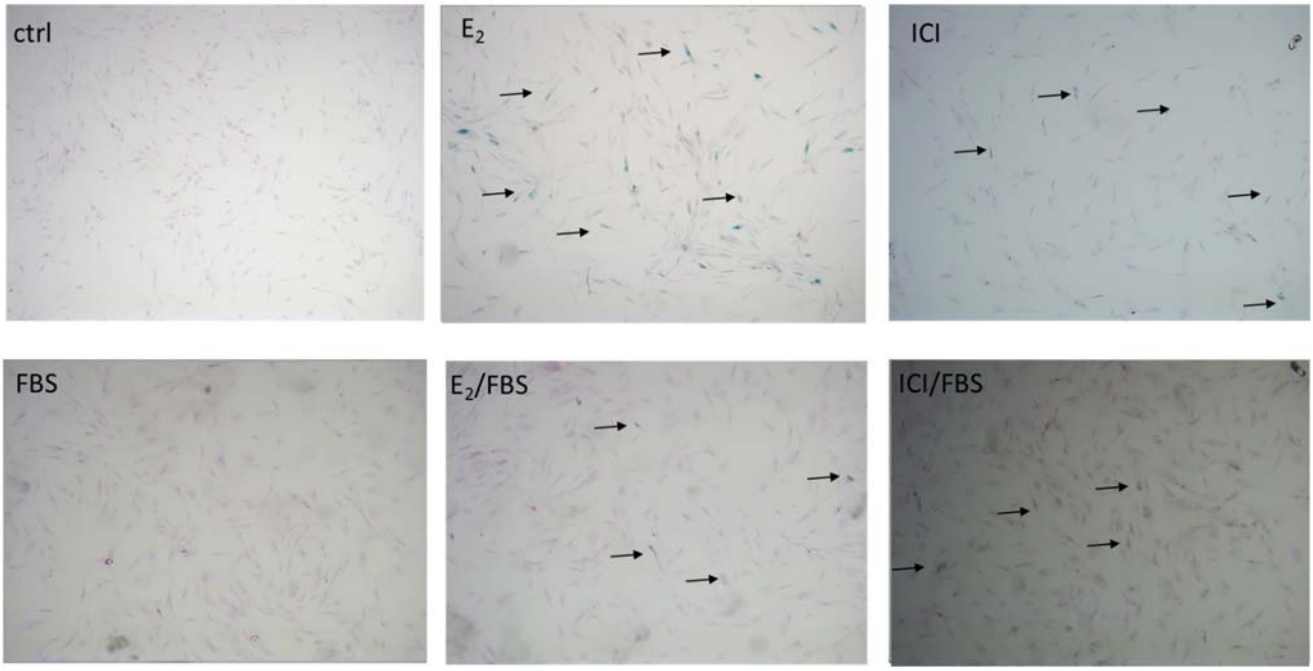
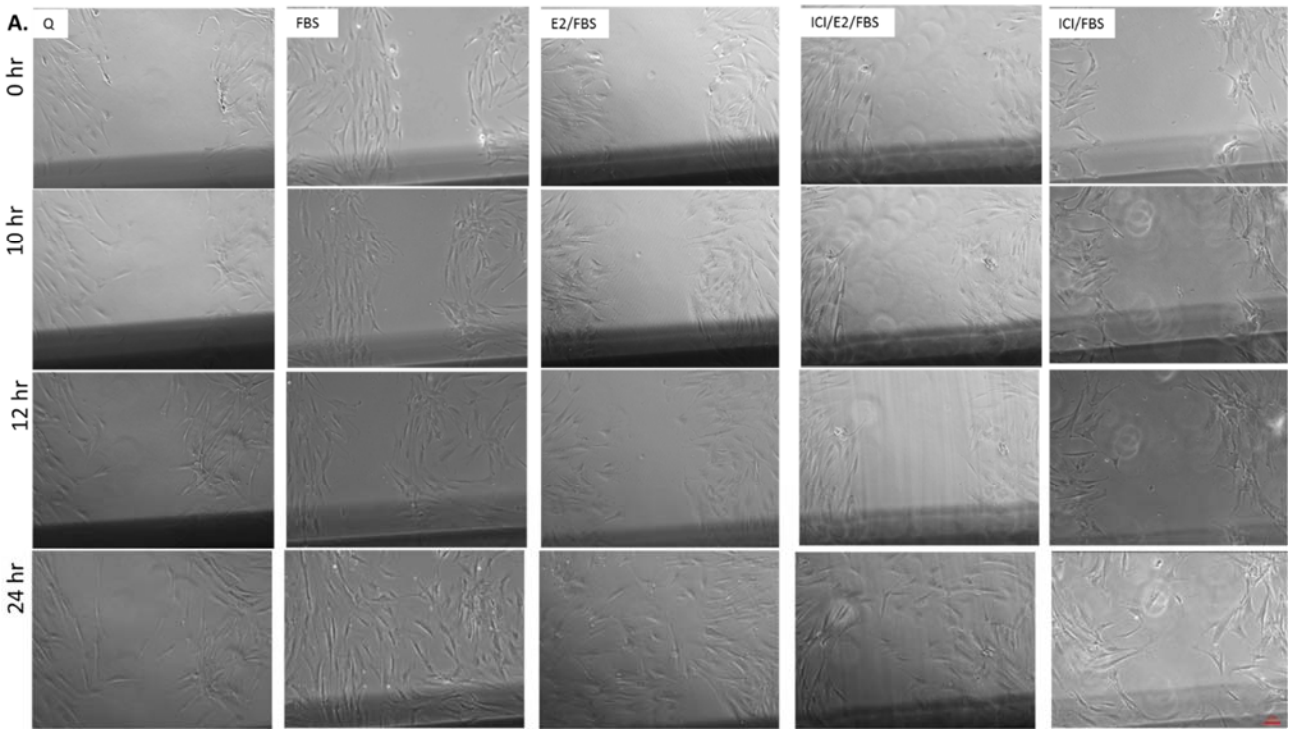


Figure 15. Effect of estrogen on cell senescence in VSMCs.

Cells were treated with FBS, in the presence or absence of E_2 (10^{-10} M) or ICI 280,780 (5 μM). E_2 or ICI 280,780 were added 30 minutes before and during treatment of FBS. Photography image (magnification 5 X) of SA-β-gal stained cells were obtained.

4. Estrogen Attenuates FBS-induced VSMC Migration

The VSMC migration is a hallmark of vascular inflammation. Thus, we sought to assess the effect of estrogen on cell migration was evaluated using scratch assay. E_2 (10^{-10} M) had no effect on cell migration (figure 16.A). FBS significantly promoted cell migration. This FBS-induced migratory ability was significantly attenuated by E_2 (10^{-10} M) at 6 and 24 hours ($p<0.05$). ICI 280,780 (5 μM) did not refute the inhibitory effect of E_2 on FBS-stimulated cells ((figure 16.B). Furthermore, ICI 280,780 inhibited the migration of FBS-treated cells in a manner similar to that of E_2 inhibition ((figure 16.A).



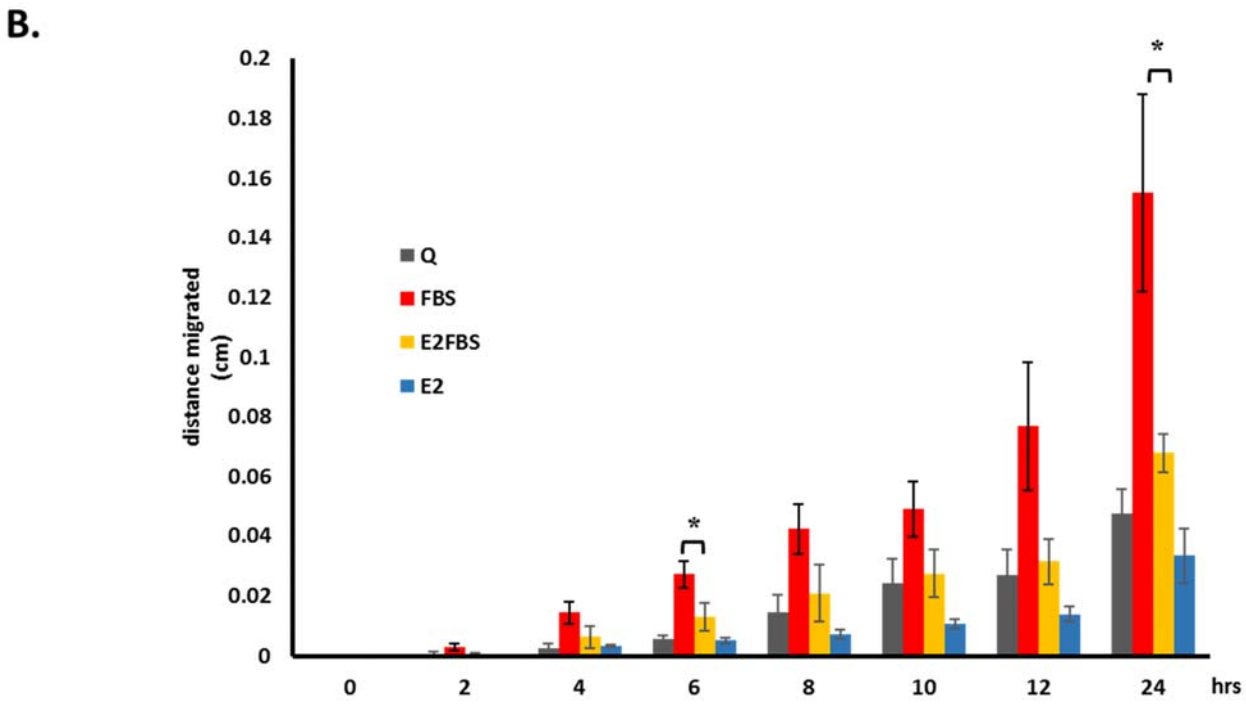


Figure 16. Effect of estrogen on VSMC migration.

A. Cells were treated with FBS in the presence or absence of E_2 (10^{-10} M), ICI 280,780, or both. E_2 was added 30 minutes before and during treatment with FBS, and ICI 280,780 was added 30 minutes before and during treatment with E_2 or FBS. Images were taken at the indicated time points. Scale bar, 50 μm. B. Values are represented as mean ± SEM of distance migrated (n = 3 replicates) (*$p<0.05$).

5. Estrogen attenuates FBS-induced ERK1/2 and FAK phosphorylation, but negates FBS-attenuated AMPK phosphorylation in VSMCs

It is well-established that the phosphorylation of ERK1/2 induces cell proliferation and migration, which is also mediated by FAK activation. In addition, We wanted to evaluate the effect of estrogen on ERK1/2 and FAK phosphorylation. Whereas FBS induced the activation

ERK1/2 of FAK (figure 17), pretreatment with E_2 (10^{-10} M) attenuated FBS-induced ERK1/2 (2.315662 ±0.07 vs 1.49 ±0.16,$p<0.05$) and FAK phosphorylation (figure 17).

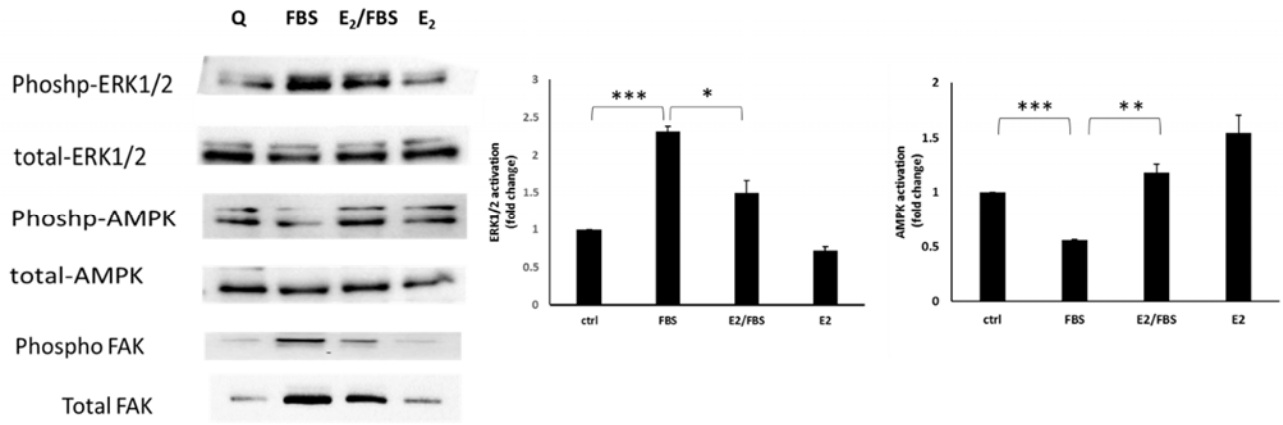


Figure 17. Effect of estrogen on ERK1/2 and AMPK in serum-induced VSMCs.

Cells were treated with FBS in the presence or absence of E_2 (10^{-10} M). E_2 was added 30 minutes before and during FBS treatment. The activation of ERK1/2, AMPK, and FAK was determined by Western blotting. The results are expressed as fold increase from the control and are represented as mean±SEM. *$p<0.05$, **$p<0.01$, ***$p<0.001$

Furthermore it has been reported that AMPK phosphorylation inhibits VSMC proliferation. Thus, we assessed the effect of estrogen on AMPK in FBS-induced cells. Serum inhibited AMPK phosphorylation (figure 17). This inhibitory effect was negated by estrogen pre-treatment (0.55 ±0.002 vs 1.17 ±0.08, $p<0.01$) (figure 17). These results indicate that estrogen inhibited cell proliferation by 2 parallel mechanisms: attenuating FBS-induced ERK1/2 and overriding FBS-attenuated MAPK. Moreover, these results suggest that estrogen inhibits cell migration via FAK inhibition.

6. Estrogen activates MAPKs, p38 and JNK, and AMPK in VSMCs

Mitogen-activated protein kinases (MAPKs) are involved in vital cell processes such as proliferation, migration, and differentiation. Thus, we wanted to study the effect of estrogen on the activation of p38 and JNK in micro-VSMCs. E_2 (10^{-10} M) stimulation increased the activation of p38 and JNK in a time-dependent manner. Maximum phosphorylation level for p38 was attained 30 minutes post-treatment (1.7 ±0.3, $p<0.05$) (figure 18). As for JNK, a maximum activation was reached after 10 minutes post-treatment (1.8 ±0.17, $p<0.01$) (figure 18).
Recent studies showed that AMPK plays an important role in CVDs. So, we wanted to study the effect the effect of estrogen on AMPK in arteriolar VSMCs. As shown in figure 18, E_2 (10^{-10} M) stimulates an increase in phosphorylation level of AMPK as early as 5 minutes (2.12 ±0.39 ,$p<0.05$).

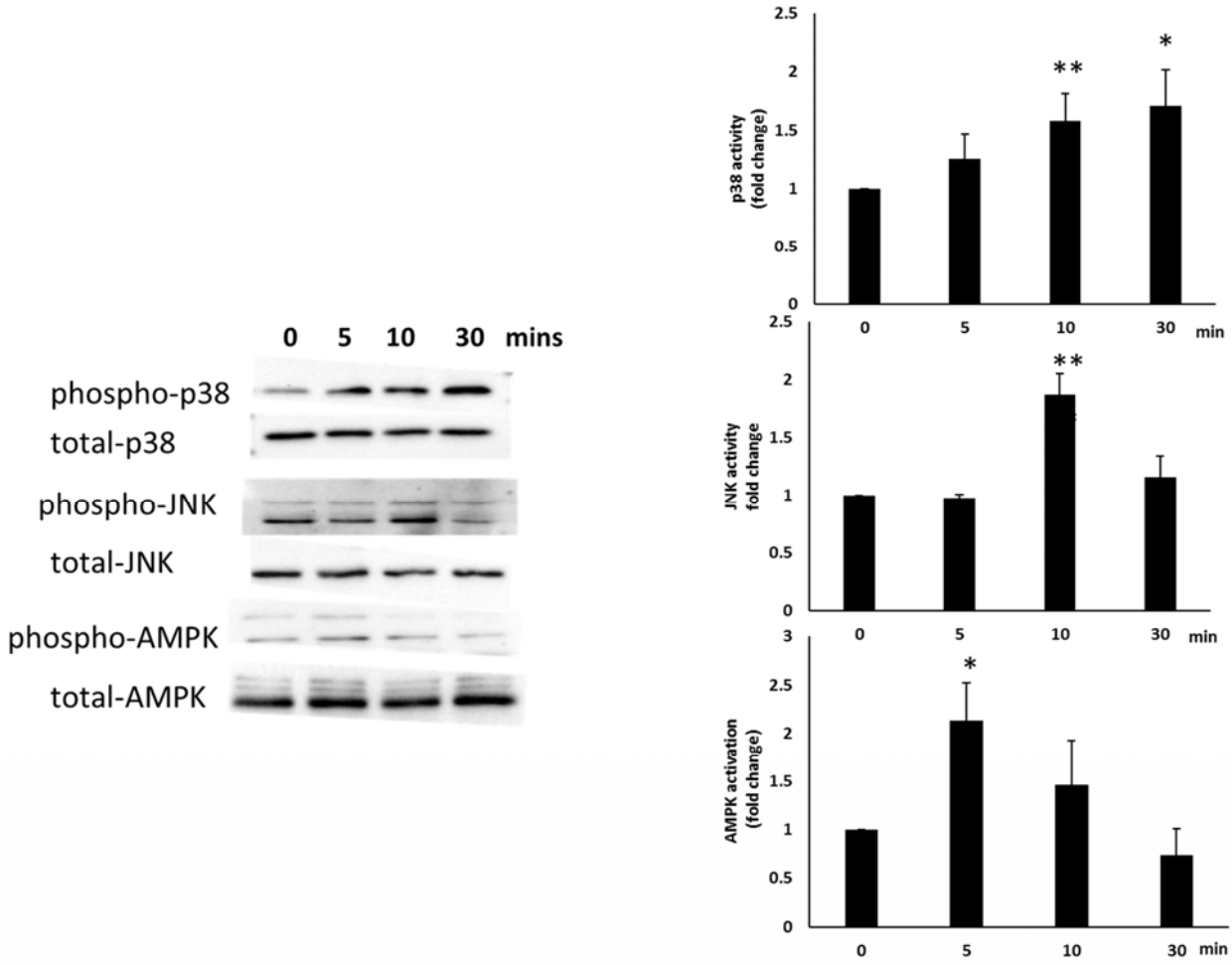


Figure 18. Effect of estrogen on p38, JNK, and AMPK in VSMCs.

Cells were treated with E_2 (10^{-10} M) for 0,5,10, and 30 minutes. The activation of p38, JNK and AMPK was determined by Western blotting. The results are expressed as fold increase from the control and are represented as mean±SEM. *p<0.05, **p<0.01, ***p<0.001

7. Estrogen increases the expression of contractile VSMC markers, calponin and caldesmon, and attenuates FBS-induced repression of these markers

Contractile VSMCs are characterized by the expression of differentiation markers, calponin and caldesmon. We sought to assess the effect of estrogen on the expression of these

markers. E_2 (10^{-10} M) lead to an increase in the expression of calponin and caldesmon in a time dependent manner (figure 19.A). This estrogen-induced upregulation reached a maximum level 72 hours post-treatment (1.49 ±0.12 and 1.89 ±0.06 fold increase for calponin and caldesmon respectively; $p<0.05$) (figure 19.A). This indicates that estrogen induces VSMC contractile phenotype.

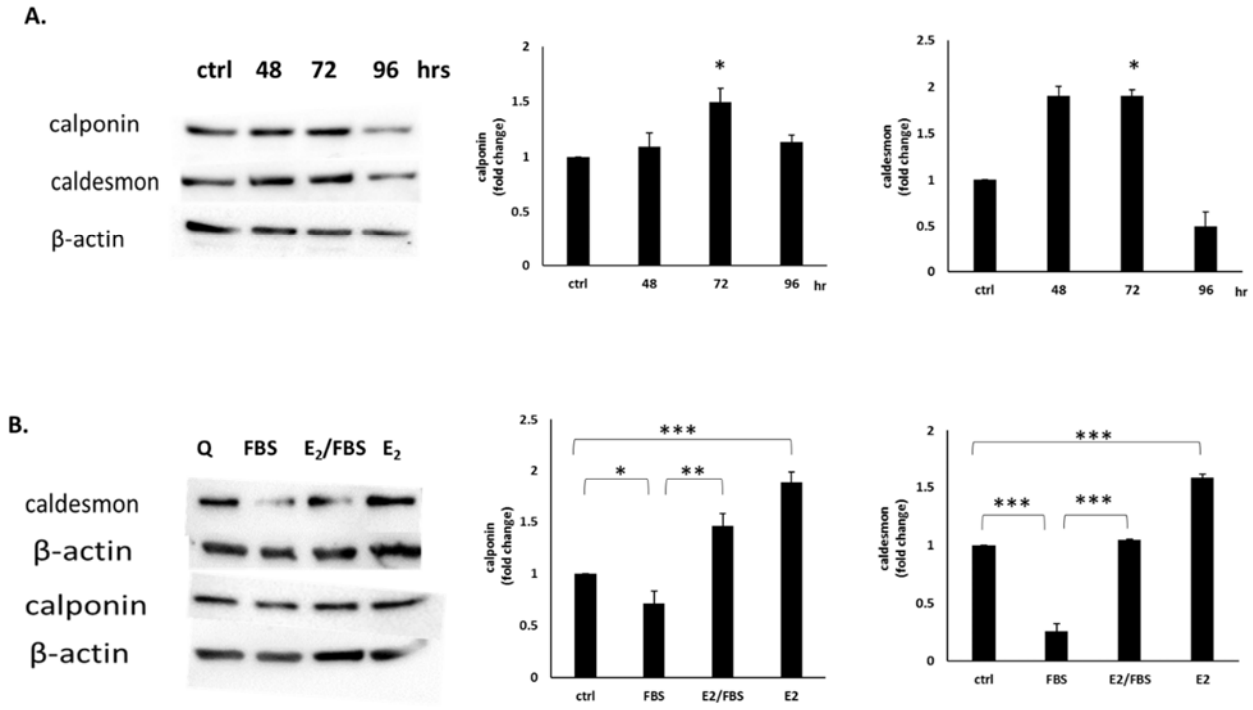


Figure 19. Effect of estrogen on differentiation markers, calponin and caldesmon in VSMCs

A. Cells were treated with E2 (10^{-10} M) for 48, 72, and 96 hours. B. Cells were treated with FBS, in the presence or absence of E2 (10^{-10} M). E2 was added 30 minutes before and during treatment with FBS. The expression of differentiation markers, calponin and caldesmon, was determined by Western blotting. The results are expressed as fold increase from the control and are represented as mean±SEM. *p<0.05, **p<0.01, ***p<0.001

FBS-treatment, which mimics mild inflammation, is known to induce synthetic VSMC phenotype. In this context, we sought to study the effect of estrogen treatment on the expression

levels of calponin and caldesmon in FBS-induced cells. As expected, FBS decreased the expression calponin and caldesmon levels (figure 19.B). This FBS-induced downregulation of differentiation markers was attenuated by pretreatment with E_2 (10^{-10} M) (0.71 ±0.11 vs 1.46 ±0.11; 0.25 ±0.06 vs 1.04 ±0.001; for calponin and caldesmon respectively, *p<0.05, **p<0.01, ***p<0.001) (figure 19.A). This indicates that estrogen attenuates FBS-induced VSMC dedifferentiation.

8. Estrogen induces actin polymerization in VSMCs

The polymerization of G-actin to F-actin is characteristic feature of contractile VSMCs. To assess the effect of estrogen on actin polymerization, we used phalloidin, which stains F-actin. E_2 (10^{-10} M) induced the formation of defined actin polymers, with higher fluorescence intensity (figure 20.B). Therefore, estrogen promotes contractile phenotype of VSMCs. This result is in accordance with estrogen-induced upregulation of the contractile markers, calponin and caldesmon.

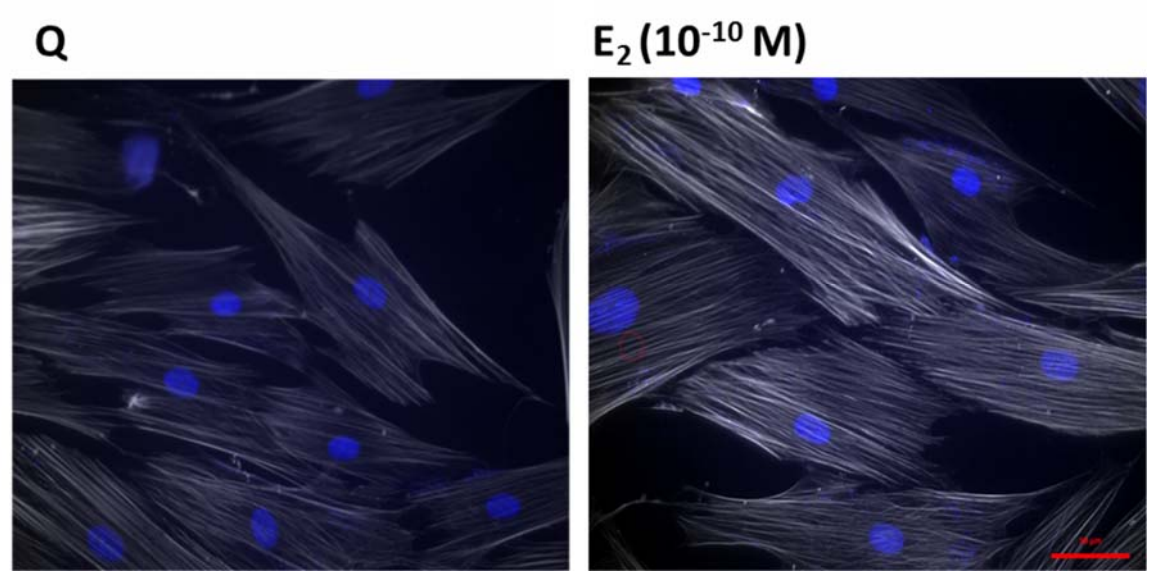


Figure 20. Effect of estrogen on actin polymerization.

Cells were treated with E_2 (10^{-10} M) then stained with phalloidin and actin polymerization was assessed. Representative micrographs showing the effect of estrogen on actin polymerization. Scale bar, 50 µm.

9. Estrogen lead to VSMC hypertrophy

We wanted to test the effect of estrogen on VSMC size. E_2 (10^{-10} M) induced a time-dependent increase in cell size, reaching a maximum increase at day 5 (2.13± 0.18 v/s 1.64± 0.05, $p<0.05$) (figure 21.B). Figure 21.A shows images of E_2 -treated and vehicle-treated VSMCs at day 5, illustrating the difference in cell size.

We then measured the effect of E_2 on cellular protein content. E_2 (10^{-10} M) increased protein content/cell in a time-dependent manner (1.6±0.11 and 1.9±0.06 fold increase, for day 4 and day5 respectively; $p<0.05$) (figure 21.C). This is in accordance with the increase in cell size upon E_2 treatment. This result indicates that estrogen induces cell hypertrophy.

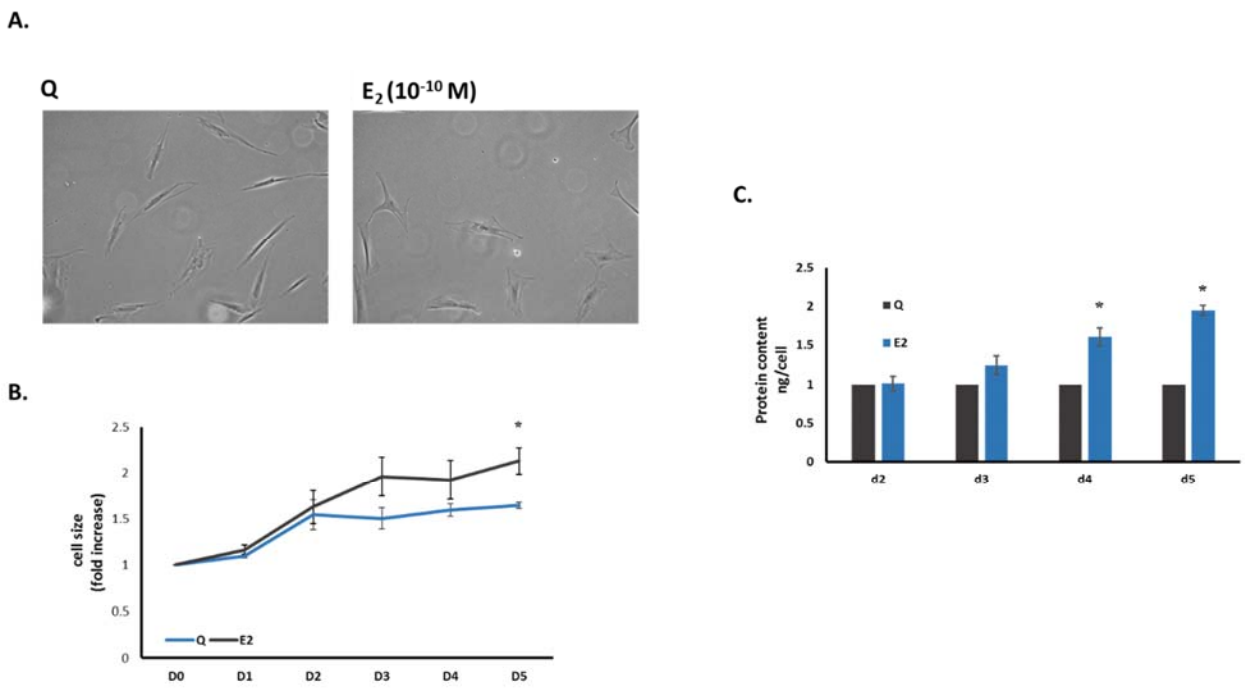


Figure 21. Effect of estrogen on size of VSMC.

A. Cells were treated with E_2 (10^{-10} M) for 5 days (replenished every other day). Representative photomicrographs of the effect of estrogen on cell size at day 5. B. Cell area was measured. Values represented are mean ± SEM of % vehicle control.(*$p<0.05$). C. cells were treated with E_2 (10^{-10} M) for 5 days (replenished every other day), and protein content/cell was determined. Values represented are mean ± SEM of % vehicle control.(*$p<0.05$).

D. Discussion:

Cardiovascular diseases are the leading cause of death world-wide[464]. Their incidence in premenopausal females is much lower compared to age-matched males[465]. This indicates a role of estrogen in vasoprotection and in the maintenance of VSMC differentiation[466]. Indeed, overwhelming evidence show that estrogen confers cardiovascular protection by playing an anti-inflammatory role, mainly attenuating the proliferation and the migration of VSMCs[467].

In this study, we reported the effect of estrogen on micro VSMCs. These VSMCs are extracted from human dermal arterioles. To our knowledge, this is the first study that documents the effect of 17-β-estradiol on VSMCs extracted from small vessels. Our results showed that estrogen increased the expression of VSMC contractile markers, calponin and caldesmon. In addition, estrogen inhibited FBS-induced VSMC proliferation and migration. The inhibition of these processes was concomitant with inhibition of FBS-induced ERK1/2. In addition, we showed that estrogen induced VSMC senescence by regulating pRb and p53.

It is well documented that VSMC proliferation and migration are key determinants of vascular inflammation[468]. This inflammation is attenuated by estrogen due to its anti-proliferative potential[450]. Surprisingly, many studies reported that estrogen promoted VSMC proliferation and migration[75,78] or at least did not attenuate VSMC inflammation[341]. Here, we showed that estrogen did not affect the proliferation and migration of quiescent VSMCs. This result is in accordance

with a previous study reporting that estrogen had no effect on the proliferative and the migratory ability of cultured un-stimulated VSMCs[76]. On the other hand, we showed that estrogen attenuated the proliferation and migration of FBS-induced VSMCs. Studies using other stimuli such as PDGF reported similar results[77,108,469].

Estrogen inhibited FBS-induced VSMC proliferation by blocking the cells in the G0/G1 phase of the cell cycle. Thus, cell cycle progression from the G0/G1 to the S-phase was attenuated. An important regulator of this transition is the Retinoblastoma protein, pRb. In fact, pRb is a tumor suppressor protein whose phosphorylation leads to its inactivation and thus cell cycle progression[470]. In quiescent VSMC, pRb is hypophosphorylated[470]. FBS-treatment induces pRb phosphorylation and thus cell cycle progression. Interestingly, estrogen pre-treatment lead to a decrease in the FBS-induced phosphorylation of pRb. This finding is in accordance with a previous study were estrogen inhibited pRb and caused G1 arrest of PDGF-stimulated human aortic SMCs[108]. Another player involved in cell cycle arrest is the tumor suppressor protein, p53[471]. The upregulation and the phosphorylation of this protein leads to cell cycle arrest[472]. In our study, estrogen induced the upregulation of p53 in FBS-stimulated VSMCs.

The regulation of the two afore-mentioned tumor suppressor proteins, pRb and p53, may lead to apoptosis or cell senescence. Although estrogen upregulated caspase-3, the terminal effector in both extrinsic and extrinsic apoptotic pathways[473], it failed to induce caspase-3 cleavage. Alternatively, estrogen induced senescence of VSMCs. This suggests that the anti-proliferative estrogenic effect is not due to apoptosis, but rather due to cell senescence.

MAPK are protein kinases that are involved in many cell processes including cell proliferation, gene expression and differentiation[474]. In addition, MAPKs regulate pRb and p53[475]. Interestingly, the vast majority of estrogen signaling pathways in VSMCs involve

MAPKs[476,477], namely, JNK[94], p38[94,478,479], and ERK1/2[100,102,478,480]. Here, we showed that estrogen induced the activation of p38 and JNK in quiescent VSMCs, and attenuated ERK1/2 phosphorylation in FBS-induced cells. This result is in line with previous research reporting estrogen-induced attenuation of ERK1/2 phosphorylation in PDGF-stimulated VSMCs extracted from mouse aorta[481], aorta of female New Zealand rabbit[480] , and human aorta[94]. Interestingly, attenuation of PDGF-induced ERK1/2 in VSMCs may be induced by G-1, GPR30 agonist[100].

5' AMP-activated protein kinase (AMPK) is a serine/threonine kinase[482]. Recent studies showed that in addition to its function as energy sensor and regulator, AMPK plays in an important role in cardiovascular diseases[483]. In rat primary VSMCs, AMPK inhibited cell proliferation and migration[483,484]. In addition, AMPK agonist, 5-aminoimidazole-4-carboxamide-1-β-d-ribofuranoside (AICAR), inhibited neo-intima formation in rat artery[483,485]. In addition, AMPK phosphorylation has been reported to mediate the vasoprotective effect of estrogen[476]. In our study, estrogen activated AMPK in quiescent and FBS-induced VSMCs. Therefore, the anti-proliferative effects of estrogen maybe mediated by two signaling pathways involving ERK1/2 and AMPK.

Estrogen may bind to cytoplasmic receptors, ERα/β to initiate a genomic response[451]. Alternatively, estrogen may elicit a rapid response by binding to the membrane receptor, GPR30[454]. In our study, estrogen initiated the activation of downstream targets in minutes. This rapid estrogenic response supports a scenario where estrogen binds to GPR30. Furthermore, ERα/β antagonist, ICI 182,780, did not inhibit the inhibitory effect of estrogen on FBS-induced proliferation and migration. This indicates that estrogen effect is ERα/β-independent, and further supports the involvement of GPR30. This result is in agreement with previous studies showing

that estrogen cardioprotective effect is mediated via GPR30 rather than ERα and ERβ. For instance, estrogen elicited a cardioprotective effect in ERα/β deficient mice[91-93]. ICI 182,780 did not affect the inhibitory effect of estrogen on post-injury neo-intima formation in aorta of female New Zealand White (NZW) rabbits[486]. In accordance to this, a recent study presented a model where the estrogenic effects on VSMCs are only GPR30-dependent[100]. This discrepancy may explained by the dominantly-expressed receptor during estrogen treatment or the crosstalk between all three receptors[100,114,120,487]. Further evidence argue for the role of GPR30 as a mediator of estrogenic effect on various cells and not only VSMCs. In pancreatic islets of female mice, ICI 182,780, did affect the hormonal pattern induced by estrogen treatment[488]. Additionally, in breast cancer cell line (SKBR3) lacking both ERα and ERβ, estrogen was able to initiate growth-factor dependent cellular responses[489]. These responses were absent in MDA-MB-231 breast cancer cells which are GPR30 deficient[490]. Interestingly, in our study, not only did not ICI 182,780 inhibit estrogen effect, but also it mimicked it, acting as an estrogen agonist. In fact, ICI 182,780 inhibited FBS-induced proliferation and migration. In addition, ICI 182,780- and E_2- pretreated FBS-induced VSMCs showed a similar cell cycle profile. This is not the first study to report the ICI 182,780 as an estrogen agonist. A previous study showed that estrogen as well as ICI 182,780 were able to decrease the proliferation of lyso-phosphatidylcholine-stimulated VSMCs extracted from rat aorta[491]. Another study done in mHippoE-18, an embryonic mouse hippocampal cell line, showed that estrogen response can be mimicked by ICI 182,780, probably by binding to GPR30[492]. Additionally, in breast cancer cells, ICI 182,780 was also reported to function as GPR30 agonist [490].

Contractile VSMCs are characterized by the expression of differentiation markers such as calponin and caldesmon[445]. These markers play an important role in the regulation of VSMC

contraction[445]. The majority of previous studies assessed the expression levels of these markers in macro VSMCs[493], we showed that estrogen increased the expression of these differentiation markers in micro VSMCs estrogen promotes contractile phenotype of VSMCs.. In fact, this is the first study to evaluate the effect of estrogen on differentiation markers. Furthermore, calponin and caldesmon are actin binding proteins[494]. In VSMCs, 80% of total actin is made up of F-actin[495], which results from the polymerization of G-actin[496]. Expectedly, estrogen increased actin polymerization, which is in line with the increased calponin and caldesmon. These results indicate that estrogen increased contractility of VSMCs.

In response to certain stimuli such as pressure, VSMCs increase in size without undergoing cell division[497]. This process is termed VSMC hypertrophy[497]. Estrogen supplementation was reported to reverse VSMC hyperplasia from ovariectomized mice[111]. However, in our study, estrogen lead to a remarkable increase in cell size. This observed VSMC hypertrophy may be in accordance the protective role of estrogen. It seems that estrogen responds to various insults in a case-dependent manner. As such, estrogen may promote rapid VSMC proliferation to repair a vessel, and it may inhibit VSMC proliferation to reduce the pathological outcomes of the repair process[83]. In our hypertrophy assay experiment, cells were seeded at a low confluency, which may be considered as an insult. In order for VSMCs to physically communicate in a starvation medium, estrogen may have promoted *in-vitro* increase in size of cell so that they can cover a larger surface area.

This study shows that estrogen attenuates the proliferative and migratory ability of FBS-induced micro VSMCSs in an ERα/ERβ-independent pathway. By potentially binding to GPR30, estrogen induces VSMC cell cycle arrest and pRb/p53-mediated cell senescence (figure 22). It is worth mentioning that hat the response and the signaling mechanism of VSMC to estrogen is

greatly affected by the vascular bed from which they are extracted. , our study presents, for the first time, the estrogen-activated pathways in microVSMCs. In addition, our results supporting a protective role of estrogen on microvasculature, may explain the lower prevalence in estrogen-replete premenopausal females.

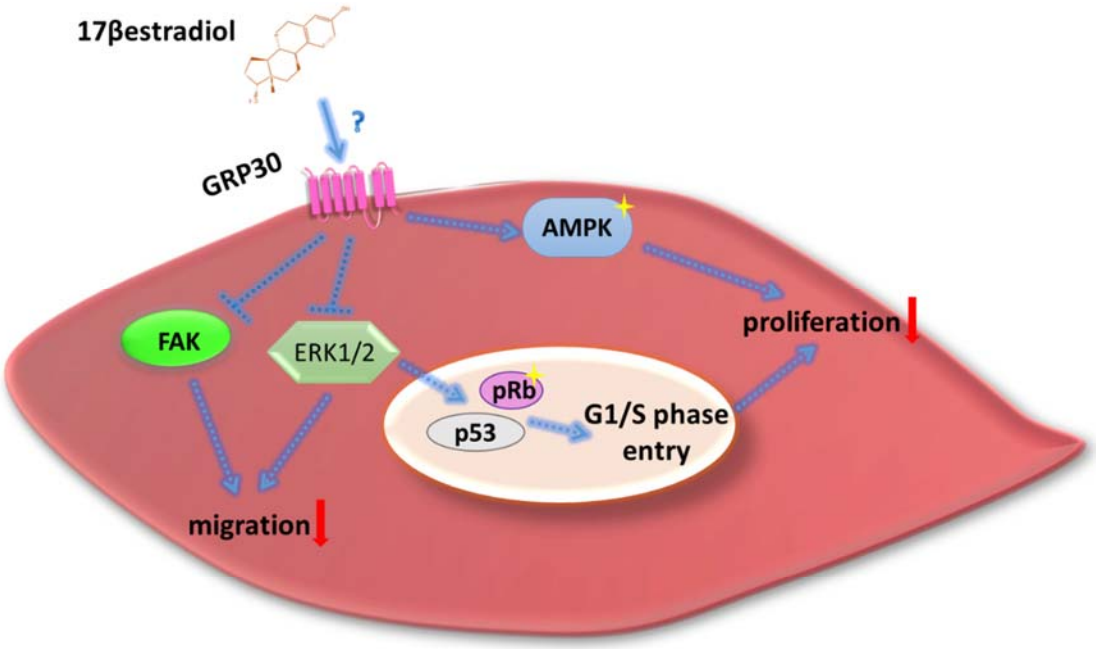


Figure 22. The proposed signaling pathway by which estrogen inhibits serum-induced VSMC inflammationof estrogen-induced effects on VSMCs.

CHAPTER IV

7-*O*-METHYLPUNCTATIN, A NOVEL HOMOISOFLAVONOID, INHIBITS PHENOTYPIC SWITCH OF HUMAN ARTERIOLAR SMOOTH MUSCLE CELLS

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Remodeling of arterioles is a pivotal event in the manifestation of many inflammation-based cardio-vasculopathologies, such as hypertension. During these remodeling events, vascular smooth muscle cells (VSMCs) switch from a contractile to a synthetic phenotype. The latter is characterized by increased proliferation, migration, and invasion. Compounds with anti-inflammatory actions have been successful in attenuating this phenotypic switch. While the vast majority of studies investigating phenotypic modulation were undertaken in VSMCs isolated from large vessels, little is known about the effect of such compounds on phenotypic switch in VSMCs of microvessels (microVSMCs). We have recently characterized a novel homoisoflavonoid that we called 7-O-methylpunctatin (MP). In this study, we show that MP decreased FBS-induced cell proliferation, migration, invasion, and adhesion. MP also attenuated adhesion of THP-1 monocytes to microVSMCs, abolished FBS-induced expression of MMP-2, MMP-9, and NF-κB, as well as reduced activation of ERK1/2 and FAK. Furthermore, MP-treated VSMCs showed an increase in early (myocardin, SM-22α, SM-α) and mid-term (calponin and caldesmon) differentiation markers and a decrease in osteopontin, a protein highly expressed in synthetic VSMCs. MP also reduced transcription of cyclin D1, CDK4 but increased protein

levels of p21 and p27. Taken together, these results corroborate an anti-inflammatory action of MP on human microVSMCs. Therefore, by inhibiting the synthetic phenotype of microVSMCs, MP may be a promising modulator for inflammation-induced arteriolar pathophysiology.

A. Introduction

Arterioles are internally wrapped with one or more layers of vascular smooth muscle cells (VSMCs) [498]. Under normal physiological conditions, VSMCs assume a contractile phenotype [447]. It is this phenotype that is largely responsible for the contractility of blood vessels, allowing them to tightly control vasotone and regulate flow both under physiologic and pathophysiologic conditions [42,447,499]. In response to inflammatory cues, VSMCs switch from the contractile to a dedifferentiated synthetic phenotype, with increased proliferative and migratory abilities [447]. This VSMC phenotypic switch plays a key role in vascular/arteriolar inflammation and remodeling [500].

The use of herbal medicine in the prevention and treatment of cardiovascular disease (CVD) has been substantially growing [501,502]. Remarkably, the PubMed database contains more than 600 clinical trials and around 3300 publications dealing with herbal drugs for CVDs [502]. These reports provide scientific evidence on the beneficial role of herbal medicine in CVD. Among the many herbal compounds associated with cardio-vasculoprotective effects are flavonoids [503-507]. For instance, the flavonoid-rich herb S*cutellaria baicalensis* was reported to confer protection against ischemic heart disease [508]. Likewise, flavonoids are known to ameliorate atherosclerosis [509] and exert an antihypertensive effect [510]. Importantly, several studies tested the effect of many chemicals/flavonoids with anti-inflammatory actions on vascular cells. Nonetheless, most of these

drugs were tested on VSMCs isolated from large vessels [511-514]. Indeed, very little is known about compounds targeting arteriolar inflammation.

A special class of flavonoids distinguished by one additional carbon atom on their carbon cytoskeleton are the homoisoflavonoids (3-benzylidenechroman-4-ones) [515]. They constitute a rare class of natural compounds [515]. More than 240 natural homoisoflavonoids have so far been reported, all restricted to only six plant families: Fabaceae, Asparagaceae, Polygonaceae, Portulacaceae, Orchidaceae, and Gentianaceae [511,515,516]. Recently, homoisoflavonoids have been receiving increased interest due to their broad spectrum of biological effects [515]. These include anti-inflammatory [517], anti-hyperglycemic [518], anti-mutagenic [519], anti-microbial [520], antiviral [521], and anti-oxidant activities [522]. The anti-oxidant effect seems to be the most important and most extensively studied owing to its potentially beneficial effects in diabetes and inflammation [523]and CVD [524]. For instance, *Ophiopogonin japonicus*, rich in anti-oxidative homoisoflavonoids, appears to be effective in treating myocardial ischemia and arrhythmias [524]. Thus, by virtue of their anti-oxidative potential, homoisoflavonoid-rich plants may be regarded as an important resource in the management or treatment of CVD.

Bellevalia eigii Feinbrun is a perennial plant belonging to the family Asparagaceae [511,525]. It is native to Mediterranean region and Sinai [526] and is widespread in Jordan, where it is known among local people as “the Jordan Valley onion” [511]. From the bulbs of *Bellevalia eigii* Feinbrun, we recently isolated, purified and characterized a new compound, 7-*O*-methylpunctatin (MP) [511] (figure 23). Here, we sought to determine the effect MP on FBS-induced inflammation of human VSMCs extracted from dermal arterioles.

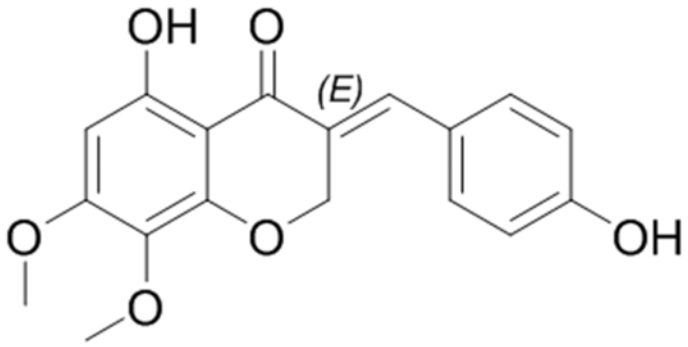


Figure 23. The chemical structure of 7-*O*-methylpunctatin (MP).

B. Materials and Methods

1. Reagents

Anti-Calponin antibody (ab46794), anti-Caldesmon antibody (ab32330), anti-β actin antibody (ab119716), anti-ERK1/2 antibody (ab17942), anti-ERK1 (phospho Y204) + ERK2 (phospho Y187) antibody (ab47339), anti-FAK antibody (ab61113), anti-FAK antibody (phospho Y397, ab81298), HRP-conjugated Goat Anti-Mouse antibody (ab97040), HRP-conjugated Goat Anti-Rabbit antibody (ab ab150080), and Tetrazolium (ab146345) were purchased from Abcam (Cambridge, UK). Anti-GAPDH antibody (2118), anti-caspase-3 (8G10), anti- NF-κB p65 antibody (3034), anti-IκB antibody (9242), and anti-IκBα (phospho Ser32/36**,** 9246) were obtained from Cell Signaling Technology (Leiden, The Netherlands). Anti-p21antibody (sc-397) and anti-p27 antibody (sc-1641) were purchased from Santa Cruz Biotechnology (Dallas, USA). Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham DMEM: F12 (BE12-719F), RPMI-1640, Penicillin/Streptomycin (17-602E), and Trypsin (BE02-007E), DMSO (0231) were obtained from Lonza (Basel, Switzerland). Fetal Bovine Serum FBS (F9665), Phosphorous Buffer Saline PBS (D1408), and Propidium Iodide (P4170) were purchased from Sigma-Aldrich (Schnelldorf, Germany). MMP-2 and MMP-9 ELISA kits

were obtained from R&D Systems (Minneapolis, MN, USA), *DC* Protein Assay kit and ClarityWestern ECL Substrate from Bio-rad (Irvine, CA, USA), BrdU kit from Roche Diagnostics (Penzberg, Germany), Luciferase Assay Kit from Promega (Fitchburg, WI, USA), Moloney murine leukemia virus reverse transcriptase (RT) from Invitrogen (Carlsbad, CA, USA), and SYBR Green fluorophore from SuperArray Bioscience Corporation (Frederick, MD, USA).

2. Cell Culture

Human arteriolar smooth muscle cells were extracted by the non-enzymatic sprouting method from post-circumcision tissue of a newborn boy. No IRB approval is needed as this source is considered clinical waste. Cells were grown in Ham's Growth medium (DMEM: F12, 50:50; supplemented with 10% FBS, and 1% penicillin/streptomycin). Only cells of passages 8–11 were used. Before treatment, cells were synchronized by starvation in a quiescent serum-free medium (DMEM: F12, 50:50, 0.5% FBS, 1% penicillin/streptomycin) for 48 h, as previously described [54]. THP-1 cells were cultured in RPMI-1640 and supplemented with 10% FBS and 1% penicillin/streptomycin. Cells were maintained in a humidified incubator at 37 °C with 5% CO_2 atmosphere.

3. Preparation of 7-O-methylpunctatin

Extraction, characterization, and purification of MP was done as we recently reported [511]. MP was stored at −20 °C, and for cell treatment, it was dissolved in DMSO. The dissolved compound was stored in the dark at −20 °C.

4. MTT Assay

VSMCs were grown in 96-well plate until they reached 30–40% confluence. Then cells were starved in serum-free medium for 48 hrs. Following starvation, cells were treated with increasing concentrations of MP for 24, 48, and 72 h. MTT solution (20 µL, 5 mg/mL) was added to each well, and cells were incubated for an hour in a 5% CO_2 incubator. The medium was then removed, and 200 µL DMSO was added to each well. The plate was placed on a shaker for 15 min to allow for the dissolution of formazan crystals. Using an ELISA Multiscan EX Reader (ThermoFisher, Vantaa, Finland), optical density was read at 550 nm. Absorbance is directly proportional to cell viability.

5. BrdU Incorporation Assay

Here, five thousand cells/well were seeded into 96-well plates. Cells were then starved for 48 h before commencing any treatment. Cell proliferation was then measured with BrdU kit (Roche Diagnostics, Penzberg, Germany) following the manufacturer's protocol. Optical density was measured using a microplate reader spectrophotometer at excitation wavelength 450 nm.

6. Cell Cycle Analysis

Cells were made quiescent by culturing in starvation medium for 48 h. After starvation, cells were treated for 48 h with complete medium in the absence or presence of MP. They were then washed with PBS, trypsinized, and collected by centrifugation. After washing twice with ice-cold PBS, cells were re-suspended in 500 µL PBS. For permeabilization and fixation, 2 mL of ice-cold pure ethanol was added for 15 min. The cell suspension was centrifuged, and the cell pellet was washed twice with PBS. Cells were then incubated for 10 min in 1 mg/mL of

propidium iodide in PBS. Propidium iodide (PI) fluorescence was read using Guava EasyCyte8 Flow Cytometer (Luminex, Hayward, CA, USA). Cell cycle analysis was done using Guava Soft 2.7 software.

7. RT-PCR

Cells were seeded and allowed to grow in complete medium, then starved for 48 h. Total RNA was extracted using Nucleospin RNA II kit as per the manufacturer's protocols (Machery Nagel, Germany). cDNA was then synthesized using 1 µg of total RNA by RevertAid 1st strand cDNA synthesis kit (Thermo Fisher Scientific, USA). RT-PCR was then performed using the iQ SYBR green supermix. Using serial dilutions of cDNA of positive controls for each gene of interest, standard curves are determined and plotted, and then the threshold cycle value (Ct) obtained for each gene and normalized to the housekeeping gene GAPDH (internal control). The ΔΔCt method was used to analyze expression changes between the different conditions, where the control untreated group value is set to one. Gene sequences were amplified using the following primers:

Cyclin D1F: TCCTGTGCTGCGAAGTGGAAAC;

Cyclin D1R: AAATCGTGCGGGGTCATTGC;

cdk4F: AAGAGTGTGAGAGTCCCCAATGG;

cdk4R: GATTTTGCCCAACTGGTCGG;

Myocardin F: GAGAGGTCCATTCCAACTGC;

Myocardin R: GGGCTGTGAGGCTGAGTC;

SM-22α F: TCCAGGTCTGGCTGAAGAATGG;

SM-22α R: CTGCTCCATCTGCTTGAAGACC;

SM-α F: ACTGAGCGTGGCTATTCCTCCGTT

SM-α R: GCAGTGGCCATCTCATTTTCA;

GAPDH F: CGCTCTCTGCTCCTCCTGTTC;

GAPDH R: TTGACTCCGACCTTCACCTTCC.

8. Phase Contrast Microscopy

VSMCs were grown in 6-well plates. Cells were then starved for 48 h then treated with complete medium in the absence or presence of MP. Images were acquired using a phase-contrast microscope (Zeiss, Oberkochen, Germany) after 24, 48, and 72 h.

9. Scanning Electron Microscopy

Cells were cultured in complete medium on coverslips in 12-well plates. At 80% confluency, cells were starved for 48 h, then treated with complete medium in the absence or presence MP for 48 hrs. Wells were then washed and cells fixed with 2.5% glutaraldehyde for 45 min at 4 °C. After washing with PBS, cells were dehydrated with increasing ethanol concentrations (25, 50, 75, 95, and 100%) for 5 min per incubation. The coverslips were mounted on scanning electronic miscoscope (SEM) stub, cells were sputtered with gold and images were acquired using Tescan SEM (MIRA3 software; Brno; Czech Republic).

10. Wound Healing (Scratch) assay

Cells were cultured in 12-well plates until 90–95% confluent. They were then incubated in quiescent medium (0.5% FBS) for 48 h. Using a 10 µL sterile pipette tip, a scratch was made on the cellular monolayer. Wells were washed with PBS to remove cellular debris, and medium

was replenished in the absence or presence of MP. Wound healing was monitored at 0, 2, 4, 6, 8, 12, and 24 h, and photomicrographs were taken using a Zeiss phase contrast microscope. ZEN imaging software (blue edition) from Zeiss was used to measure the width of the scratch.

11. Invasion Assay

Transwell inserts were coated with matrigel and allowed to dry overnight under ultraviolet light. Cells in serum-free media were seeded onto the rehydrated upper transwell chamber in the absence or presence of MP. The lower chamber was loaded with complete medium, acting as a chemotactic attractant. Cells were then incubated at 37 °C for 24 h. After treatment, the medium was aspired, and wells were washed with PBS. Non-invading cells were removed from the upper surface with a cotton swab, whereas invading cells were fixed with methanol and stained with DAPI. The membrane was cut with a blade and mounted on an anti-fade agent. Slides were observed under Zeiss Axio fluorescent microscope. Cells from at least five different fields were counted.

12. Cell Adhesion Assay

Cells in starvation medium were seeded in 6-well plates and allowed to adhere for 1 h at 37 °C. Then, wells were gently washed with PBS to remove non-adherent cells. Images were acquired using Zeiss phase contrast microscope and adherent cells were counted.

13. Monocyte Adhesion Assay

Cells were grown in complete medium until confluence. Cells were then treated with MP for 1hour followed by treatment with PMA for 20 h. THP-1 cells labelled with NucBlue

(Thermo Fischer Scientific) were added over the VSMC monolayer and allowed to adhere for 30 min. Non-adherent THP-1 cells were removed by washing the wells with PBS. Images of the adherent THP-1 cells were acquired using Zeiss Axio fluorescent microscope. In addition, quantitative analysis was done by measuring the fluorescence intensity of five random fields of photomicrographs.

14. Measurement of MMP-2 and MMP-9

Cells were grown to a subconfluent level and then starved for 48 h. Following treatment with the respective conditions, medium of each condition was collected for MMP-2 and MMP-9 detection using ELISA kits (R&D Systems), as recommended by the manufacturer and we recently reported [62].

15. Actin Staining

At 30–40% confluence, cells were starved in quiescent medium for 48 h. Following treatment, cells were washed twice with PBS, fixed with 4% paraformaldehyde, and permeabilized with 0.1% Triton X-100. Cells were then washed again and incubated with Rhodamine phalloidin stain for 1 h in the dark. Cells were washed again, and nuclei were counter-stained with DAPI for 15 min at room temperature. Cells were mounted on an anti-fading agent and visualized using Zeiss Axio fluorescent microscope.

16. Luciferase Reporter Assay

As we previously reported [304], cells were transiently transfected with NF-κB-driven promoter luciferase using Amaxa Nucleofector (Amaxa Biosystems, Gaithersburg, MD, USA)

according to the manufacturer's protocol. Renilla luciferase vector was used as an internal control, to which firefly luciferase values were normalized. Following transfection, cells were allowed to recover overnight, and then starved for 48 h. After treatment, cells were washed and lysed in luciferase lysis buffer (Promega), and luciferase activity was measured.

17. Western Blotting

Cells were washed with PBS and then lysed using 2% SDS, 60 mM Tris lysis buffer (pH 6.8) as previously described [527]. Proteins were quantified using *DC* Protein Assay and equal amounts of protein (20–30 µg) were loaded and separated using 5–11% SDS-PAGE. Proteins were then transferred onto polyvinylidene difluoride (PVDF) membrane (Biorad). After blocking in 5% fat-free milk in TBS-T, 1 h at room temperature, the membrane was incubated overnight with the relevant primary antibody at 4 °C. The membrane was then washed thrice with TBS-T for 10 min each and incubated with the appropriate HRP-conjugated secondary antibody for 1 h at room temperature. The membrane was washed again (three times with TBS-T, 10 min each) and then developed using enhanced chemiluminescence (ECL clarity, Biorad) and quantified using Chemidoc MP Imaging system (Bio-rad).

18. Statistical Analysis

Statistical analyses were performed by student's *t*-test for either paired or unpaired observations. For multiple comparisons, ANOVA was used—either one-way ANOVA (with Dunnett's post hoc test) or two-way ANOVA (with Tukey-Kramer's post hoc test). Except for Western blotting, experiments were performed at least three times, and each time was made of triplicate wells. The average of the triplicate from each experiment (individual mean) was

calculated, and these means were then averaged. Data were presented as mean ± SEM. A *p*-value of less than 0.05 was considered as significant.

C. Results

1. MP Inhibits FBS-Induced VSMC Proliferation

Cells were stimulated with FBS then were treated with or without MP. MP inhibited proliferation in a concentration- and time-dependent manner (figure 24.A). At the concentrations of 200 and 300 μM, MP significantly reduced the number of viable cells at 24, 48, and 72 h. However, the lowest MP concentration (100 μM) caused a significant reduction only at 48 and 72 h (100 vs 83% and 100 vs 77% respectively; $p < 0.05$ for both). A similar result was observed when FBS-unchallenged (i.e., quiescent) cells were treated with increasing concentrations of MP (100, 200, and 300 μM) (figure 24.B).This suggests that MP inhibits basal and FBS-induced proliferation of these cells.

In order to determine whether the anti-proliferative effect of MP is associated with a change in DNA synthesis, BrdU incorporation assay was employed. MP slightly but significantly decreased basal BrdU incorporation (100 vs 88%; $p < 0.05$) (figure 24.C). As expected, FBS induced a significant increase in DNA synthesis (100 vs 258 ± 28%; $p < 0.05$) (figure 24.C). This increase was potently diminished by MP (258 ± 28% vs 140 ± 20%; $p < 0.01$). These results are in line with the anti-proliferative effect of MP evaluated by MTT.

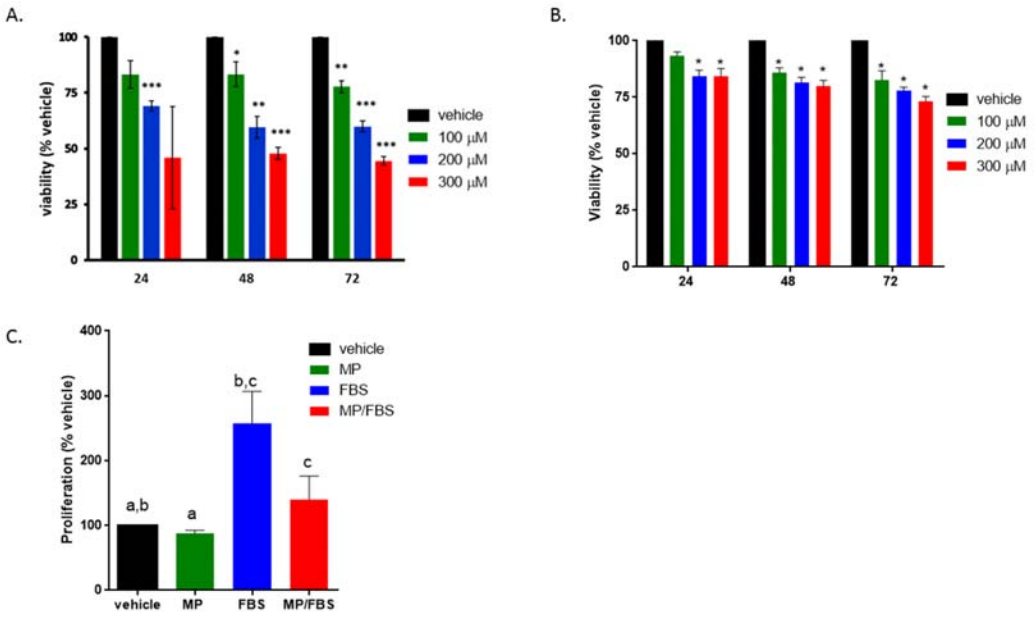


Figure 24. MP attenuates basal and FBS-induced vascular smooth muscle cells (VSMC) proliferation.

Cells were treated with increasing concentrations of MP (100, 200, and 300 µM) for 24, 48, and 72 h in the presence (**A**) or absence (**B**) of FBS. Cell viability was assessed by the overall metabolic activity measured by MTT. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.005$. Two-way ANOVA was performed. (**C**) Cells were grown in starvation or complete medium, in presence or absence of MP (100 µM). DNA synthesis was assessed BrdU incorporation assay. Values are calculated as % of the corresponding vehicle control value and represented as mean ± SEM of three different experiments, each run in triplicate. Bars with same letters are statistically significant. One-way ANOVA followed by Tukey's test was performed.

2. MP Induces Cell Cycle Arrest of FBS-Induced VSMC

Having established that MP attenuates FBS-induced cell proliferation, we next sought to assess the effect of MP on cell cycle progression. As shown in figure 24. A, MP increased the G_0/G_1 cell population, while decreasing those in the S and G_2/M phases. Expectedly, treatment with FBS decreased the percentage of cells in G_0/G and increased the percentage of cells in the S phase (figure 24. A). These FBS-induced changes in the cell cycle profile were attenuated by

pretreatment with MP. This indicates that MP inhibited cell proliferation by arresting the cells in the G_0/G_1 phase.

3. MP Downregulates the Expression of Cyclin D1 and CDK4 and Upregulates the Expression of CDK Inhibitors, p21 and p27, in VSMCs

To further validate our results, we sought to characterize the changes in the expression of factors directly involved in cell cycle regulation. Here, we looked at the expression level of cyclin D1, CDK4, p21 and p27. RT-PCR analysis showed that MP significantly decreased the mRNA level of cyclin D1 and CDK4 (figure 24.B and C). As expected, treatment with FBS induced an increase in the transcript level of both cyclin D1 (100 vs 252 ± 21%; $p < 0.05$) and CDK4 (100 vs 259 ± 49%; $p < 0.05$) (figure 24.B and C). This increase was abrogated by MP (252 ± 21% vs 127 ± 12% for cyclin D1 or 259 ± 49% vs 125 ± 26% for CDK4) (figure 24.B and C). Furthermore, MP induced an increase in the expression of p21 and p27 (figure 24.D). While the upregulation of p21 was noticeable at 24 and 48 h (figure 24.D), p27 was upregulated only after 48 h of treatment with MP (figure 24.D). These results further reinforce our hypothesis that MP attenuates FBS-induced cell cycle progression by inhibiting the escape from G_1 phase.

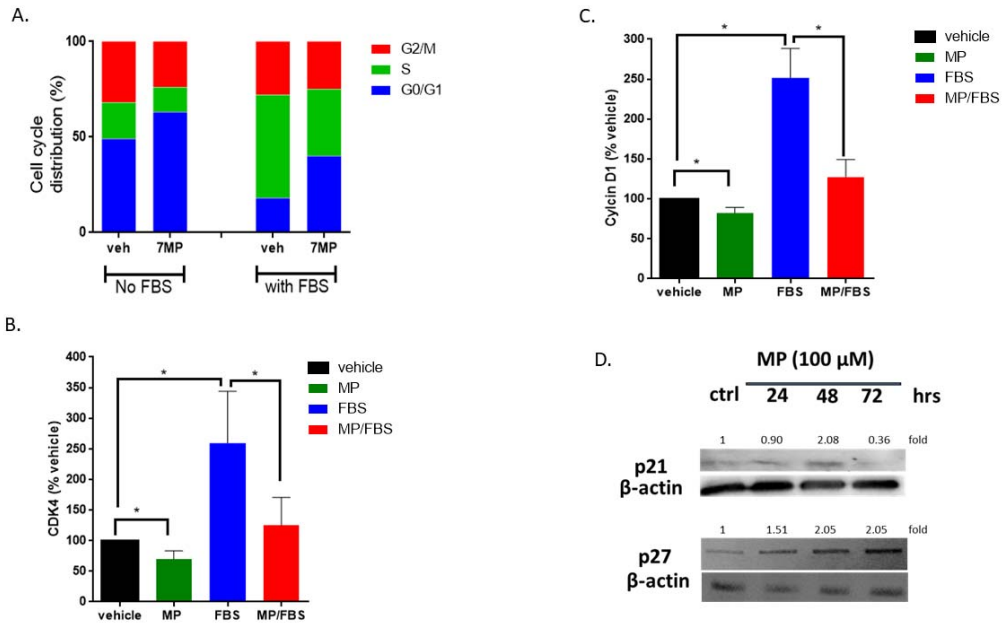


Figure 25. MP blocks VSMCs in G_0/G_1 phase of cell cycle.

(**A**) Cells were treated with MP (100 µM) for 48 hrs, stained with PI, then sorted by flow cytometer. Data represent the mean of three independent experiments. (**B**) and (**C**) Cells were grown in starvation or complete medium, in presence or absence of MP (100 µM). The expression levels of CDK4 (**B**) and Cyclin D1 (**C**) were determined by RT-PCR. Values are calculated as % of the corresponding vehicle control value and represented as mean ± SEM of three replicates. (*$p < 0.05$). (**D**) Cells were treated with MP (100 µM) for 24, 48, and 72 hrs. The expression of p21 and p27 was detected by Western blotting. Values mean fold change of three replicates. One-way ANOVA followed by Tukey's test was performed for all panels.

4. MP Induces VSMC Apoptosis

It is well-established that cellular morphologic changes greatly reflect as well as affect cellular function [528-530]. For instance, cells undergoing apoptosis show distinguished morphological features such as cell shrinkage and cytoplasmic extensions [531]. Light microscopic examination of MP-treated cells revealed morphological changes indicative of a loss in the VSMC characteristic spindle shape. These changes occurred 48 and 72 h post treatment (figure 24.A). Indeed, cells adopted a round translucent morphology (figure 24.A), likely indicative of cell death. Higher magnification showed shrunken and smaller-sized cells (figure 24.B). Moreover, treated

cells exhibited long string-like cytoplasmic extensions with blebs attached to their ends (figure 24.C; red arrows). All these changes are characteristic of apoptotic cells [532,533]. Using SEM, the cellular ultrastructure revealed the presence of cytoplasmic protrusions known as apoptopodia (figure 24.D; red arrows). Collectively, these observations indicate that MP induces apoptosis of microvascular smooth muscle cells.

To validate our finding, we determined the effect of MP on caspase-3 cleavage and Bax/Bcl2 ratio. We found that caspase-3 was not activated in MP-treated VSMC for 24 h (figure 24.E). This result is in agreement with cell viability results and cell morphology images. However, longer exposure (48 and 72 h) to MP activated apoptotic cell death revealed by the cleavage of caspase 3 (figure 24.E). In addition, MP induced an increase in the Bax/Bcl2 ratio after 48 or 72 h of treatment (figure 24.E), suggesting the activation of the intrinsic apoptotic pathway.

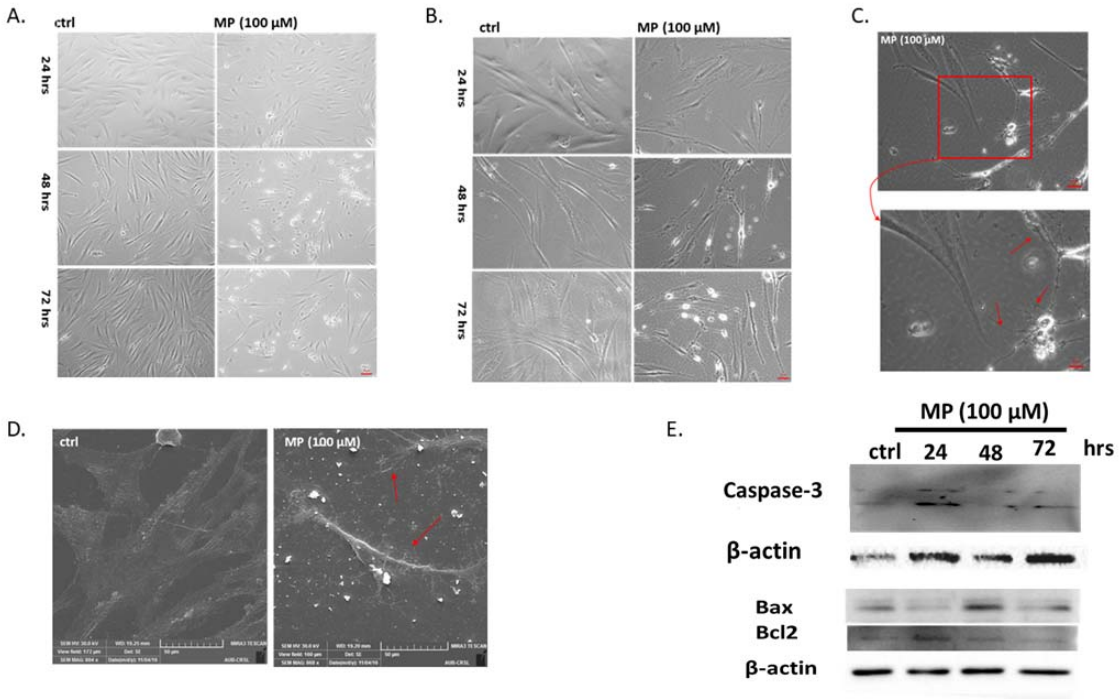


Figure 26. MP induces VSMC apoptosis. Cells were treated with MP (100 µM) for 24, 48, and 72 hrs.

Micrographs were captured at magnifications of (**A**) 4X, (**B**) 10X, and (**C**) 20X. (**C**) The area in the red square was captured at 100× magnification (lower micrograph). (**D**): Representative SEM images of MP-treated cells. Scale bars, 50 μm. In (**C**) and (**D**): Red arrows point at cytoplasmic extensions. (**E**) Cells were treated with MP (100 μM) for 24, 48, and 72 hrs. Activation of caspase-3 and the expression levels of Bax and Bcl2 were detected by Western blotting. Values mean fold change of three independent experiments. One-way ANOVA followed by Tukey's test was performed.

5. MP Attenuates FBS-Induced VSMC Migration, Invasion, and Adhesion

The effect of MP on FBS-induced migration was examined using scratch assay. MP, at a concentration of 100 μM, significantly attenuated wound-healing ($p < 0.05$) after 12 h (figure 24.A and B). At this time point, no cytotoxic effect of MP was observed (data not shown) (figure 24.A and B), indicating that the anti-migratory capacity is independent of MP's anti-proliferative effect. In addition, we evaluated the effect of MP on the invasive capacity of VSMCs using Matrigel-coated Boyden chambers. Our results showed that MP inhibited FBS-induced invasiveness (figure 24.C).

Because cell adhesion to its substratum is critical for cell migration and invasion, we next determined the effect of MP on VSMC adhesion. We found that MP significantly inhibited VSMC adhesion as shown in figure 24 D and E.

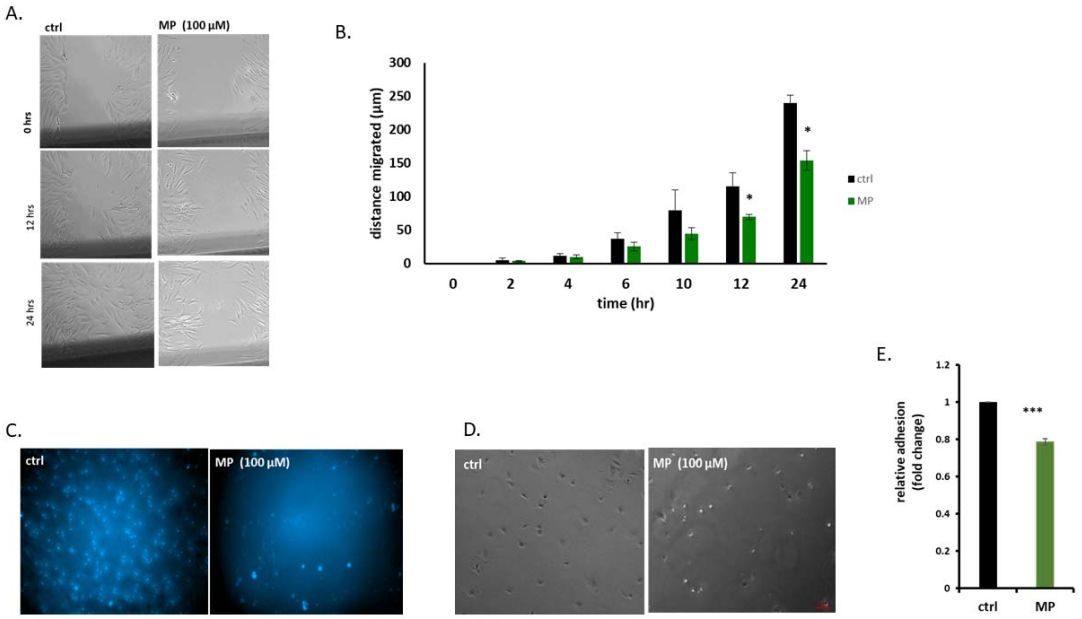


Figure 27. MP inhibits VSMC migration, invasion, and adhesion.

(**A**) Cells were treated with MP (100 µM) and cell migration was assessed by scratch assay. Images were taken at the indicated time points (Scale bar, 50 µm). (**B**) Values are represented as mean ± SEM of distance migrated ($n = 3$ replicates) (*$p < 0.05$). (**C**) Cells were treated with MP (100 µM). Cell invasion was evaluated using invasion assay. Representative photomicrographs showing the effect of MP on invading cells. (**D**) Cells were treated with MP (100 µM) and allowed to adhere for 1 hr. Representative photomicrographs of the effect of MP on VSMC adhesion. Scale bar, 50 µm. (**E**) Values are represented as mean ± SEM of relative fold inhibition of vehicle-treated cells (*$p < 0.05$). One-way ANOVA was performed for all panels.

6. MP Inhibits MMP-2 and MMP-9 Secretion in VSMCs

Matrix metalloproteases-2 and -9 (MMP-2 and MMP-9) are known to play a major role in vascular remodeling. Specifically, the activation of MMP-2 and MMP-9 in response to inflammatory stimuli leads to ECM degradation, thus facilitating VSMC migration and invasion [534-536]. Here, our results show that MP significantly decreased the levels of secreted MMP-2 and MMP-9. Specifically, MP slightly but significantly reduced basal levels of secreted MMP-2 (100 vs 89 ± 3%; $p < 0.05$) (figure 24A) and MMP-9 (100 vs 83 ± 2%; $p < 0.05$) (figure 24.B). Stimulation with FBS induced a profound increase in MMP-2 (100 vs 283 ± 12%; $p < 0.01$) and

MMP-9 (100 vs 307 ± 9%; $p < 0.01$) (figure 248.A and B). This increase was significantly attenuated by pretreatment with MP (283 ± 12% vs 150 ± 11% or 307 ± 9% vs 157 ± 22%, for MMP-2 and MMP-9 respectively; $p < 0.01$ for both) (figure 24.A and B).

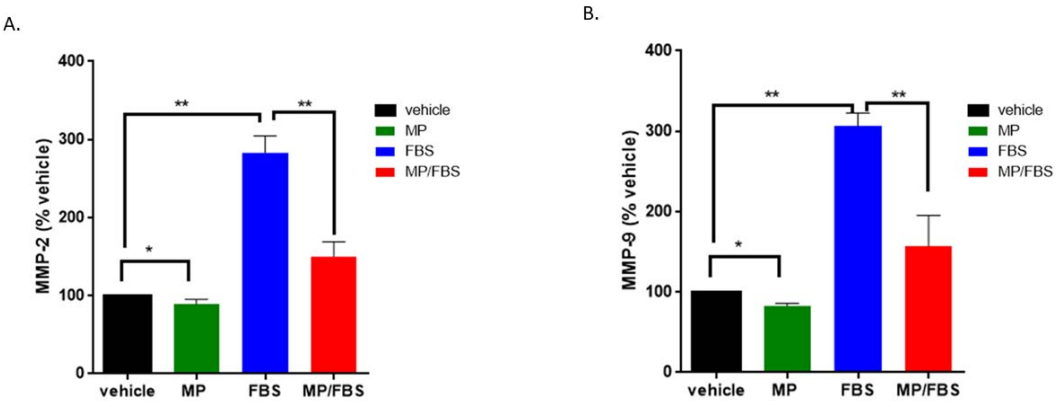


Figure 28. MP inhibits basal and FBS-induced MMP-2 and MMP-9 secretion.

Cells were grown in starvation or complete medium, in presence or absence MP (100 µM). Levels of secreted MMP-2 (**A**) and MMP-9 (**B**) were evaluated by ELISA. Data represented are mean ± SEM of % MMP level in the corresponding vehicle-treated well. (*$p < 0.05$, **$p < 0.01$). One-way ANOVA followed by Tukey's test was performed.

7. MP Decreases the Phosphorylation of ERK1/2 and FAK

Activation of the ERK1/2 pathway plays a key role in VSMC proliferation and migration [537-539]. In addition, FAK activation is associated with cell migration and adhesion [540]. Thus, we investigated the effect of MP on the phosphorylation of ERK and FAK using Western blotting. We found that MP induced a decrease in ERK1/2 phosphorylation in a time-dependent manner (figure 24). Moreover, FAK phosphorylation decreased as early as 10 min post-MP treatment (figure 24).

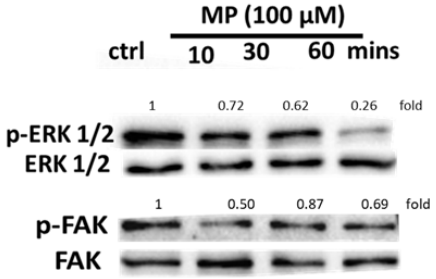


Figure 29. MP attenuates ERK1/2 and FAK phosphorylation in a time-dependent manner.

Cells were treated with MP (100 μM) for 10, 30, and 60 min. The phosphorylation levels of ERK1/2 and FAK were determined by Western blotting. Values mean fold change of three independent experiments.

8. MP Increases the Expression of Early and Mid-Term Differentiation Markers and Decreases the Expression of a De-differentiation Marker

VSMC phenotype, whether contractile or synthetic, may be defined by the level of expression of specific markers. The contractile phenotype is characterized by differentiation markers that are grouped into early, mid-term, and late differentiation markers [541]. On the other hand, synthetic VSMCs secrete many ECM proteins, including osteopontin and osteonectin [542,543].

Here, we show that MP significantly increased the expression of the early differentiation markers, SM22-α, SM α-actin, and myocardin (100 vs 197 ± 16%; 100 vs 181 ± 20%; or 100 vs 222 ± 22%; for SM22-α, SM α-actin, and myocardin respectively; $p < 0.01$ for all) in quiescent cells (figure 30.A–C). As expected, treatment with FBS induced a decrease in the expression of these markers. This decrease was greatly attenuated by MP (FBS alone versus FBS plus MP: 43 ± 8% vs 110 ± 14%; 51 ± 8% vs 142 ± 12%; or 41 ± 10% vs 160 ± 19%; for SM22-α, SM α-actin, or myocardin respectively; $p < 0.01$ for all). Similar results were obtained for mid-term differentiation markers, calponin and caldesmon (figure 30.D) Moreover, MP

abrogated the basal (100 vs 67 ± 14%; $p < 0.01$) and FBS-induced (223 ± 18% vs 118 ± 9%; $p < 0.01$) expression of osteopontin, a glycoprotein secreted by synthetic VSMCs (figure 30.E). These results indicate that MP drives VSMCs towards a contractile phenotype via increasing the expression of differentiation markers and decreasing the expression of osteopontin in quiescent and FBS-induced cells.

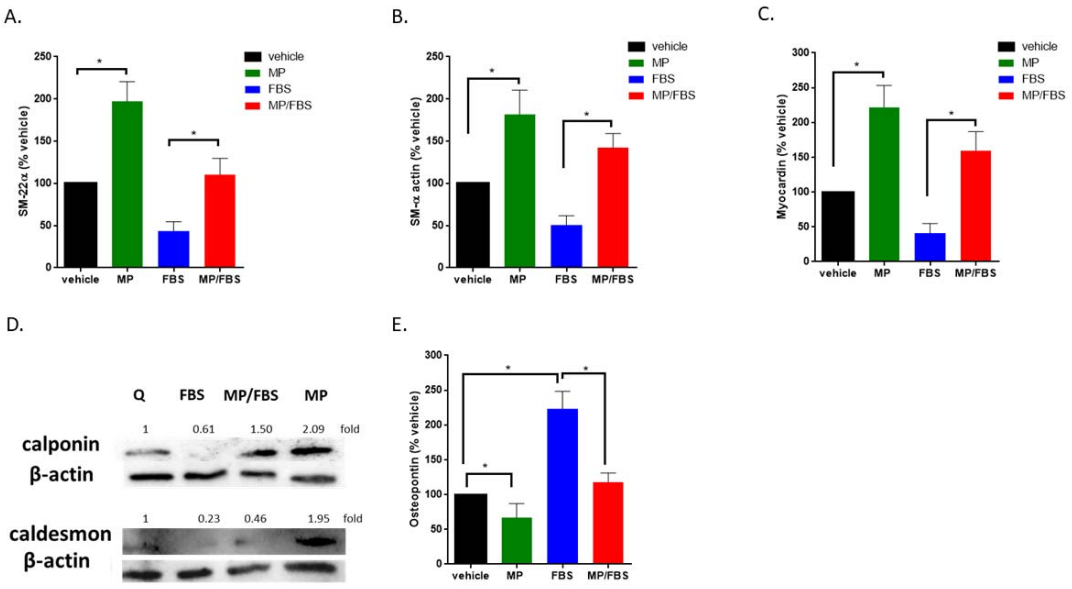


Figure 30. MP increases the expression of basal and FBS-attenuated differentiation markers and decreases basal and FBS-induced expression of osteopontin.

Cells were grown in starvation or complete medium, in presence or absence MP (100 µM). Expression levels of contractile differentiation markers (**A**) SM-22α, (**B**) SM-α, (**C**) myocardin were evaluated by RT-PCR. Values represented are mean ± SEM of % vehicle control. (*$p < 0.05$). (**D**) Cells were grown in starvation or complete medium, in presence or absence MP (100 µM). The expression of differentiation markers, calponin and caldesmon, was determined by Western blotting. Values mean fold change of three independent experiments. (**E**) Cells were grown in starvation or complete medium, in presence or absence MP (100 µM). The expression level of osteopontin was asssessed by RT-PCR. Values represented are mean ± SEM of % vehicle control. (*$p < 0.05$). One-way ANOVA followed by Tukey's test was performed.

9. MP Inhibits Actin Polymerization

The actin cytoskeleton in VSMCs is dynamic and responds to external stimuli by polymerization of globular (G) actin to filamentous (F) actin [495]. To test the effect of MP on FBS-induced actin polymerization, phalloidin stain was employed. Phalloidin binds to F-actin and prevents their depolymerization. As expected, FBS treatment induced actin polymerization. This FBS-induced polymerization was inhibited by MP pretreatment as shown in figure 31.

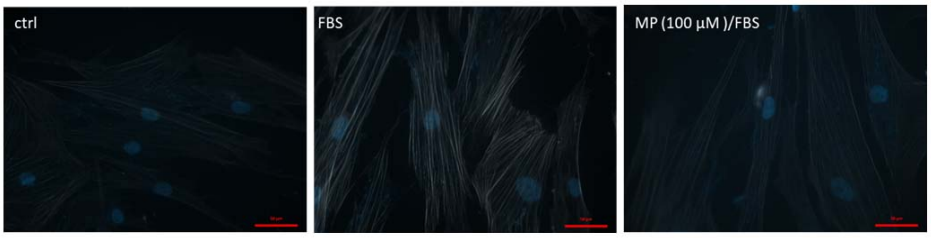


Figure 31. MP attenuates FBS-induced actin polymerization.

Cells were treated with FBS for 24 h in the presence or absence of MP (100 µM). Cells were then stained with phalloidin and actin polymerization was assessed. Representative micrographs showing the effect of MP on FBS-induced actin-polymerization. Scale bar, 50 µm.

10. MP Inhibits PMA-induced Adhesion of THP-1 Monocytes on VSMCs

Monocyte adhesion to VSMC takes place in many vasculopathies including atherosclerosis, thrombosis, and restenosis [544]. We sought to determine the effect on MP on monocyte adhesion to PMA-induced VSMCs. Toward this, THP-1 cells were incubated with PMA-stimulated VSMCs, with or without pretreatment with MP. In the absence of PMA, the number of THP-1 cells adhered to VSMCs was expectedly minimal. However, stimulation of VSMCs with PMA for 20 h lead to significant increase in the number of adherent THP-1 cells (figure 31.A). Indeed a 3-fold increase in monocyte adhesion was observed under these conditions (figure 31.B). Interestingly, this increase was completely abolished when VSMC were pretreated with 100 µM of MP (figure 31.A and B).

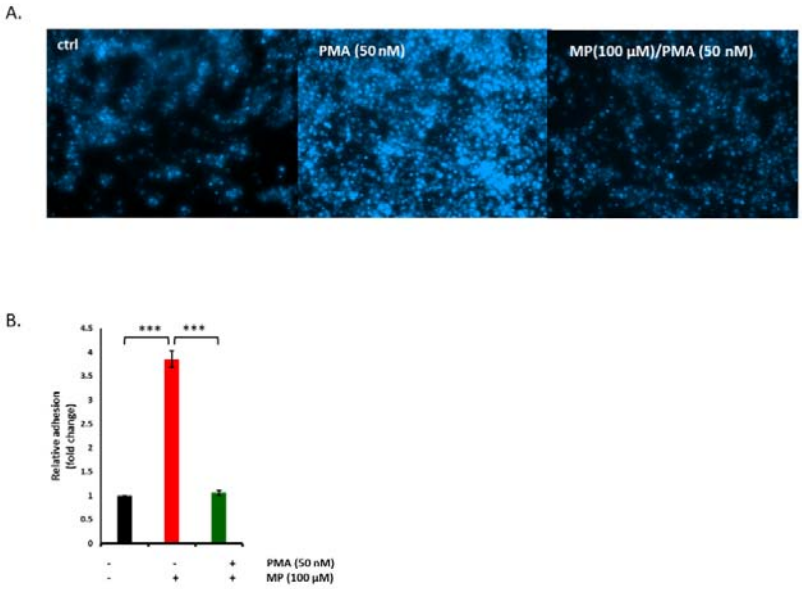


Figure 32. MP abolishes PMA-evoked adhesion THP-1 cells to VSMCs.

Cells were treated with PMA (50 nM) in presence or absence of MP (100 µM), and adhesion of THP-1 cells was examined by monocyte adhesion assay (**A**). Representative photomicrographs of the effect of MP on THP-1 adhesion to VSMC. (**B**) Quantitation of mean fluorescence intensity of adherent THP-1, assessed at 5 different regions; *** $p < 0.001$. Values are represented as mean ± SEM of relative fold adhesion of vehicle-treated cells. One-way ANOVA followed by Tukey's test was performed.

11. MP Inhibits FBS-Induced Expression of NF-κB in a Concentration-Dependent Manner

NF-κB transcription factor is a key regulator of vascular inflammatory responses [545]. Here, the effect of MP on the expression of NF-κB and the phosphorylation of its inhibitor protein, IκB, were evaluated. As shown in figure 31.A, FBS evoked a significant increase in NF-κB transcription (100 vs 288 ± 62%; $p < 0.05$). This increase was attenuated by MP in a concentration-dependent manner (288 ± 62% vs 138 ± 19%, 111 ± 6% or 87 ± 9% for 100, 200, and 300 µM respectively; $p < 0.01$ for all). Western blotting analysis showed that whereas FBS induced an increase in NF-κB expression, pretreatment with MP abolished its activation in a

time-dependent manner (figure 31.B). Furthermore, pretreatment with MP inhibited FBS-induced phosphorylation of IκBα (figure 31.B).

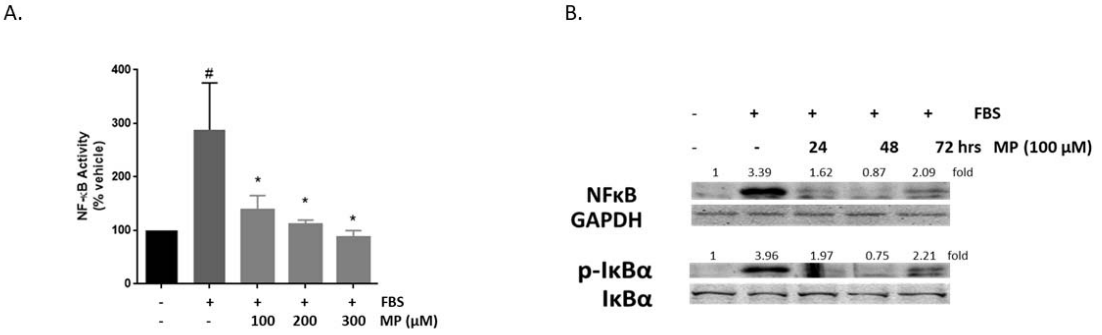


Figure 33. MP inhibits FBS-induced NF-κB expression in a time- and concentration-dependent manner, and attenuates the activation of its inhibitor, IκBα.

(**A**) Cells were treated with FBS in the absence or presence of increasing concentrations of MP (100, 200, and 300 μM). NF-κB expression was determined by luciferase assay. Values represented are mean ± SEM. # denotes $p < 0.05$ (FBS versus vehicle) and * denotes $p < 0.05$ (MP + FBS vs FBS)). (**B**) Cells were treated with FBS in the presence or absence of MP (100 μM) for 24, 48, and 72 h. Expression of NF-κB and phosphorylation of IKBα were detected by Western blotting. Values mean fold change of three independent experiments. One-way ANOVA followed by Tukey's test was performed.

D. Discussion

Inflammation of arterioles has recently emerged as a key event in the manifestation of many diseases [498,546,547]. These include diabetes and inflammation-based disorders such as chronic obstructive pulmonary disease, inflammatory bowel diseases, cystic fibrosis, and atherosclerosis. Importantly, studies show that changes in arterioles in response to hypercholesterolemia predate the formation of atherosclerotic plaques in large arteries [548]. Moreover, cardiovascular risk factors such as obesity and hypertension induce inflammatory responses at the level of arterioles [549-553]. In response to inflammatory cues, VSMCs acquire increased proliferative, migratory and invasive capabilities. Accordingly, inhibiting these dedifferentiation hallmarks would confer anti-inflammatory effects on arterioles.

In this study, we assessed the vasculoprotective role of MP against FBS-induced arteriolar SMC inflammation, a model mimicking mild arteriolar inflammation. Our results showed that MP, a novel homo-isoflavonoid that we isolated and characterized, inhibited FBS-induced proliferation and migration of human arteriolar smooth muscle cells. Moreover, MP attenuated VSMC adhesion and invasion as well as monocyte adhesion to VSMCs. This inhibition was concomitant with a decrease in matrix metalloproteases, MMP-2 and MMP-9, as well as an increase in the expression of myocardin, SM-22α, SM-α actin, calponin and caldesmon. On the other hand, MP decreased the expression of osteopontin, and abolished FBS-induced NF-κB expression and IκB phosphorylation.

The significance of this study stems from three points: the novelty of the studied compound, the concentration used to conduct the experiments, and the relevance of the employed model. First, MP is a newly isolated and characterized homoisoflavonoid. Its potential vasculoprotective effects are established for the first time in this study. Moreover, MP proved to be potent at a sub-cytotoxic concentration. This may be promising especially in developing a noncytotoxic drug, which may have no or fewer side effects. To our knowledge, this is the first study using human arteriolar SMC as a model for arteriolar inflammation. Previous studies addressing vascular inflammation had used VSMCs extracted from large vessels. This is likely due to the challenging technical difficulty of isolating and maintaining a culture of microvascular smooth muscle cells isolated from human arterioles.

The origin of the VSMC greatly affect its response to various stimuli. For instance, studies reporting the effect of estrogen on VSMCs differ in different vascular beds. To address this, we recently published a paper where we elaborated on the effect of the vascular bed on various functional responses [243]. Another example is that whereas Epac activates JNK in

arteriolar SMCs (unpublished data), it inhibits JNK activation in rat aortic vascular smooth muscle cells [554]. More relevantly, certain diseases such as retinopathies and kidney diseases affect arterioles rather than large vessels. Accordingly, microvascular SMCs would be a better model to recapitulate many aspects of these pathophysiological cues. However, whether MP effects mirror this vascular bed discrepancy remains to be established.

Overwhelming evidence shows that increased cell proliferation is a hallmark of VSMC phenotypic switch, especially in response to inflammation [468,555]. In our study, MP attenuated FBS-induced VSMC proliferation by blocking cells in the G_0/G_1 phase and inhibiting their progression to S phase. Progression from the G1 to S phase requires cyclin D1 [556,557]. Early in G1, cyclin D1 binds to CDK4. The resulting cyclin D1-CDK4 complex inhibits retinoblastoma protein, thus facilitating the transcription of S-phase genes [558]. Moreover, the activity of cyclin D1-CDK4 complex is inhibited by CDK inhibitors such as p21 and p27 [558]. Here, MP-induced cell cycle arrest was concomitant with an increase in the expression of CDK inhibitors, p21 and p27, as well as a decrease in both cyclinD1 and CDK4.

The anti-proliferative effect of MP is in accordance with the results of two previous studies. The first study reported that brazilin, a homoisoflavonoid, inhibited PDGF-induced proliferation of rat aortic VSMCs and induced cell-cycle arrest at G_0/G_1 phase [559]. The other study showed that the homoisoflavonoid-rich plant, *Ophiopogon japonicas*, exhibited anti-proliferative effects on thrombin-induced rat aortic VSMCs [560]. Similar to brazilin and *Ophiopogon japonicas*, MP induced the upregulation of p27 [559,560]. This suggests that homoisoflavonoids attenuate VSMC proliferation by targeting cell cycle regulators. Notably, p27 and p21 are regulated by ERK1/2 [561], which is a mitogenic factor itself [562]. Indeed, pharmacological [563] and genetic [539] inhibition of ERK1/2 inhibit VSMC proliferation. Here, MP

attenuated FBS-induced phosphorylation of ERK1/2. This inhibition is in line with the effect of brazilin in VSMCs [559].

Clinically, arteriolar SMC proliferation has been previously assessed in two pathological conditions: hypertension [564] and menorrhagia [565]. VSMCs cultured from rat renal preglomerular arterioles showed that Angiotensin II significantly increased VSMC proliferation, indicating that VSMC hyperplasia may be associated with hypertension [564]. In menorrhagia, endometrial biopsies showed that proliferation of arteriolar SMCs varied between healthy and menorrhagic females [565]. Moreover, arterioles are responsive to pro-inflammatory cues [546], and thus are also remodeled during atherosclerosis by changing their cellular function and phenotype [546,548,566]. It is only reasonable to assume that inhibiting VSMC proliferation may aid in the ameliorating microvasculature in these pathological conditions. In this sense, MP, owing to anti-proliferative effect on arteriolar SMCs, presents a promising therapeutic potential at the level of microcirculation.

Many vascular complications arise from the imbalance in the proliferation/apoptosis ratio of VSMCs [567]. For example, VSMCs of diabetic patients have an increased level of the anti-apoptotic protein, Bcl2, and exhibit high proliferative rate [568]. This “failure to die” leads to alteration in the vessel microarchitecture [569]. In our study, MP-treated VSMCs became shrunk and translucent, with fine cytoplasmic extensions ending with blebs. All these features are characteristic of apoptotic cells [570]. These microscopic observations were confirmed by the fact that MP increased Bax/Bcl2 ratio and upregulated the level of activated caspase-3. Thus, MP induced apoptosis in FBS-induced micro VSMCs. It would thus be tempting to test whether MP can decrease thickening of muscularized arterioles in pulmonary artery hypertension or chronic obstructive lung disease [571,572].

Migration, invasion and adhesion of VSMCs are also major determinants of the dedifferentiated phenotype, and play a major role in vascular pathogenesis [573]. In our study, MP potently attenuated these hallmarks. Knowing that both ERK1/2 and FAK mediate cell migration [538,574], we may assume that MP-attenuated migration is achieved via inhibiting ERK1/2 and FAK phosphorylation. Moreover, in addition to its role in mobilizing adrenergic receptors to the cell surface [166], actin polymerization plays a key role in VSMC migration [495]. During migration, integrins are activated and clustered along with adhesion molecules at the migrating edge of the cell [575]. These cytoskeletal rearrangements along with actin polymerization facilitate cell crawling [576]. In our study, MP attenuated FBS-induced actin polymerization. Furthermore, MP reduced the invasive capacity of VSMCs by attenuating the expression of the matrix metalloprotease of MMP-2 and MMP-9. Previous studies report that VSMC migration was attenuated by the homoisoflavonoid, brazilin, and the homoisoflavonoid-rich plant, *Ophiopogon japonicas* [559,560]. However, these studies neither used human arteriolar cells nor assessed the effects on VSMC adhesion or invasion.

Clinically, arteriolar SMC migration was observed during cardiac transplantation in a condition termed transplant arteriosclerosis [577]. This condition is characterized by inflammation and intimal thickening due to the accumulation of SMCs from both donor and recipient [577]. A study using post-transplantation cardiac biopsy specimens from allograft patients showed that higher arteriolar SMC migration was associated with rejection grade, and thus with inflammation [577]. Here, MP attenuated arteriolar SMC migration. Hence, we may postulate that MP may contribute to reversing arteriolar inflammation and decreasing rejection grade.

Monocyte adhesion to blood vessels is a defining feature of vascular inflammation [578,579]. In response to inflammatory cues, arterioles become more permissive to monocytes, allowing them interact with endothelial cells and VSMCs [546]. Monocyte adhesion may also precipitate arteriolar barrier dysfunction [580]. Indeed, in response to increased luminal shear stress, monocytes are recruited to then adhere onto collateral arterioles [581]. Furthermore, direct evidence of monocyte adhesion to arterioles in response to Angiotensin II has been reported [582]. In our study, we showed that MP attenuated monocyte adhesion to PMA-activated arteriolar SMCs. Because PKC is a direct target of PMA [583], it may be postulated that MP attenuates PMA-provoked adhesion by inhibiting PKC. Indeed, PKC is inhibited by many flavonoids [584]. However, whether MP inhibits PKC is yet to be investigated.

VSMCs are not terminally differentiated, but are rather characterized by plasticity that allows phenotypic switch [585]. The extent of VSMC differentiation is determined by the expression level of differentiation markers. These markers are divided in to early, mid-term, and late differentiation markers, according to their order of appearance during embryogenesis [541]. The early differentiation markers include SM-α actin, myocardin, and SM22-α [541]. Caldesmon and calponin are considered to be mid-term differentiation markers [586]. Finally, desmin and smoothelin are among late differentiation markers [586]. Contractile VSMCs are characterized by the elevated expression of these markers [445]. In response to inflammation, VSMCs reduce the expression of these markers and adopt the expression of synthetic dedifferentiated phenotype [445]. VSMCs then become active in secreting ECM molecules such as osteopontin and osteonectin [543,587,588]. As such, high levels of these proteins indicate VSMC switch to synthetic phenotype.

Myocardin is a transcriptional co-activator that acts upstream of calponin and caldesmon. It is selectively expressed in cardiomyocytes and contractile SMCs [589-591]. It is a potent activator of the Serum Response Factor (SRF) where it stabilizes its binding at the CC(A/T-rich)$_6$GG (CArG) cis-elements of CArG-dependent genes. Interestingly, expression of VSMC differentiation genes, such as SM22, MHC, SM α-actin and caldesmon requires CArG box in their promoter region and/or intronic sequences [446,592]. Nonetheless, some studies report that myocardin-driven expression of CArG-dependent SMC marker genes is not sufficient for the initiation of complete SMC differentiation [593].

In response to stimuli, myocardin regulation of SMC markers expression is attenuated mainly through 2 distinct pathways, both involving ERK1/2 activation. Activated ERK1/2 leads to Elk-1 phosphorylation, which competes with myocardin for SRF, leading to attenuated SMC marker expression [594]. Alternatively, myocardin may be directly phosphorylated by ERK1/2. This ERK1/2-induced myocardin phosphorylation hinders its ability to bind SRF and induce marker genes expression [595]. Several other effector pathways have been reported. For example the JAK/STAT signaling mediates VSMC switch to the synthetic phenotype via the interaction between STAT3 and myocardin [596]. Furthermore, Yap1 upregulation promotes synthetic phenotype by reducing myocardin-SRF interaction [597]. Similarly, NF-κB (p65) interacts with myocardin hindering myocardin-SRF interaction necessary for contractile genes expression [598]. Interestingly, myocardin attenuates the expression of NF-κB(p65)-driven genes [598]. In this sense, the protective role of myocardin lies in orchestrating the expression of SMC differentiation genes and in attenuating the expression of inflammatory genes.

Osteopontin is a glycoprotein secreted by many cell types including osteoblasts, monocytes, and VSMCs [599,600]. Specifically, its expression is increased during the switch of VSMCs to the synthetic phenotype as well as during vascular remodeling [601,602], as it is involved proliferation and migration [603-605]. Osteopontin induces migration by phosphorylating FAK, dephosphorylating downstream ILK, and by disrupting FAK-ILK interaction [605]. In addition, osteopontin leads to decreased expression of differentiation markers such as α-SM actin, and calponin [606]. Furthermore, studies show that osteopontin plays a role in vascular inflammation by inducing leukocyte chemotaxis and macrophage adhesion to endothelial cells [602]. Transcription of osteopontin is regulated by NF-κB [607,608].

Whereas previous reports have focused almost exclusively on assessing the expression level of these differentiation markers in macro-VSMCs [493,606,609], our study assessed the expression of these markers in micro-VSMCs. We showed that MP induced an elevation in myocardin, SM-α, SM-22α, calponin, and caldesmon, and reversed the FBS-attenuated expression of these markers in arteriolar SMCs. In fact, one previous study assessed the phenotypic switch of arteriolar SMCs in the context of benign nephrosclerosis (bN), a common hypertensive kidney damage characterized by fibrosis of renal arterioles [610]. Using renal tissue specimens, this study showed that arteriolar SMCs undergo a phenotypic switch in bN [610]. Surprisingly, their findings did not suggest an inverse correlation between caldesmon and dedifferentiated VSMCs [610]. Conversely, our results showed the caldesmon and calponin were upregulated by MP in FBS-induced arteriolar SMCs. Moreover, MP effectively decreased the expression of basal and FBS-induced expression osteopontin, further emphasizing the maintenance of a contractile phenotype of VSMCs. In 2018, Lin et al. showed that the flavonoid, (−)-epigallocatechin gallate (EGCG), attenuated Ag-II induced proliferation and migration in

vitro and neointimal formation in vivo [611]. These inhibitory effects were mediated by myocardin. Therefore, this and other studies present myocardin as a molecular therapeutic target in vascular inflammation. In light of our results showing its ability to modulate myocardin expression, MP may thus be expected to possess a much-desired anti-inflammatory capacity. Indeed, our unpublished observations strongly suggest such an effect.

NF-κB is a member of the Rel-family of transcription factors [612]. Under normal physiological conditions, NF-κB is attached to its inhibitor protein IκB which traps it in the cytosol [612]. In response to inflammatory stimuli, IκB gets phosphorylated by its kinase, IκK, then ubiquitinated and degraded [133]. NF-κB then becomes free to translocate to the nucleus and trigger the transcription of pro-inflammatory genes [613,614]. In vascular inflammation, such as atherosclerosis, NF-κB upregulates the expression of adhesion molecules (ICAM-1and VCAM-1) and matrix-metalloproteases (MMP-2, and MMP-9) [615], further exacerbating vascular inflammation [616]. The vast majority of research show that NF-κB activates the inflammatory signaling in VSMCs of large vessels [613]. Except for one study showing that NF-κB is expressed and activated in arterioles [617], no previous studies assessed the modulation of NF-κB expression specifically in microVSMCs. Here, we show that NF-κB expression and IκB phosphorylation were attenuated by MP in FBS-activated micro VSMCs. This supports the vascular anti-inflammatory actions of MP and presents NF-κB and IκB as major molecular targets in the involved signaling pathway. By doing so, MP may be suppressing many genes such as MMP-2 and MMP-9, in addition to other genes which remain to be investigated.

E. Conclusions

To sum up, our data are consistent with the model shown in figure 31, which illustrates different lines of action of MP and the involved molecular players. All depicted MP effects serve to attenuate VSMC dedifferentiation and thus may amend arteriolar inflammation. Further research is needed to better dissect the molecular mechanisms implicated in MP signaling. For instance, some flavonoids and isoflavonoids have been shown to regulate cAMP signaling in VSMCs [618,619]. Given the important and broad range of cAMP effects in human microvascular smooth muscle cells [54,166,304,305], if MP appears to modulate this pathway in these cells lines, one would expect to find diverse effects of MP on arteriolar physiology and pathophysiology. Further investigations are warranted to better determine the potential of MP as anti-inflammatory drug especially as pertains to vascular anti-inflammatory therapies. Knowing that modern drug therapy is still insufficient in preventing or treating CVD [620], the use of alternative medicine such as flavonoids and homoisoflavonoids may provide an important resource for potential new drugs.

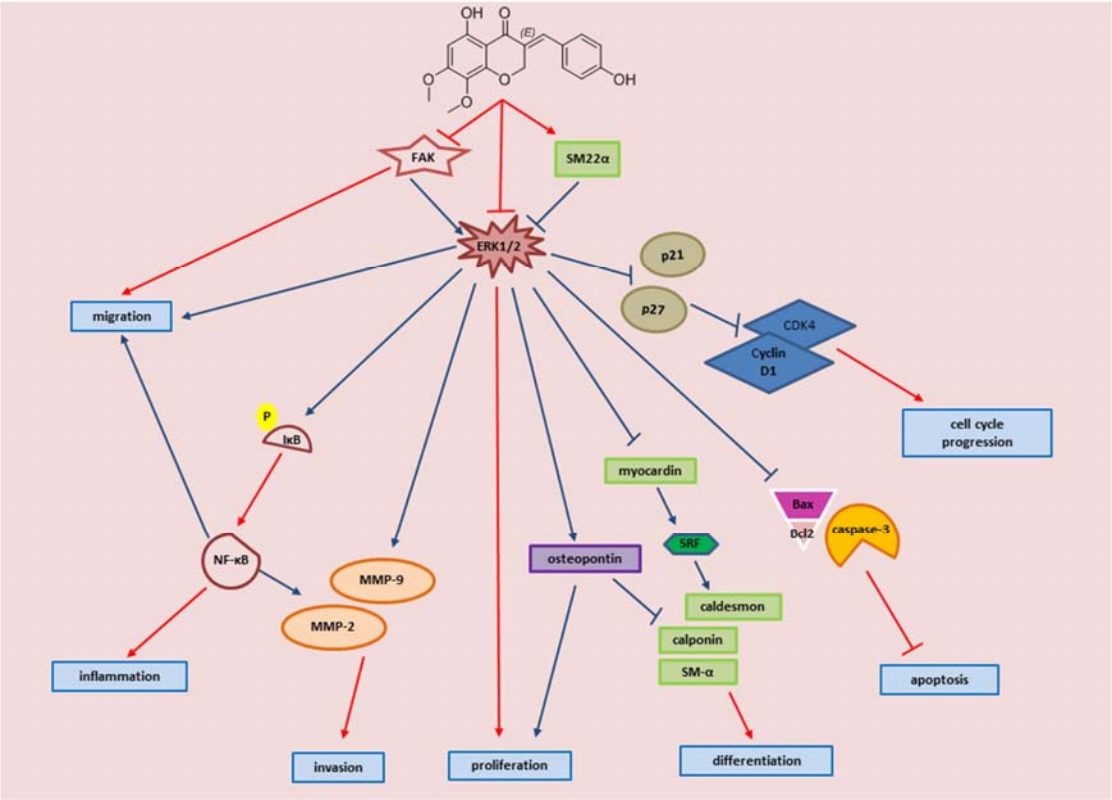


Figure 34. Schematic representation of the proposed signaling pathway by which MP attenuates FBS-induced inflammation of arteriolar SMC.

The diagram displays key events of VSMC inflammation, and the molecular effectors by which MP attenuates these events. Red arrows are established by our study, blue arrows are established as referenced below: FAK → ERK1/2 [621,622]; SM22α → ERK1/2 [623]; ERk1/2 → osteopontin [624]; ERK1/2 → p21/p27 [561]; ERK1/2 → caspase-3, BAX/Bcl2 [625,626]; Osteopontin → proliferation [627]; Osteopontin → α SM and calponin [606]; p21, p27 → Cyclin D1/CDK4 [628,629]; NF-κB → MMP-2, MMP-9 [630-632]; NF-κB → proliferation, migration [633]; NFκB → cyclin D1 [613,634].

CHAPTER V

ESTROGEN POTENTIATES COLD-INDUCED VASOCONSTRICTION BY INCREASING ALPHA 2C ADRENOCEPTOR EXPRESSION THROUGH THE CAMP/EPAC/JNK/AP-1 PATHWAY

Authors: Manal M. Fardoun, Khodr Issa, Dina Maaliki, Suzanne A. Nasser, Elias Baydoun, Ali H. Eid

Cutaneous cold-induced vasoconstriction is a normal physiological reaction mediated by alpha 2C-adrenergic receptors (α_{2C}-ARs) expressed in vascular smooth muscle cells (VSMCs). When this reaction is exaggerated, Raynaud's phenomenon (RP) ensues. RP is more prevalent in females compared to age-matched men. We previously established that 17-β estradiol (estrogen) upregulates α_{2C}-ARs in human VSMCs via a cAMP/Epac/Rap pathway. We also showed that cAMP acts through JNK to increase α_{2C}-AR expression. However, whether estrogen employs JNK to regulate α_{2C}-AR is not investigated. Knowing that the α_{2C}-AR promoter harbors an activator protein-1 (AP-1) binding site that can be potentially activated by JNK, we hypothesized that estrogen regulates α_{2C}-AR expression through an Epac/JNK/AP-1 pathway. Our results show that estrogen (10^{-10} M) activated JNK in human VSMCs extracted from cutaneous arterioles. Pretreatment with ESI09 (10 μM; an Epac inhibitor), abolished estrogen-induced JNK activation. In addition, pre-treatment with SP600125 (2 μM; a JNK specific inhibitor) abolished estrogen-induced expression of α_{2C}-AR. Importantly, estrogen-induced activation of α_{2C}-AR promoter was attenuated with SP600125. Moreover, transient transfection of VSMCs with an Epac dominant negative mutant (Epac-DN) abolished estrogen-

induced activation of α_{2C}-AR promoter. However, co-transfection of constitutively active JNK mutant overrode the inhibitory effect of Epac-DN on α_{2C}-AR promoter. Moreover, estrogen caused a concentration-dependent increase in the activity of AP-1-driven reporter construct. Mutation of AP-1 site in the α_{2C}-AR promoter abolished its activation by estrogen. This *in vitro* estrogen-increased α_{2C}-AR expression was mirrored by an increase in the *ex vivo* functional responsiveness of arterioles. Indeed, estrogen potentiated α_{2C}-AR-mediated cold-induced vasoconstriction, which was abolished by SP600125. Collectively, these results indicate that estrogen upregulates α_{2C}-AR expression via an EPAC-mediated JNK/AP-1- dependent mechanism. These results provide an insight into the mechanism by which exaggerated cold-induced vasoconstriction occurs in estrogen-replete females and identify Epac and JNK as potential targets for the treatment of RP.

A. Introduction

Cold-induced vasoconstriction at the level of the extremities is a normal physiological reaction [128]. It aims at reducing heat loss and redirecting blood towards internal, more vital body organs [128]. Exaggerated cold-induced vasoconstriction leads to a pathological condition termed Raynaud's phenomenon (RP) [130]. Due to increased cold sensitivity, affected individuals suffer from cold-induced vasospastic attacks and triple color change at the level of the digits [130].

Epidemiological studies show a significantly greater incidence of RP in pre-menopausal females compared to age-matched males [46,131]. In addition, post-menopausal women receiving unopposed ERT (estrogen replacement therapy) are at a higher risk of RP than those not receiving ERT [169]. Moreover, noradrenaline-mediated vasoconstriction is higher in

premenopausal females at their mid-menstrual cycle [170]. This stage is characterized by higher estrogen level as compared to other stages of the cycle [170]. Furthermore, evidence supports a direct role of estrogen in vasoreactivity. For instance vascular responsiveness is higher in human and rat females in their reproductive age as compared to their counter males [171]. Supplying males with estrogen enhances their vascular responsiveness [171]. In addition to its role in vasoreactivity, estrogen plays a role in regulating body temperature [172]. Since RP is a vascular thermoregulatory control disorder, the implication of estrogen in the disease becomes obvious. Taken together, these observations imply that there is a positive association between the female hormone, 17β-estradiol (estrogen) and RP [42].

Mechanistically, cold-induced vasoconstriction results from a reflex reaction mediated by neuronal (norepinephrine) and local effectors that increase vascular sensitization to cold [128,635]. The latter effect is mediated by adrenergic receptors located on the surface of vascular smooth muscle cells (VSMCs) of cutaneous arterioles [128,635]. VSMCs express α_1, α_2, and β_2 adrenergic receptors [636]. While β_2-ARs are involved in vasodilation [637], α_1-ARs and α_2-ARs are known to exert a vasoconstrictive effect [638]. In arterioles, α_1-ARs do not play a role in cold-induced vasoconstriction evident by the lack of any effect of their blockers on this constriction [52]. α_{2A}-ARs and α_{2C}-ARs, but not α_{2B}-ARs, are expressed in human cutaneous VSMCs. Interestingly, inhibition of α_{2C}-ARs, but not α_{2A}-ARs, abolished cold-induced vasoconstriction [52]. This clearly shows that α_{2C}-AR is the sole mediator of the entirety of cold-induced vasoconstriction.

A distinctive characteristic of α_{2C}-ARs is their intra-cellular localization in the ER-Golgi apparatus [158]. These entrapped α_{2C}-ARs are functionally competent, as they successfully bind to radioligands [639,640]. Interestingly, α_{2C}-ARs were observed on the cell membrane of PC12

and At-T20 neuroendocrine cells, indicating that their mobilization to the cell surface could be regulated [640]. Relevantly, certain stimuli such as cold induces the mobilization of α_{2C}-ARs to the cell surface [55], where they become available to their ligand [55,158]. This spatial translocation of α_{2C}-AR is prerequisite to unmask their functional ability to induce vasoconstriction [52,53].

We have previously reported that estrogen increases α_{2C}-AR expression in VSMCs via a cAMP-mediated pathway [51]. Moreover, elevated cAMP levels in VSMCs increases the expression of α_{2C}-AR by activating JNK [155]. Activated JNK is known to initiate the transcription of many AP-1-dependent genes [641]. Interestingly, the promoter region of α_{2C}-AR harbors an AP-1 binding site [642]. However, whether estrogen employs JNK and AP-1, or whether Epac acts through AP-1, to regulate α_{2C}-AR expression is still unknown. In this study, we investigated the role of JNK and AP-1 in estrogen-induced or Epac-mediated α_{2C}-AR expression.

B. Materials and Methods:

1. Reagents:

DMEM:F12 (D8437), phenol red free DMEM (D6434), L-Glutamine (G7513), Fetal Bovine Serum (F9665), Phosphate Buffer Saline (D1408), L-Glutamine (G7513), and Epac inhibitor ESI09 (SML0814) were purchased from Sigma-Aldrich (Schnelldorf, Germany). Penicillin/Streptomycin (17-602E), Trypsin (BE02-007E) and Amaxa Nucleofector (VPC-1001) were obtained from Lonza (Basel, Switzerland). Anti-JNK1 + JNK2 + JNK3 (phospho T183+T183+T221) (ab124956), Anti-JNK1+JNK2+JNK3 antibody (ab179461), anti-alpha 2C adrenergic receptor (ab123368), anti-β-actin antibody (ab119716), HRP-conjugated Goat Anti-Mouse antibody (ab97040), HRP-conjugated Goat Anti-Rabbit antibody (ab150080), 17-β estradiol (ab120657), JNK inhibitor SP600125 (ab120065) and UK 14,304 (ab120773) were

purchased from Abcam. *DC*™ Protein Assay kit and Clarity™ Western ECL Substrate were purchased from Biorad (CA, USA). Insulin-Transferin-Selenium (41400045) was obtained from Thermofischer Scientific (USA). Luciferase assay kit (E1500) and pRL-CMV [*Renilla* luciferase gene driven by cytomegalovirus (CMV) promoter/enhancer] were purchased from Promega (Wisconsin, USA). The activator protein (AP)-1-luciferase reporter plasmid was obtained from Stratagene (California, United States). The α_{2C}-AR promoter-reporter plasmid (−1,915/+5, relative to the transcription start site +1) was a kind gift from Dr. Herve Paris [152,305].

2. Cell Culture:

Human arteriolar SMCs were extracted by non-enzymatic sprouting method from dermal arterioles of a post-circumcision tissue of a newborn boy. No IRB approval is needed as this source is considered clinical waste; however, ethical approval was obtained. Cells were used between passages 6 and 11, during which the expression and regulation of α_{2C}-ARs is similar [152]. VSMCs were maintained in Ham's Growth medium (DMEM: F12; 50:50) supplemented with 10% FBS and 1% penicillin/streptomycin. Prior to treatment, cells were made quiescent for 48 hrs in phenol red free DMEM supplemented with L-glutamine, insulin-transferrin-selenium, and 1% penicillin/streptomycin. Cells were grown in a 5% CO_2 incubator at 37 °C.

3. Western Blotting:

Cells were washed with PBS and then lysed using lysis buffer (2% SDS, 60 mM Tris (pH 6.8), as previously described [155]. Proteins were quantified using *DC*™ Protein Assay and

equal amounts of protein (20-30μg) were loaded on SDS-PAGE. After running, proteins were transferred onto polyvinylidene difluoride (PVDF) membrane (Biorad). The membrane was blocked with fat-free milk (5% in TBS-T) for 1 hr at room temperature, then incubated overnight with primary antibody at 4°C. Then, the membrane was washed with TBS-T, 3 times for 10 minutes each, and incubated with the relevant HRP-conjugated secondary antibody for 1hr at room temperature. The membrane was washed again, developed using enhanced chemiluminescence (ECL clarity, Biorad), and quantified using Chemidoc MP Imaging system.

4. Site directed mutagenesis:

As we previously reported [155], site-directed mutagenesis of AP-1 site was performed using the QuickChangeTM XL-site-directed mutagenesis kit (Stratagene, La Jolla, CA). Mutant oligonucleotide primers and double stranded DNA were employed to mutate wild-type AP-1 site (ATGATTCAT, −346/−338, relative to the transcription start site) in the α_{2C}-AR promoter-luciferase reporter construct to ACTGTTTGT.

5. Transient transfections:

VSMCs were transiently transfected by nucleofection through the Nucleofector device according to the manufacturer's instructions (Lonza) as we previously described [305]. Optimum transfection level (80% transfection efficiency, minimal toxicity) was attained with nucleofection of $400x10^3$ cells with 4 μg of nucleic acid. Total amount of nucleic acid was kept constant for all conditions. When needed, appropriate empty plasmid was used for adjustment. After transfection, cells were allowed to recover overnight in complete media. *Renilla*

luciferase CMV immediate early enhancer/promoter (pRL-CMV) was used as an internal control to normalize the firefly luciferase units.

For luciferase analysis, cells were washed with PBS and lysed with luciferase lysis buffer, snap frozen, and then thawed at room temperature water bath. Cell lysates are centrifuged at 10,000 g for 10 minutes and the supernatant was used to determine luciferase activity.

6. Vasomotor activity of isolated arterioles:

Male mice (C57BL6) aged 10 to 12 weeks were sacrificed by CO_2 asphyxiation, and the tail artery was isolated and placed in cold Krebs buffer (in mM: 118.3 NaCl, 4.7 KCl, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 2.5 $CaCl_2$, 25.0 $NaHCO_3$, and 11.1 glucose). Arterial segments were mounted in a microvascular chamber, and maintained at constant temperature (37 °C) and pressure (60 mmHg), with continuous perfusion in Krebs solution. Live vessel images were acquired through a video camera connected to an inverted microscope, focused on the vessel chamber. The vessel diameter was directly determined using acquisition system (LabChart, ADInstruments, UK). Vessel stabilization was done using increasing concentrations of α_2-AR selective agonist (UK-14304). The effect of the agonist was stopped by successive washes of the artery, allowing it to return to its baseline level. For cold temperature studies, the temperature of the chamber medium was decreased to 28 °C for 30 minutes before assessing α_2-AR vasoconstriction. Concentration-effect curves were analyzed by comparing the agonist concentration causing 20% constriction (CC_{20}), determined by regression analysis.

The use of male mice (C57BL6) was approved by American University of Beirut animal care and use committees in compliance with the national institute of health guide for the care and use of laboratory animals.

7. Immunofluorescence-confocal microscopy:

Cells were washed with PBS then fixed in 3% paraformaldehyde for 20 minutes at room temperature. Cells were then washed with PBS and blocked with 3% normal goat serum (NGS) for 1 hr at room temperature. Next, cells were washed and probed overnight at 4 °C using primary antibody in 1% NGS. After washing, cells are incubated with secondary antibody prepared in 1% NGS for 1 hr at room temperature. Nuclear staining was done using Hoechst dye for 15 min at room temperature RT. T Cells were then mounted on a slide using anti-fade medium. Cells were visualized by laser scanning confocal microscopy (×40 oil objective, 512 × 512 pixels, LSM510, Zeiss, Germany). Quantitation of mean fluorescence intensity of cell surface α_{2C}-ARs was assessed at the cell boundary of each cell using the Image J software.

8. Statistical Analysis:

Data was statistically evaluated using student's t-test for either paired or unpaired observations using GraphPad Prism version 5.0 and InStat3 Software (GraphPad software, Inc. San Diego, CA). For the luciferase assay, experiments were performed at least three times, and each time was made of triplicate wells. The average of the triplicate from each experiment (individual mean) was calculated, and these means were then averaged. Data was presented as mean ± SEM, where *n* is equal to the number times an experiment was repeated for *in vitro* experiments, and number of subjects for *ex vivo* experiments. For the comparison of more than

two means, ANOVA was used: either one-way ANOVA (with Dunnett's post hoc test) or two-way ANOVA (with Tukey-Kramer's post hoc test).

C. Results:

1. JNK mediates estrogen-induced activation of α_{2C}-AR promoter.

We sought to assess the role of JNK in estrogen-induced activation of full length α_{2C}-AR promoter. As expected, treatment of cells with increasing concentrations of 17-β estradiol (10^{-11}- 10^{-7} M) increased the transcriptional activity of α_{2C}-AR promoter-reporter construct in a concentration-dependent manner (figure 31). The highest activation was attained at an estrogen concentration of 10^{-9} M (4.2 ± .2; $p<0.01$) (figure 31). This estrogen-induced increase in the α_{2C}-AR promoter activity was attenuated by pretreatment with SP600125, a JNK inhibitor (2 μM) (figure 31). These results indicate that estrogen-induced transcription of α_{2C}-AR is mediated by JNK.

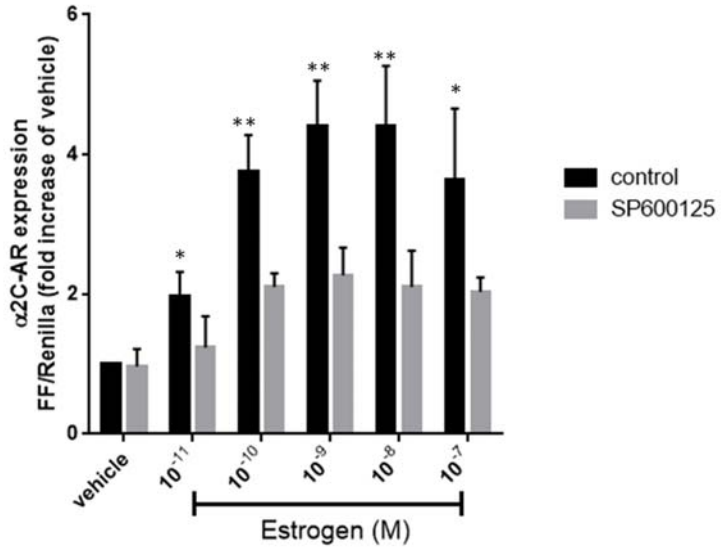


Figure 35. JNK mediates estrogen-induced activation of α_{2C}-AR promoter in VSMCs. Cells were transiently transfected with the α_{2C}-AR promoter-reporter construct and internal *Renilla* control construct. After recovery, cells were starved for 48 hours then treated with

increasing concentrations of estrogen (10^{-11} to 10^{-7} M, 24 hours), in the presence or absence of SP600125 (2 μM; a JNK specific inhibitor). SP600125 was administered 30 minutes before and during exposure of the cells to estrogen. Promoter activity was assessed by luciferase assay. Results are expressed as fold increase in the firefly (FF)/*Renilla* luminescent signal from the control level and are presented as means ± SEM. n=3, * $p<0.05$

2. Estrogen induces α_{2C}-AR expression via JNK activation.

We next sought to determine whether estrogen activates JNK. Indeed, 17β-estradiol (10^{-10} M) caused a time-dependent activation of JNK (figure 31A). The maximal JNK phosphorylation was attained 10 minutes post-estrogen treatment.

We further wanted to assess the role of JNK activation in estrogen-induced expression of α_{2C}-AR. Whereas estrogen increased α_{2C}-AR expression ($p<0.05$), pretreatment with SP600125 (2 μM) abolished this estrogen-induced increase ($p<0.05$) (figure 31B). This indicates that JNK mediates estrogen-induced expression of α_{2C}-AR.

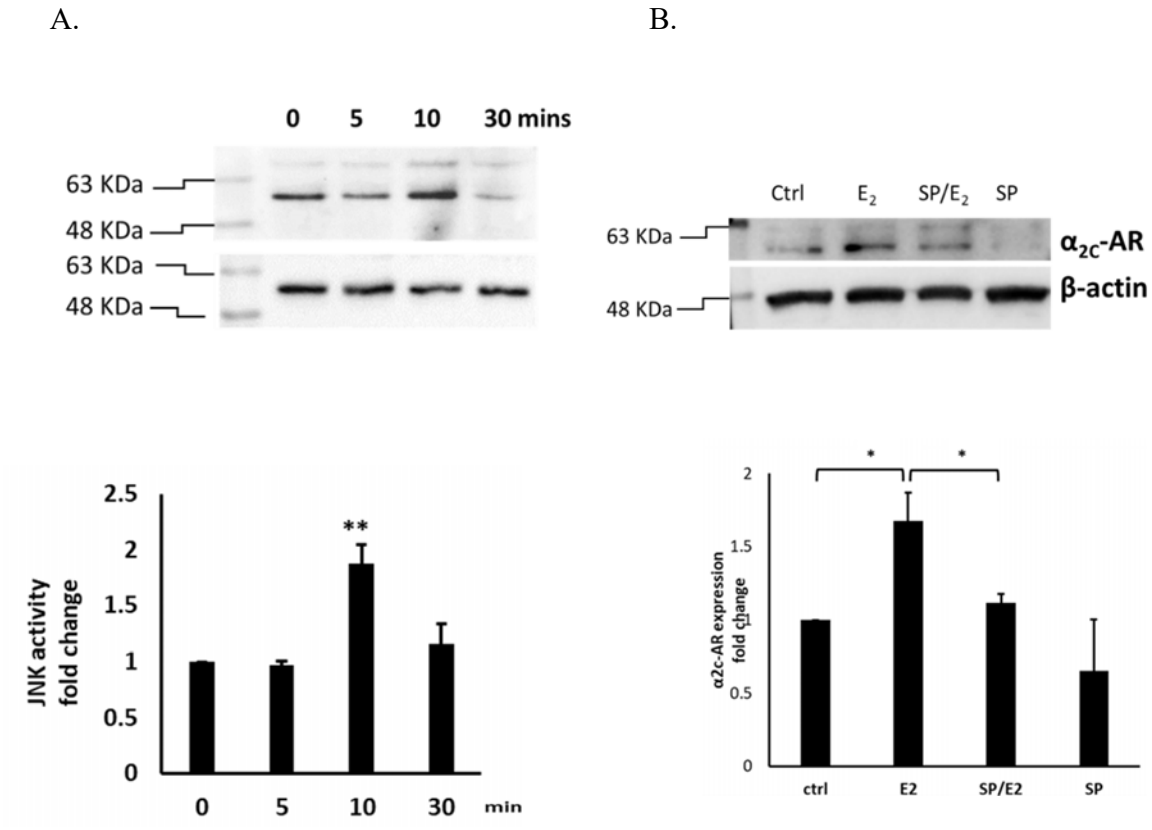


Figure 36. JNK mediates estrogen-induced expression of α_{2C}-AR in VSMCs.

A. Cells were treated with estrogen (10^{-10} M) for 5, 10 and 30 minutes. Total and phosphorylated JNK were assessed using Western blotting. Top: blot representing the effect of estrogen (10^{-10} M) on phospho-JNK levels. The results are expressed as fold increase from the control at time 0 and are presented as means ± SEM. n=3, ** $p<0.001$. B. Cells were treated with estrogen (10^{-10} M) for 24 hours, in the presence or absence of SP600125 (2 μM; a JNK specific inhibitor), and cell lysates were assessed for α_{2C}-AR expression using Western blotting. SP600125 was administered 30 minutes before and during exposure of the cells to estrogen. Top: blot representing the effect of estrogen (10^{-10} M) on α_{2C}-AR expression in the absence or presence of SP600125. The results are expressed as fold increase from the control and are presented as means ± SEM. n=3, *$p<0.05$

3. Epac mediates estrogen-induced JNK activation.

It has been reported that estrogen-induced α_{2C}-AR expression is mediated through the activation of Epac/Rap signaling [51]. Having established that α_{2C}-AR expression in response to

estrogen is also dependent on JNK, we wanted to evaluate the role of Epac in estrogen-induced JNK activation. Estrogen-induced increase in JNK activity was inhibited by pretreatment with ESI09 (10 μM), a specific Epac inhibitor ($p< 0.05$) (figure 31). This indicates that estrogen-induced JNK phosphorylation in mediated through Epac signaling.

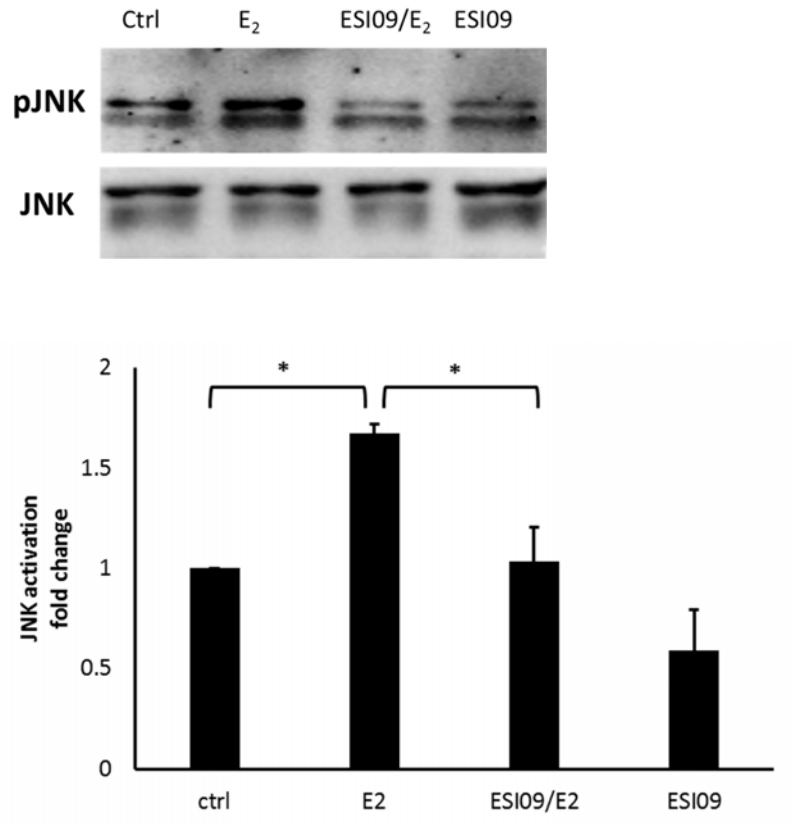


Figure 37. Epac mediates estrogen-induced JNK activation.

Cells were treated with estrogen (10^{-10} M) for 10 minutes, in the presence or absence of ESI09 (10 μM; an Epac specific inhibitor). ESI09 was administered 30 minutes before and during exposure of the cells to estrogen. Cell lysates were assessed for JNK phosphorylation using Western blotting. Top: blot representing the effect of estrogen on JNK phosphorylation, in the presence or absence of ESI09. The results are expressed as fold increase from the control and are presented as means ± SEM. n=3, *$p < 0.05$

4. JNK acts downstream of Epac to mediate estrogen-induced activation of α_{2c}-AR promoter.

To determine whether JNK acts downstream of Epac in estrogen-induced α_{2C}-AR signaling, transient transfection were used and the activity of α_{2C}-AR promoter: reporter was assessed. Transfection with a dominant negative mutant form of Epac (Epac DN) completely abrogated estrogen-induced activation of the promoter (figure 31). However, co-transfection of Epac DN along with the constitutively active form of JNK (JNK CA) overweighed the inhibitory effect of Epac DN (3.8±0.25 fold v/s 1.83±0.24 fold for estrogen concentration of 10^{-10} M ($p< 0.01$). These findings suggest that estrogen elicits its effects on the α_{2C}-AR promoter by increasing the activity of Epac and JNK successively. This means that JNK acts downstream of Epac to mediate estrogen-induced α_{2C}-AR expression.

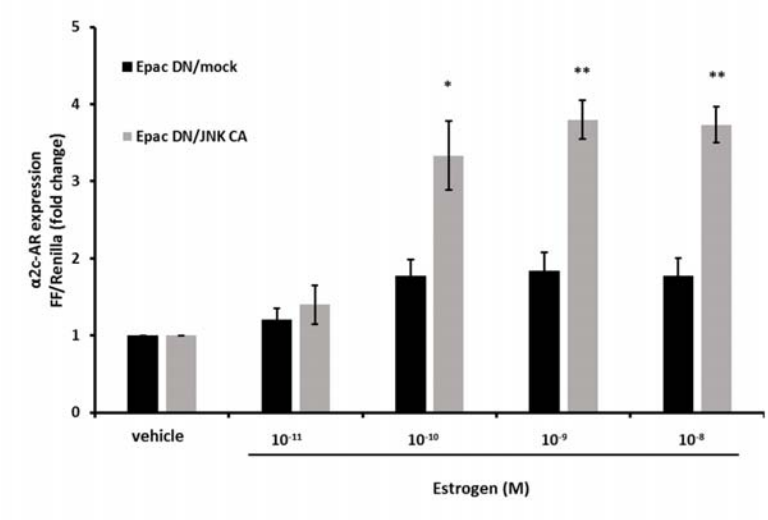


Figure 38. JNK acts downstream of Epac to mediate estrogen-induced activation of α2c-AR promoter.

Cells were transiently co-transfected with α_{2C}-AR promoter: reporter along with expression plasmids for Epac DN, without or with another expression plasmid for a constitutively active mutant of JNK (JNK CA). Internal *Renilla* plasmid was used as an internal control. After recovery, cells were starved for 48 hours then treated with increasing concentrations of estrogen (10^{-11}-10^{-8} M, 12 hours). Promoter activity was assessed using luciferase assay. Results are expressed as fold increase in the firefly (FF)/*Renilla* luminescence and are presented as means ± SEM. n=3, * denotes $p<0.05$ and ** denotes $p<0.01$.

5. AP-1 site is necessary for estrogen-induced α_{2C}-AR expression.

Estrogen induces α_{2C}-AR promoter activation through JNK, which in turn is known to activate AP-1 dependent genes [305]. Knowing that the promoter region of α_{2C}-AR harbors an AP-1 consensus binding site located at −346/−338 (relative to the transcription start site) [642], we wanted to assess the effect of estrogen on the AP-1 site activity. Treatment with increasing concentrations of 17β-estradiol (10^{-11} - 10^{-7} M, 12 hrs) caused a concentration-dependent increase in AP-1 activity (figure 31A). This increase was attenuated by mutation of the AP-1 site in the α_{2C}-AR promoter (3.43±0.33 fold v/s 1.8±0.11 fold, $p< 0.01$) (figure 31.B). These results show that the AP-1 site is necessary for estrogen-induced α_{2C}-AR expression.

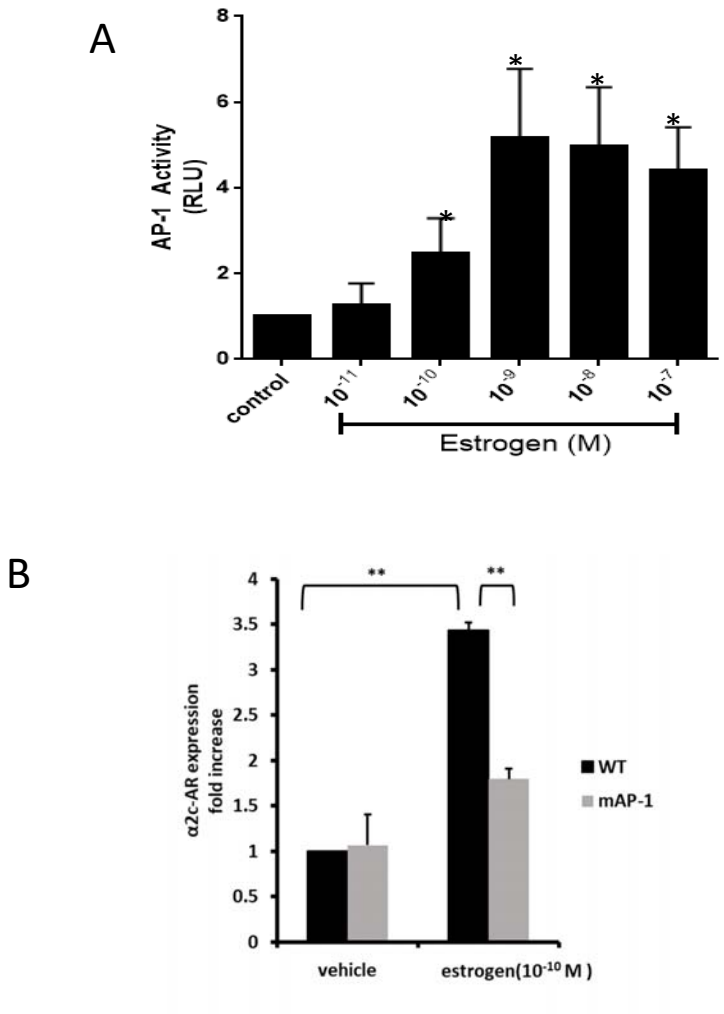


Figure 39. AP-1 site is necessary for estrogen-induced α2C-AR expression.

A. Cells were transiently transfected with the AP-1 site-reporter construct and internal *Renilla* control construct. After recovery, cells starved for 48 hours then treated with increasing concentrations of estrogen (10^{-11} to 10^{-7} M, 48 hours). AP-1 site activity was assessed using luciferase assay. Results are expressed as fold increase in the firefly (FF)/*Renilla* luminescencel and are presented as means ± SEM. n=3, $p<0.05$

B. Cells were transiently transfected with α_{2C}-AR promoter-reporter construct (wild type, WT, or the AP-1 site mutant, mAP-1) and internal *Renilla* control construct. After recovery, cells starved for 48 hours then treated estrogen (10^{-10} M, 48 hours). Promoter activity was assessed using luciferase assay. Results are expressed as fold increase in the firefly (FF)/*Renilla* luminescent signal from the control level and are presented as means ± SEM .n=3, ** $p<0.01$

6. Effect of JNK on estrogen potentiated cold-induced vasoconstriction:

We then sought to determine whether JNK-mediated α_{2C}-AR expression is associated with functional rescuing. In accordance with our previously published results, at 37 ℃, 17β-estradiol (10^{-10} M, 24 hrs) did not significantly affect the constriction induced by activation of α_{2C}-ARs with UK-14304 (10^{-9} -10^{-5} M) (figure 40.A) [51]. However, estrogen significantly increased vasoconstriction to UK-14304 at 28 ℃. This cold-induced estrogen-potentiated vasoconstriction was abolished when JNK was inhibited by SP600125 (figure 40.B). No effect of SP00125 alone on vasoreactivity was noted (data not shown). It is worth mentioning that we have already established that the entirety of cold-induced vasoconstriction is solely mediated by α_{2C}-ARs [52].

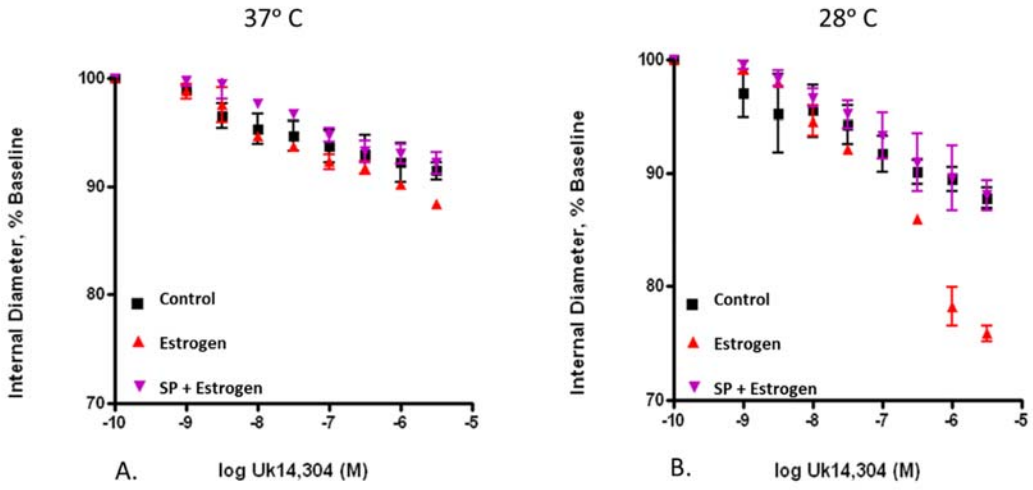


Figure 40. Effect of estrogen (10^{-8} M, 24 hours) on the function of α_{2C}-ARs in the presence or absence of SP600125, a JNK inhibitor, in mouse isolated tail arteries.

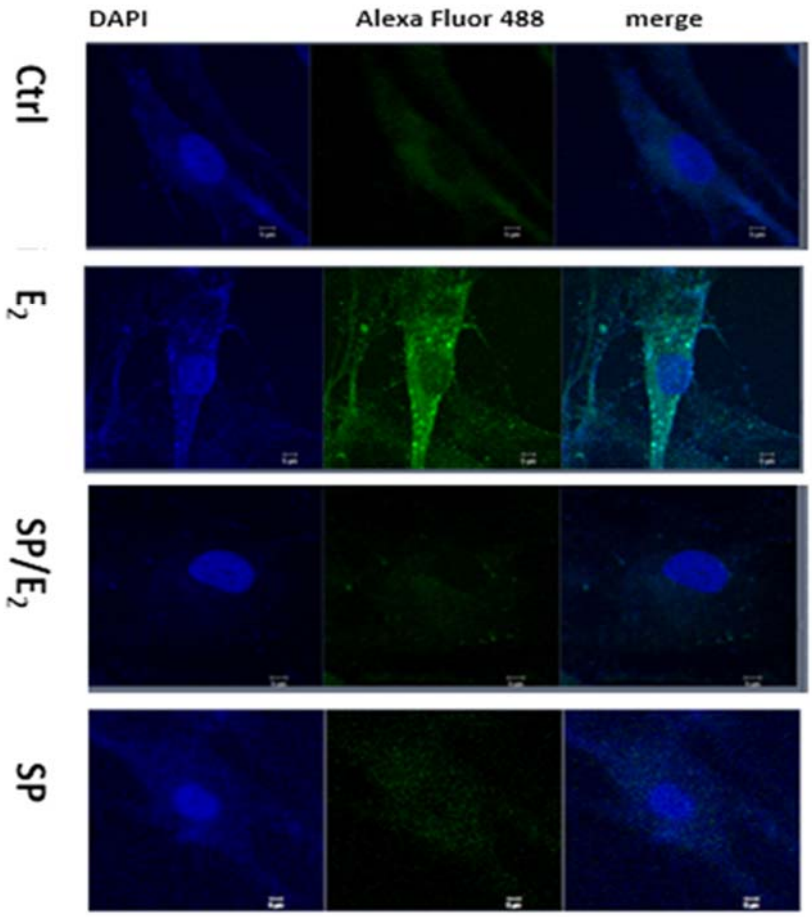
SP600125 (50 μM) was administered 30 minutes before and during exposure of the cells to estrogen. Vasoconstriction to the α_2-AR agonist, UK-14304 (10^{-9}-10^{-5} M) was assessed at 37 °C (A.) and 28 °C (B.). Responses to the agonist were expressed as a percentage of the stable baseline diameter and are presented as means± SEM (n≥5). No significant differences were

noted in A. In B, significant differences ($p<0.05$) were noted for the following comparisons: estrogen versus control, and estrogen versus SP+estrogen.

7. JNK mediates estrogen-augmented α_{2C}-AR translocation at cold temperature.

Having established that JNK mediates estrogen-induced α_{2C}-AR expression, we wanted to test whether JNK affects α_{2C}-AR translocation in human microVSMCs. Cells were treated with estrogen in the presence or absence of SP600125 (2 μM), and the localization of α_{2C}-AR was determined. Indeed, our results show that estrogen induced cell-surface localization of α_{2C}-AR (figure 40). This estrogen-potentiated cold-induced translocation of α_{2C}-AR was inhibited by SP600125 (figure 40). This means that JNK mediated estrogen-induced spatial rescue of α_{2C}-AR.

A.



B.

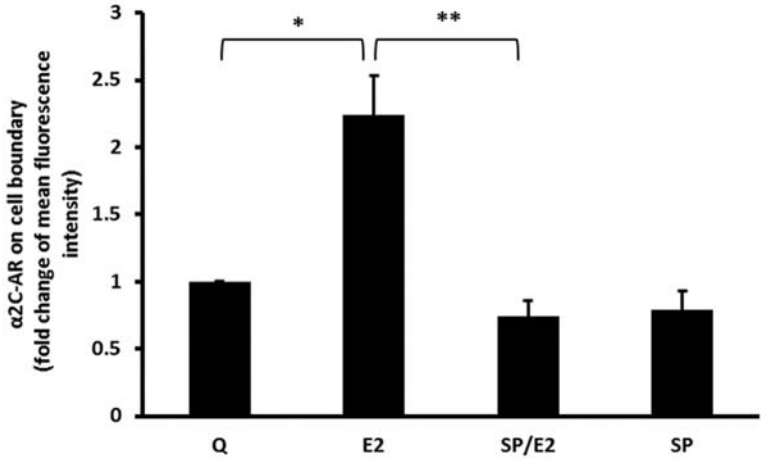


Figure 41. JNK mediates cold-induced α_{2C}-AR localization in human micro VSMCs.

A.Localization of α_{2C}-ARs in micro VSMCs (green, Alexa Fluor 488) by indirect immunofluorescence-confocal microscopy at cold (28 °C) conditions. Nuclei were stained with Hoechst dye (blue). Following 48 hours starvation, cells were treated with estrogen (10^{-10} M) for 1 hour, in the presence or absence of SP600125 (2 μM; a JNK specific inhibitor), and incubated for 1h at cold (28 °C) conditions. Cells were then fixed and stained for membrane α_{2C}-ARs. SP600125 was added 30 minutes prior to treatment. Scale bars, 20 μm. The optical slices obtained by this approach allowed spatial visualization of cell surface α_{2C}-ARs. For fluorescence comparisons, the highest intensity observed was used as reference for each set of experiments and identical settings and conditions were used to capture and process all images. Nuclei were visualized with Hoechst stain dye (blue). B. Quantitation of the mean fluorescence intensity of α_{2C}-AR on cell boundary. Data presented as relative mean intensity of fluorescence from 3 replicates of the experiment are shown. *$p < 0.05$, **< 0.01

D. Discussion

In this study, we showed that estrogen acts through the Epac/JNK/AP-1 signaling pathway to induce α_{2C}-AR expression (figure 40). We also showed that pharmacological inhibition of JNK attenuated estrogen-induced spatial and functional rescue of α_{2C}-AR at cold temperature . These findings present an insight on a signaling cascade that may explain exaggerated cold-induced vasoconstriction in estrogen-replete females.

In the vasculature, cAMP regulates α_{2C}-AR expression, positively via Epac or negatively via PKA [51,305], with the former effect being more dominant in microVSMCs [152,305]. Notably, estrogen induces an increase in cAMP levels, leading to Epac-mediated Rap-2 dependent increase in α_{2C}-AR expression, or Rap-1 dependent change in cell adhesion to the substratum [51,304]. These selective and rather divergent activation of Epac could be explained by caveolae microdomains, where adynelate cyclase, Epac, and Rap are compartmentalized [643,644]. Here we show that this Epac mediates estrogen-induced activation of JNK in microVSMCs. We speculate that JNK is present in the vicinity of Epac, probably in the same microdomain. On the other hand, our results appear to disagree with a previous study reporting that Epac synergizes with PKA to inhibit the JNK activation in macroVSMCs [554]. Knowing that our experiments are

carried in microVSMCs, the discrepancy in Epac effect may be due to the difference in the vascular bed from which VSMCs were isolated. Thus, it is not safe to extrapolate results between micro and macro VSMCs.

In the present study, we show that pharmacological inhibition of JNK attenuated estrogen-induced transcription and expression of α_{2C}-AR. In cells transfected with Epac-DN, estrogen failed to induce α_{2C}-AR expression. However, this inhibitory effect was overridden by co-transfection of VSMCs with Epac-DN and constitutively active JNK. These results show that JNK activation is necessary and sufficient for estrogen-induced α_{2C}-AR expression. Furthermore, we showed that JNK spatially and functionally rescued α_{2C}-AR in estrogen-replete environment. Indeed, while estrogen potentiated cold-induced vasoconstriction [51], inhibiting JNK abolished this effect, probably by reducing α_{2C}-ARs mobilization to the cell membrane. This mobilization is likely facilitated via Rho A/Rho-mediated actin rearrangement as we and other recently reported [55,166] . Here, we propose that JNK is a mediator of estrogen-induced α_{2C}-AR translocation. Importantly, this would not be the first report to incriminate JNK in cytoskeleton-dependent events. Indeed, in Ang-II-induced VSMC migration, the effect of Rho/Rock signaling on cytoskeletal rearrangement was mediated by JNK [645]. Thus, it may be postulated that a similar cross talk between estrogen-activated Rho/ROCK and JNK lead to α_{2C}-AR translocation in VSMCs. Further analysis is needed to validate this hypothesis.

To the best of our knowledge, this is the first study to show that estrogen increases the expression and translocation of α_{2C}-AR via JNK. In fact, JNK plays a significant role in regulating the vascular tone. Inhibiting JNK leads to a dose-dependent relaxation of norepinephrine-pre-constricted aortic rings and abolishes their norepinephrine-induced vasoconstriction [646]. In addition, JNK inhibition reduced Ang II-induced increase in systolic

blood pressure [646]. Contextually, this blood pressure regulation is most critically regulated at the levels of small rather than large vessels. Knowing that Ang II has the potential to activate JNK [647], we may suggest that the hypotensive effect resulting from JNK inhibition may be due to decreased α_{2C}-AR expression and/or translocation. Alarmingly, the aforementioned experiments were done on aortic rings, which is a major limitation. The role of JNK in vascular contractility was further determined by genetic inhibition of JNK. Whereas vascular contraction was not affected in single JNK knock-out mice, it was significantly increased in JNK2+3 double knock-outs [648]. This implies that there is a gene-dosage effect and that JNK1 is responsible for enhanced vascular contractility, especially that JNK1-deficient mice have lower blood pressure than wild type mice [649], and that JNK-1 is an Epac target [650]. This finding also supports our notion that JNK is implicated in vasoconstriction. Further studies are warranted to test whether JNK1 particularly mediates α_{2C}-AR expression/mobilization.

Several previous studies assessed the interplay between JNK and adreneroceptors. For example, activation of α_2-adrenoceptor lead to JNK phosphorylation in rat aortic SMCs [651,652] and enhanced contraction in rat aortic rings [652]. In addition, stimulation of α_1-adrenergic receptor activated JNK in rat myocytes [653]. Whether this adrenoceptor/JNK interplay is translated to α_{2C}-AR is yet to be determined. If this hypothesis is validated, then it will reveal a positive feedback loop between JNK and α_{2C}-AR, contributing to α_{2C}-AR re-sensitization.

Estrogen elicits its effects through the classical genomic pathway, involving intracellular estrogen receptors α and β (ERα and ERβ), or by rapid non-genomic response mediated by membranous G-protein estrogen receptor (GPER) [654]. In the context of α_{2C}-AR expression, we report that estrogen induced JNK activation as early as 10 minutes. Thus, it is only reasonable to assume that this activation is due to a rapid non-genomic effect. Notably, ERα and ERβ agonists

were able to increase the expression of α_{2C}-AR in cutaneous VSMCs [51]. Likewise, 17β-estradiol:BSA, a cell-impermeable form of estrogen, mimicked estrogen effect [51]. Therefore, estrogen may act through a cell membrane receptor to activate JNK.

JNK acts through c-Jun, which dimerizes with c-Fos to form AP-1, a transcription factor that drives AP-1 mediated expression [655]. Interestingly, the promoter region of α_{2C}-AR harbors an AP-1 binding site at −346/−338 relative to transcription start site [642]. Previous findings showed that cAMP increased the transcriptional activity of the α_{2C}-AR promoter through JNK/AP-1 signaling [155]. However, in that study, cAMP levels were elevated by forskolin, a non-physiological agonist of adenylate cycles. Here, we showed that estrogen, a physiologic agonist, activates AP-1 via JNK, leading to increased transcriptional activation of the α_{2C}-AR promoter. Therefore, the JNK/AP-1 interaction may represent a therapeutic target in treating RP. Certainly, this approach proved to be successful in other CVDs. For example, interrupting JNK/AP-1 interaction decreased VSMC hypertrophy [656]. This JNK/AP-1 blockage did not affect other mitogen-activated protein kinases (MAPKs) such as ERK1/2 and p38. Thus, other cellular pathways mediated by these MAPKs may not be affected. Another endeavor is JNK inhibition, which has been used to treat diabetes. Indeed, intraperitoneal injection of cell-permeable JNK inhibitory peptide improved insulin resistance and glucose tolerance in mice. Since JNK is a key mediator in estrogen-induced α_{2C}-AR regulation, such inhibitory peptide might have a potential for the treatment of RP [649]. It is tempting to envision such drugs for RP treatment. However, further studies must be carried on to ensure their efficiency and safety.

Notably, estrogen-induced α_{2C}-AR upregulation is insufficient and likely independent of a functional rescue of the receptor. We have previously established that cold mobilizes α_{2C}-AR to the cell membrane of VSMCs [51]. Here, we showed that estrogen potentiated this cold-induced

translocation via JNK activation. Thus, the two pathways employed by estrogen to regulate α_{2C}-AR expression and functional rescue converge at the level of JNK. This JNK activation is achieved within minutes post treatment, supporting the notion that the two aforementioned pathways are a result of rapid nongenomic effect of estrogen.

It is worth mentioning that we used VSMCs and tail arteries of male origin to test the effect of estrogen. This is called the crossover approach. Knowing that hormones have a relatively long half-life, the aim of this approach is to decrease the carryover of the tested hormone from the donors, in an attempt to decrease the background noise [27]. However, a limitation to this approach is that cells/arteries extracted from a given sex are not exposed to the high concentrations of the hormone of the other sex. Notably, the estrogen level in female mice varies during estrous cycle. Knowing that estrogen increases α_{2C}-AR expression in VSMCs [51], using female mice will affect our results, as the exact phase of the estrous cycle cannot be maintained during all experiments.

Our study presents new insights on the signaling pathway by which estrogen regulates α_{2C}-ARs expression and activity, and may thus explain the prevalence of RP in premenopausal females. Initially, α_2-AR blockers were used to alleviate RP symptoms [657]. However, no definitive drug for RP has been yet approved by the US Food and Drug association [196]. The challenge in finding a specific treatment arises from the fact that α_{2C}-AR is expressed in many brain regions and is implicated in the presynaptic regulation of the heart. Therefore, targeting α_{2C}-AR in an attempt to cure RP might interfere with α_{2C}-AR function in other organs, thus causing deleterious side effects. It would be tempting to speculate that RP management may involve targeted therapies against Epac/Rap/JNK pathway especially that Epac-induced activation of Rap occurs in microVSMCs but not human aortic smooth muscle cells

(unpublished observations). Indeed, although aortic SMCs express Rap, its activation in these cells appears to be Epac-independent. Therefore, targeting microVSMC-specific Epac/Rap/JNK pathway may be successful in the realm of RP therapy.

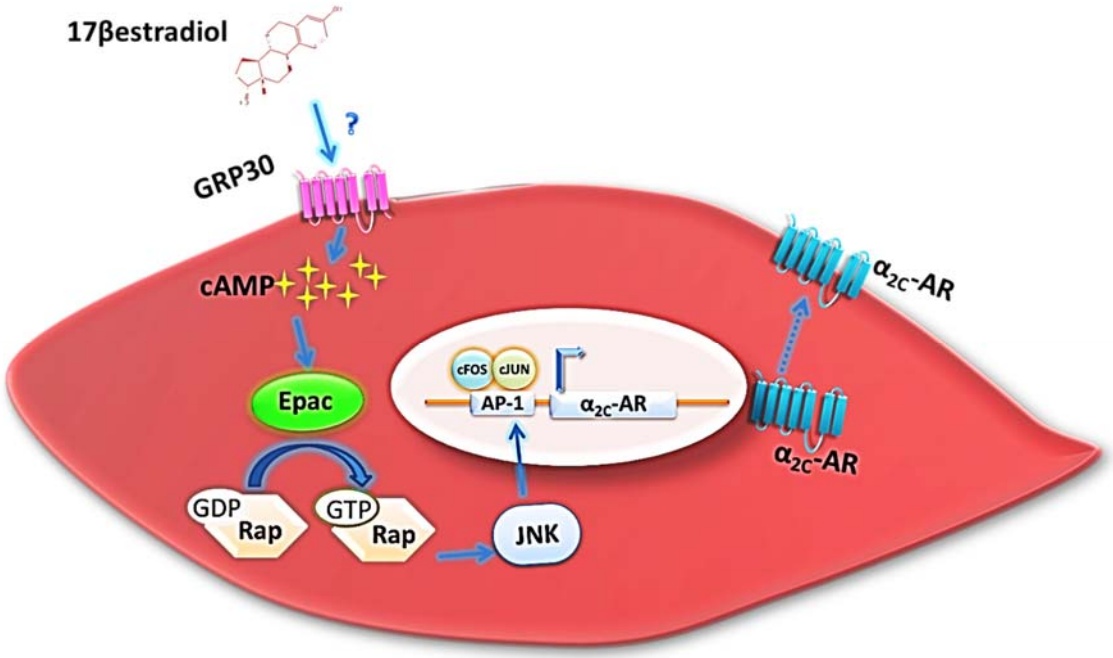


Figure 42. Schematic representation of the proposed mechanism for α_{2C}-AR expression and translocation in VSMCs.

Estrogen induces an increase in cAMP level, which activates its downstream target Epac. In turn, activated Epac catalyzes the exchange of GDP to GTP on Rap, thus activating it. The now active Rap induces JNK phosphorylation, leading to the dimerization of the cFus and cJun to form the AP-1 transcription factor. AP-1 binds to AP-1 site in the α_{2C}-AR promoter, initiating its transcription. Furthermore, estrogen-activated JNK mediates α_{2C}-AR trafficking to the cell membrane.

CHAPTER VI

MITOGEN-ACTIVATED PROTEIN KINASE P38 MEDIATES ESTROGEN-INDUCED CONTRACTION THROUGH MYOSIN LIGHT CHAIN PHOSPHORYLATION IN HUMAN MICROVASCULAR SMOOTH MUSCLE CELLS

Authors: Manal Fardoun, Khodr Issa, Elias Baydoun, Ali H. Eid

Cardiovascular diseases (CVDs) are the leading cause of death worldwide. Hypertension remains the major contributor to these diseases. The prevalence of hypertension in premenopausal women is lower compared to age-matched men, suggesting a vasorelaxant effect of estrogen. However, recent evidence showed that estrogen-based hormone replacement therapy (HRT) may lead to an increase in blood pressure (BP) in postmenopausal females, arguing against the hypotensive effect of estrogen. In fact, estrogen was reported to mediate vascular smooth muscle cell (VSMCs) contraction by stimulating myosin light chain (MLC) phosphorylation. However, the mechanism by which estrogen induces VSMC contraction is yet to be investigated. It has been shown that estrogen induces reactive oxygen species (ROS) and activates the MAPK, p38. Furthermore, MLC was reported to be activated by p38 and by ROS activated Rho-kinase in VSMCs. In addition, evidence show that Rho activates p38 in these cells. In this study, we hypothesized that estrogen induces MLC phosphorylation through ROS/Rho/p38 signaling pathway. Our results showed that estrogen (10^{-10} M) induced ROS production. Furthermore, estrogen induced Rho mobilization to the plasma membrane.

Pretreatment with NAC (5 μM, ROS scavenger) attenuated estrogen-induced Rho translocation. In addition, estrogen induced p38 activation in a time-dependent manner. This estrogen-induced-p38 activation was inhibited by NAC. Furthermore, estrogen (10^{-10} M) stimulated MLC phosphorylation, which was attenuated by NAC (ROS scavenger, 5 μM) or SB2021190 (p38 inhibitor, 3 μM). Moreover, *ex-vivo* results showed that estrogen-induced vasoconstriction of endothelium-denuded mouse tail artery was attenuated by SB2021190. Taken together, these results show that estrogen induces VSMC contraction by phosphorylating MLC via ROS/Rho/p38 pathway. This pathway presents a novel insight to the mechanism underlying estrogen-induced hypertension, and provides therapeutic targets for the treatment of the disease.

A. Introduction:

Cardiovascular disease (CVD) remains the leading cause of morbidity and mortality worldwide [222]. Hypertension is implicated in almost 50% of these CVD-related deaths [658]. In fact, hypertension affects around 40% of the general population [659]. According to the WHO, hypertension is defined as elevated blood pressure, higher than 130 over 80 mmHg (WHO, AHA). Mostly, hypertension is asymptomatic [229] and results as a secondary complication to other pathological conditions such as type-2 diabetes, obesity, and chronic kidney disease [660]. Among the risk factors of hypertension are diet, exercise, family history, age and sex [661]. Women are thought to be at a lower risk of developing hypertension than men [253]. This sex difference fades at menopause, after which more women are affected by hypertension [253]. This suggests a hypotensive role of estrogen.

Lately, the vasorelaxant effect of estrogen has been a matter of controversy as recent evidence supported a vasoconstrictive effect of estrogen. In fact, some studies reported that post-

menopausal females on hormone replacement therapy had increased BP [341]. A study by Women Health Initiative (WHI) in 2003 showed that post-menopausal females on Estrogen-alone therapy had higher blood pressure [662]. This study is a significant as it is randomized, double-blinded and has a great number of participants over a long period of time. However, it was not the first study to unravel the hypertension-promoting effect of estrogen. Several previous studies indicated that estrogen elevated BP in post-menopausal females [368,663-666]. Furthermore, the use of contraceptives by premenopausal females promoted hypertension [368,667,668]. Moreover, women in their third trimester of pregnancy, during which estrogen reaches its maximum level, show increased ambulatory blood pressure [669]. Collectively, these observational studies reflect a blood pressure elevating effect of the estrogen.

Several studies were carried in an attempt to elucidate the mechanism by which estrogen induces vasoconstriction. It has been demonstrated that estrogen induces the phosphorylation of myosin light, a key mediator of VSMC contraction [351]. Moreover, MLC may be phosphorylated via the MAPK p38 [670]. In addition, the activation of MLC may be mediated in a ROS/Rho-dependent manner [671]. Notably, estrogen induces ROS release in non-VSMCs [672]. Knowing that in VSMCs, estrogen activates p38 [95], which is also activated by ROS [673], we hypothesized that estrogen induces MLC activation and consequently VSMC contraction via ROS/Rho/p38 signaling pathway.

B. Materials and Methods:

1. Reagents:

DMEM: F12 (D8437), phenol-red free DMEM (D6434), L-Glutamine (G7513), Fetal Bovine Serum (F9665), Phosphate Buffer Saline (D1408), N-Acetyl-L-cysteine (A7250) were

purchased from Sigma Aldrich (Schnelldorf, Germany). Rabbit monoclonal antibody to anti-phospho p38 (ab38238), rabbit monoclonal antibody to total p38 (ab7952), rabbit monoclonal antibody to Rho (ab178027), rabbit polyclonal antibody to beta-actin (ab119716), Goat polyclonal Secondary antibody to Rabbit IgG (ab6721), 17-β estradiol (ab120657), p38 inhibitor SB 2021190 (ab120638) were obtained from Abcam®. Rabbit polyclonal antibody to anti-phospho MLC (3674) and rabbit polyclonal antibody to total MLC (3672) were purchased from Cell Signaling TECHNOLOGY®. Insulin-Transferin-Selenium (41400045) and Dihydroethedium (D23107) were obtained from ThermoFischer Scientific. DC™ Protein Assay kit (5000112) and Clarity™ Western ECL Substrate kit (1705060) were obtained from Biorad (CA, USA). Penicillin/Streptomycin (17-602E) and Trypsin (BE02-007E) were purchased from Lonza (Basel, Switzerland).

2. Cell culture:

VSMCs, isolated from human dermal arterioles using nonenzymatic sprouting method, were grown complete DMEM: F12 medium (supplemented with 10% FBS and 1% penicillin/streptomycin). Only cells with passages 6 and 11 were used. Prior to treatment, cells were made quiescent for 48 hours in phenol red-free DMEM supplemented with L-glutamine, insulin-transferrin-selenium, and 1% penicillin/streptomycin. Cells were maintained in a 5% CO_2 incubator at 37 ºC.

3. DHE stain:

Intracellular ROS production was detected using DHE stain. Cells were seeded on coverslips in 12-well plates in complete medium. At 80% confluency, cells were starved for 48 hrs. After treatment, medium was aspirated and cells were washed with PBS. Cells were then incubated with DHE (5 μM) for 1 hour in dark. After staining, cells were washed and observed under Zeiss Axio observer microscope at 518 nm excitation wavelength.

4. Western blot:

Cells were washed with PBS and the lysed using 2% SDS, 60 mM Tris lysis buffer (pH 6.8). Proteins were quantified using DC assay and equal amounts of protein (20-30μg) were loaded and separated using SDS-PAGE. The proteins were then transferred onto polyvinylidene difluoride (PVDF) membrane (Biorad). After blocking (5% fat-free milk in TBS-T, 1 hour), the membrane was incubated overnight with the relevant primary antibody at 4 ºC. The membrane was then washed 3x 10 minutes each with TBS-T, and the incubated with the appropriate secondary antibody for 1 hour at room temperature. The membrane was washed again (3x, TBS-T, 10minutes), and then developed using enhanced chemiluminescence (ECL clarity, Biorad) and quantified using Chemidoc MP Imaging system (Bio-Rad, USA).

5. Rho translocation:

Cells were lysed using cold homogenization buffer (mM): 100 Tris- HCl (pH 7.4), 1 EGTA, 1 EDTA, 1 PMSF, and 1 Na3VO4 EDTA, Tris-HCL, EGTA, PMSF and NA3VO4. The homogenates were centrifuged at 100,000 g and 4 °C for 20 minutes. The supernatant (cytosolic

fraction) was collected, and the pellet (membrane fraction) was re-suspended in homogenization buffer containing 1% Triton X-100. Membrane and cytosolic levels of Rho were determined by Western blot using monoclonal Rho antibody.

6. Pressure myography:

Male mice (C57BL6) were sacrificed by CO_2 asphyxiation. The tail artery was isolated and placed in cold Krebs buffer (in mM: 118.3 NaCl, 4.7 KCl, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 2.5 $CaCl_2$, 25.0 $NaHCO_3$, and 11.1 glucose). Following treatment, arteries were mounted on a microvascular chamber, and maintained at constant temperature (37 °C) and pressure (60 mmHg), with continuous perfusion in Krebs solution. Live vessel images were acquired through a video camera connected to an inverted microscope, focused on the vessel chamber. The vessel diameter was directly determined using acquisition system. Vessel stabilization was done using increasing concentrations of α_1-AR selective agonist (phenylephrine). The effect of the agonist was stopped by successive washes of the artery, allowing it to return to its baseline level. Concentration-effect curves were analyzed by comparing the agonist concentration causing 20% constriction (CC_{20}), determined by regression analysis.

7. Statistical analysis:

Statistical analysis was performed using a student's t-test or ANOVA (GraphPad Prism version 5.0). Data was presented as mean ± SEM, where n is equal to the number an experiment is repeated (n will be ≥3). A p value less than 0.05, was considered significant.

C. Results:

1. Estrogen induces ROS production in VSMCs

Estrogen induce ROS production in several cell lines [346 674 675, 672]. We sought to test the effect of estrogen on ROS production in micro VSMCs using DHE stain. Estrogen (10^{-10} M) induced ROS production in a time-dependent manner (figure 40). The maximal ROS production was achieved 5 minutes after treatment.

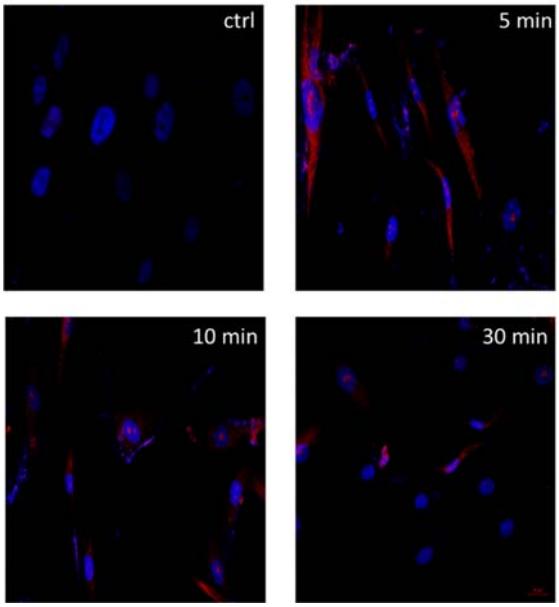


Figure 43. Effect of estrogen (10^{-10} M) on ROS production in VSMCs.

Cells were treated with estrogen (10^{-10} M) for 5, 10, and 30 minutes. After treatment, cells were washed and incubated with DHE stain for 30 mins. Later, cells were washed and observed microscope at 518 nm excitation wavelength. Scale bar, 50 µM.

2. ROS mediates estrogen-induced Rho translocation

It is well established that elevated ROS level activates Rho in micro VSMCs [676 677 161 55]. Having established that estrogen induces ROS in micro VSMCs, we wanted to study the effect of estrogen on Rho translocation. Estrogen induced Rho translocation in a time-dependent manner (figure

40.A). The optimal Rho translocation to the plasma membrane was noted 5 minutes post-treatment. This estrogen-induced Rho translocation was attenuated by NAC (ROS scavenger, 5 mM) (figure 40.B). These results indicate that estrogen leads ROS-dependent Rho activation.

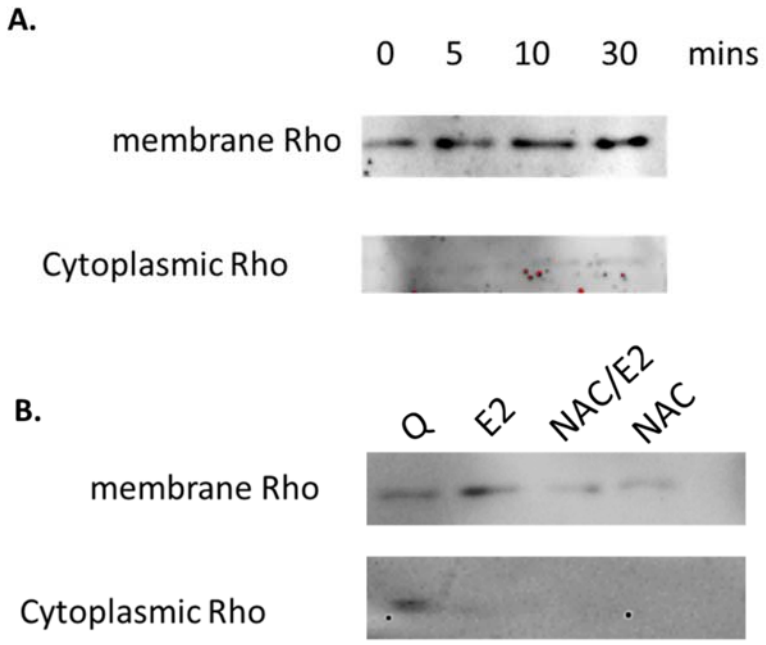


Figure 44. Effect of estrogen (10^{-10} M) on Rho translocation.

A. Cells were treated with estrogen (10^{-10} M) for 5, 10, and 30 minutes. Cells were lysed in homogenization buffer and Rho partitioning was assessed by western blot.
B. Cells were treated with estrogen (10^{-10} M), in the absence and presence of NAC (5 μM, ROS scavenger). NAC was administered 30 minutes before and during exposure of the cells to estrogen.

3. Estrogen activates p38 in a ROS-dependent manner

It has been reported that estrogen induces p38 phosphorylation in rat aortic VSMCs [94]. We wanted to determine whether estrogen activates p38 in micro VSMCs as well. Our results show that estrogen caused a time-dependent p38 activation (figure 40.A). Maximal p38 phosphorylation in activation was attained 30 minutes after treatment. This estrogen-induced p38 activation was

inhibited by NAC (5 μM) (figure 40.B). This indicates that ROS mediates estrogen-induced p38 phosphorylation.

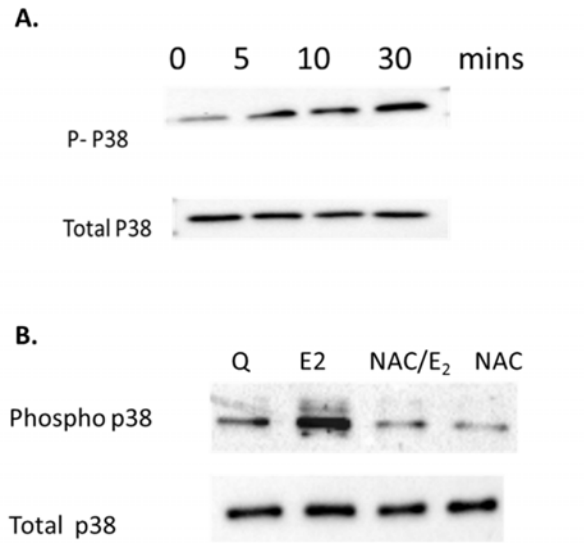


Figure 45. Effect of estrogen (10^{-10} M) on p38 activation.

A. Cells were treated with estrogen (10^{-10} M) for 5, 10, and 30 minutes. The phosphorylation levels of p38 was determined by Western blotting. B. cells were treated with estrogen (10^{-10} M) for 30 minutes, in the absence and presence of NAC (5 μM). NAC was administered 30 minutes before and during exposure of the cells to estrogen.

4. Estrogen activates MLC in a ROS- and p38-dependent manner

Previous reports showed that estrogen induces MLC phosphorylation in rat aortic SMCs [351]. Similarly, estrogen induced MLC phosphorylation in a time-dependent manner, with maximal phosphorylation level attained 10 minutes post-treatment (figure 40.A). This estrogen-induced MLC phosphorylation was attenuated with SB2021190 (3 μM) or NAC (5 μM) pretreatment (figure 40.B). This indicates that estrogen activates MLC via ROS/p38 signaling.

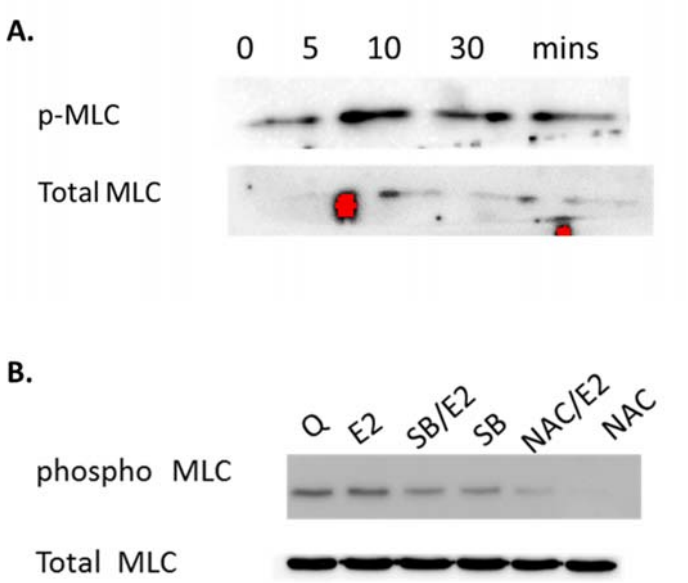


Figure 46. Effect of estrogen (10^{-10} M) on MLC activation.

A. Cells were treated with estrogen (10^{-10} M) for 5, 10, and 30 minutes. The phosphorylation levels of MLC was determined by Western blotting. B. Cells were treated with estrogen (10^{-10} M), in the absence or presence of NAC (5 μM), or SB2021190 (3 μM). NAC or SB2021190 was administered 30 minutes before and during exposure of the cells to estrogen.

5. p38 mediates estrogen-potentiated of phenylephrine (PE)-stimulated constriction of isolated endothelium-denuded mouse tail artery

Having established that estrogen-induced MLC phosphorylation is mediated via p38, we sought to determine the role of p38 in estrogen potentiated PE-induced vasoconstriction. Estrogen (10^{-4} M) significantly increased vasoconstriction to PE at 37 ℃ (figure 40). This estrogen-induced increment in the vasoconstriction was attenuated by pretreatment with SB2021190 (10 μM) (figure 40). Therefore, estrogen emplys p38 to mediate the vasoconstriction of denuded arteries.

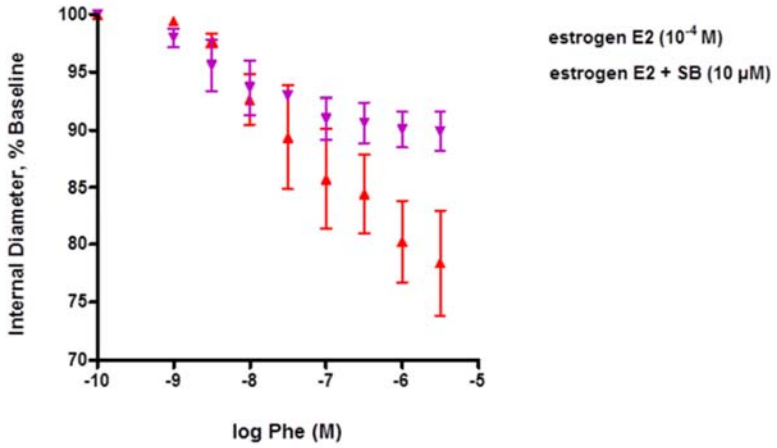


Figure 47. Role of p38 in estrogen-potentiated of phenylephrine (PE)-stimulated constriction of isolated endothelium-denuded mouse tail artery.

Arterial segments were denuded then incubated with estrogen (10^{-4} M, 24 hours) in the presence or absence of p38 inhibitor, SB2021190 (10 µM). SB2021190 was administered 30 min before and during exposure of the cells to estrogen. Vasoconstriction to the α1-AR agonist, PE (0.1–10.10^{3} nmol/l) was assessed at 37°C. Responses to the agonist were expressed as a percentage of the stable baseline diameter and are presented as means± SEM.

D. Discussion:

The vasorelaxant effect of estrogen is overwhelmingly documented. However, this beneficial effect has been recently questioned, as the Women's Health Initiative reported increased CVD in post-menopausal females on HRT. Indeed, women on HRT and oral contraceptives, both of which contain estrogen, showed increased blood pressure, suggesting a vasoconstrictive potential of estrogen. However, the mechanism by which estrogen elicits this effect is still obscure. Here, we showed that estrogen activates MLC, the major effector in VSMC contraction, via a ROS-initiated Rho/p38-dependent signaling pathway (figure 40. Thus, this study gives an insight to the mechanism underlying the vasoconstrictive potential of estrogen and may explain the increase in BP in females on ERT or contraceptives.

It is well-established that BP is regulated by resistance vessels. Due to small lumen, resistance vessels greatly slow the blood flow from arteries. In our study, we used VSMCs extracted from human arterioles, the main site of resistance in the entire vascular network. These cells are the optimal model mimicking the physiology of arteriolar VSMCs. To our knowledge, we are the only laboratory using human arteriolar VSMCs to study hypertension.

Evidence show that vasoconstriction is the result of VSMC contraction, which is mediated by MLC activation. MLC is under the regulation of myosin light chain kinase (MLCK), which phosphorylates MLC, and myosin light chain phosphatase (MLCP), which induces MLC inactivation. Here, we showed that estrogen phosphorylated MLC. This result is in accordance with a previous study showing that estrogen lead to MLC activation in rat aortic smooth muscle cells [351]. However, in urethral smooth muscle cells, estrogen attenuated MLC phosphorylation in a concentration-dependent manner [678]. In addition, estrogen receptor agonist, G1, attenuated MLC activation in porcine coronary artery smooth muscle cells [313]. This discrepancy in estrogen effect on MLC seems to be cell-type dependent.

Estrogen binds to the cytoplasmic estrogen receptors, ERα and ERβ, to elicit genomic effect. In addition, estrogen binds its membrane receptor, GPR30, to initiate rapid non-genomic response. In this study, the effect of estrogen was rapid, inducing ROS production in as early as 5 minutes. This clearly suggests the implication of GPR30 in mediating estrogenic effect. Further studies employing GPR30 antagonist, G-15, are warranted to confirm the role of this membrane receptor in estrogen-induced MLC activation. Interestingly, GPR30 re-expression enhanced estrogen-mediated contraction of VSMCs [351]. However, a limitation of the aforementioned experiment is that it was conducted in VSMCs extracted from large vessel (rat aorta), which are not the pimary regulators of BP. Surprisingly, G1, GRP30 agonist, induced porcine coronary

artery relaxation [313]. This further highlights the differential response of VSMCs extracted from diverse vascular beds, and mandates the execution of these experiments in VSMCs extracted from arterioles. It is worth mentioning that the expression of GPR30 is attenuated by prolonged culture of VSMCs [351]. Thus, the passage of VSMC may be another factor affecting the differential response of MLC to estrogen.

Great line of research indicates that estrogen plays an antioxidative role in VSMCs. For example, estrogen attenuated progesterone-induced ROS production in rat aortic VSMCs [679]. In addition, the activation of ERα attenuated ROS production in HG-treated human aortic VCMSs [104]. Similarly, the activation of estrogen receptor, GRP30, inhibited angiotensin II-induced ROS in embryonic rat aortic VSMCs [680]**.** However, in our study we showed that estrogen induced ROS production. The role of this ROS spark was not to not to induce oxidative stress, but rather to initiate a signaling pathway. In fact, we are not the first to demonstrate a ROS-producing potential of estrogen. In fact, estrogen was reported to induce superoxide production in VSMCs of porcine coronary artery [402]. The authors of the latter study suggested that estrogen activates nNOS, which is expressed in VSMCs [402]. Depending on its microenvironment, nNOS may lead to the production NO, which mediates vasorelaxation, or the release of superoxide, leading to vasoconstriction. Interestingly, aging decreases the levels of L-arginine and tetrahydrobiopterin (BH_4), cofactors needed for nNOS-mediated NO production. As such, nNOS has increased propensity towards superoxide production. This may explain increased BP in postmenopausal females on estrogen-based HRT. In addition, increased BP in this population may also be due to compromised function of endothelium of resistance arteries in post-menopausal women [372] .

A downstream target of ROS is the Rho GTPase. Rho plays a key role in regulating VSMC cytoskeleton[681].. In this study, we showed that estrogen-induced ROS activated Rho in micro VSMCs. Our result is in line with previous studies showing that ROS activated Rho pathway to induce vasoconstriction [161,671]. Notably, the Rho effector, Rho kinase, increases the phosphorylation of MLC by attenuating MLCP [682]. This supports our finding that estrogen employs Rho to activate MLC.

Overwhelming evidence highlight the role of mitogen-activated protein kinases (MAPKs) in the mediating the effect of estrogen in VSMCs. Particularly, p38 MAPK has been involved in estrogen-modulated VSMC proliferation, migration, differentiation and apoptosis. In addition, p38 has been involved in VSMC contraction [670,683-685]. Here, we report that p38 is mediates estrogen-induced MLC activation. In addition, *ex vivo* functional assay showed that the inhibition of p38 attenuated the vasoconstriction of estrogen-treated mouse tail artery. These results support the role of p38 in estrogen-induced vasoreactivity. It is also worth mentioning that p38 acts downstream ROS. This result is in line with previous studies showed that ROS played the role of a messenger to activate p38 in VSMCs [686,687].

Our results showed that both p38 and ROS mediate MLC activation. Previous studies showed that Rho/ROCK and p38 pathways independently mediated MLC phosphorylation in rat aortic VSMCs [670]. Other studies showed that p38 is not involved in MLC activation. For instance, MLC activation was attenuated by the inhibition of Rho kinase but not p38 in VSMC line derived from the brain basilar artery of guinea pigs [688]. Other study showed that ROCK activated MLC in hindpaw skin tissues of mice [671]. In our study, ROS inhibition attenuated p38 activation; however, whether Rho/ROCK pathway is needed for p38 activation is yet to be determined.

Taken together, these results unravel a signaling pathway by which estrogen induces MLC activation and augments vasoconsriction. Targeting molecular players in this patway may present a therapeutic approach to hypertension in females on HRT and oral contraceptives. Considering the traditional vasorelaxant role of estrogen, it seems that VSMC contractility is under coexisting positive and negative regulatory effects initiated by estrogen.

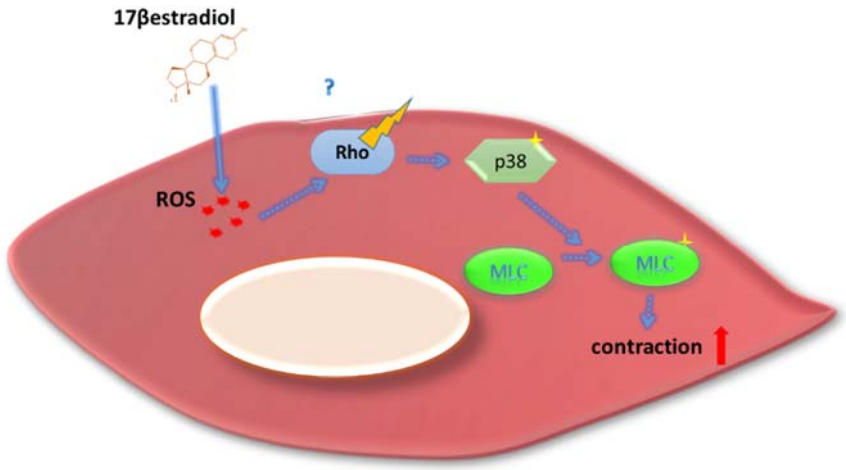


Figure 48. A schematic representation of the pathway of estrogen-induced activation of MLC in VSMCs.

CHAPTER VII

DISCUSSION AND CONCLUSION

The role of of estrogen in CVD has always been an intriguing topic. Substantial amount of research has been undertaken to better assess this role and to dissect the underlying molecular mechanism. However, what is unraveled seems to be the tip of the iceberg, as much more research in this aspect is still warranted. For instance, the majority of studies reported the effect of estrogen on VSMCs of large vessels. Knowing that VSMCs' response to estrogen is affected by the vascular bed from which they are extracted, estrogen effect on microvasculature has to be evaluated. Another instance urging more studies on effect of estrogen on CVD is the lately questionable vasorelaxant role of estrogen. This controversy is raised by recent evidence supporting a vasoconstrictive effect of the hormone.

In this dissertation, we first reported the effect of estrogen on VSMCs extracted from human arterioles. Our results indicate that estrogen, at a physiological concentration, attenuated VSMC proliferation by inducing cell senescence, via an ERα/β-independent p53/pRb-mediated mechanism. In addition, estrogen increased the expression of the contractile differentiation markers, calponin and caldesmon. Thus, the potential of estrogen to promote a contractile phenotype may in part explain its vasculoprotective effect in estrogen-replete females. Knowing that diabetes is accompanied by arteriolar inflammation at the level of the kidneys and the retina, it would interesting to monitor and compare the healing of inflamed arterioles between of male and female mouse models after induction of diabetes.

Estrogen's beneficial effect seems to be mediated by the membrane estrogen receptor, GPR30. It would be interesting to validate this assumption by using GPR30 agonist, G1, and

atagonist, G-15. Moreover, a GPR30 knock out mouse model can be employed to assess the role of GRP30 in the protective effect of estrogen at the level of microvasculature.

In the realm of protection of microvasculature, we showed that the novel homoisoflavonoid, 7-O-methylpunctatin (MP), had an anti-inflammatory effect on VSMCs extracted from human arterioles. In fact, MP attenuated the synthetic phenotype of microVSMCs and upregulated the expression of contractile markers. As such, it would be interesting to investigate the anti-inflammatory effect of this homoisoflavonoid *in vivo*, probably using diabetic mouse model. The recovery of arteriolar inflammation can be tested then. In addition, it would be tempting to use *in silico* tools to specify molecular targets of this drug.

Exaggerated cold-induced vasoconstriction brings about Raynaud's phenomenon. This condition is much more prevalent in premenopausal females. My mentor's laboratory has previously shown that the female hormone estrogen upregulates α_{2C}-AR expression, the sole mediator of cold-induced vasoconstriction. In this dissertation, we showed that estrogen employs the mitogen-activated protein kinase, JNK, and the AP-1 site to induce α_{2C}-AR expression. These results provide an insight into the mechanism by which exaggerated cold-induced vasoconstriction occurs in estrogen-replete females.

Initially, ER-antagonists were effectibve in treatment of RP, but these would block all estrogen-induced pathways, including beneficial ones. Dissecting the molecular mechanim underlying estrogen-induced α_{2C}-AR expression presents Epac and JNK as potential targets for the treatment of RP. This approach has the advantage of being more specific for RP treatment, without disturbing other estrogen-provoked signaling pathways.

Ongoing research in our laboratory is investigating the effect of stress and smoking on α_{2C}-AR expression. It would tempting to study vasoreactivity in a smoker female or a stressed

female. Would the estrogen synergize with stress or smoking to increase α_{2C}-AR expression and consequently cold-induced vasoconstriction? This is an interesting question to be answered. Another exciting question would be related to pregnancy. Having established that estrogen augments cold-induced vasoconstriction, is the increase in estrogen level during pregnancy related to maintaining a proper temperature to the embryo?

My mentor has previously established the implication of the SP-1 site in estrogen-induced α_{2C}-AR expression. Here, we showed this expression is also dependent on AP-1 site. How are these two site related? Do they have a compensatory role? Do they interact to lead to maximal α_{2C}-AR expression? Genetic tools including site-directed mutagenesis may be employed to answer these questions.

It has been reported that α_{2C}-AR plays a role in cell differentiation. However, its effect in modulating VSMC phenotype is still not determined. It would be interesting to study whether α_{2C}-AR upregulation plays a role in modulating VSMC phenotype? If so, how does this modulation affect the progression of RP?

The primary site of estrogen production is the ovaries. However, adipose tissue may be a source of estrogen biosynthesis in fat women. Is it valid to postulate that overweight females are at higher risk of RP? Is RP more prevalent in fat females? If so, would a special diet be recommended for RP treatment?

Estrogen induces α_{2C}-AR expression via the cytosolic receptors, ERα and ERβ. Interestingly, BSA-conjugated estrogen, a membrane impermeable form of estrogen, was able to α_{2C}-AR expression as well. These results strongly corroborate the involvement of the membrane estrogen receptor GPR30 in estrogen-induced α_{2C}-AR expression. This is further supported by a recent study showing that estrogen facilitates cold-induced vasoconstriction through the

activation of GPR30 [689]. Thus, it is interesting to evaluate the role of GPR30 in estrogen-induced α_{2C}-AR expression, and to dissect the underlying mechanism.

Given the role of estrogen and estrogen receptors in α_{2C}-AR expression, it would also be interesting to assess vasoreactivity in an *in vivo* model of ovariectomized mice and ER KO mice, with slow release-estrogen pellets.

The traditional vasorelaxant effect of estrogen has lately been a matter of controversy. Recent studies supported a rather vasoconstrictive role of the hormone. Here, we proposed a signaling pathway by which estrogen activates MLC, the effector player of VSMC contraction. This mechanism may explain the increased BP in females on HRT or contraceptives, both of which contain estrogen. Further experiments are needed to determine whether estrogen employs ROCK to activate p38.

Collectively, this dissertation focusses on the role of estrogen on microvasculature, and consequently on CVD. While estrogen played a vasculoprotective effect in vascular inflammation, it was positively associated with RP. In addition, defying its traditional vasorelaxant effect, estrogen was found to increase BP. Now the question is: does estrogen play a beneficial role in CVD? The answer is: it depends.

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