ABSTRACT

Title of Thesis: CONCENTRATION- AND TIME-DEPENDENT EFFECTS OF PROGESTERONE ON ENDOTHELIAL CELLS

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The termination of endogenous sex hormone release is thought to account for increases in cardiovascular disease (CVD) incidence in postmenopausal women. Thus, hormone replacement therapy may be a preventive measure against cardiovascular disease. To date, most research has been focused on estrogen treatment, but the effects of progesterone, a vasoactive hormone with effects on the endothelium, have received less attention. Two progesterone receptor subtypes, nuclear and membrane, are known to enact the effects of progesterone in endothelial cells which mediate the release of nitric oxide (NO). There is also some evidence that the two subtypes function in a coordinated manner. The aims of this thesis study are to assess the effects of different concentrations of progesterone on endothelial cells and isolate the actions of the progesterone receptor subtypes. Outcomes of this study include migration and proliferation assays to assess endothelial cell function and Western blotting to quantify endothelial nitric oxide synthase expression and phosphorylation. Progesterone and the membrane progesterone receptor agonist were found to inhibit migration and proliferation of human umbilical vein

endothelial cells (HUVECs), while progesterone alone or in combination with the membrane progesterone receptor agonist increased endothelial nitric oxide synthase (eNOS) phosphorylation in HUVECs after 24 hours of incubation. While increased eNOS phosphorylation is thought to be beneficial to HUVEC function, other factors released in the presence of progesterone or progesterone receptor agonists may be scavenging bioavailable NO, thus reducing the angiogenic potential of HUVECs.

CONCENTRATION- AND TIME-DEPENDENT EFFECTS OF PROGESTERONE ON ENDOTHELIAL CELLS

by

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Thesis submitted to the Faculty of the Graduate School of the
University of Maryland, College Park in partial fulfillment
of the requirements for the degree of
Master of Arts
2022

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List of Abbreviations

ANOVA	Analysis of variance
AUC	Area under the curve
AU	Arbitrary units
CVD	Cardiovascular disease
HUVEC	Human umbilical vein endothelial cell
DNA	Deoxyribose nucleic acid
EGM-2	Endothelial growth medium
eNOS	Endothelial nitric oxide synthase
FBS	Fetal Bovine Serum
HRT	Hormone replacement therapy
L	Liter
MAPK	Mitogen-activated protein kinase
mPR	Membrane progesterone receptor
mRNA	Messenger ribonucleic acid
nM	Nanomolar
nmol	Nanomoles
NO	Nitric oxide
nPR	Nuclear progesterone receptor
Org OD 02-0	10-Vinyl-19-norprogesterone
PBS	Phosphate-buffered saline
p-eNOS	Phosphorylated endothelial nitric oxide synthase\
PR	Progesterone receptor
R5020	Promegestone
RFU	Relative fluorescence units
Src	Src kinase
TBS-T	Tris-buffered saline with 10% Tween® 20
VEGF	Vascular endothelial growth factor

Chapter 1: Introduction

According to the World Health Organization (2020), cardiovascular disease (CVD) is the leading cause of mortality across the world. However, it is widely known that premenopausal females experience significantly lower CVD incidence compared to males and postmenopausal females (Burt et al., 1995; Wiinberg et al., 1995), a disparity that has been attributed to endogenous sex hormones (i.e., progesterone and estrogen) that circulate in high concentrations only in healthy premenopausal women. Once women undergo menopause, CVD risk steeply increases in a way that cannot be explained by traditional risk factors such as age or concentrations of physiological markers such as cholesterol (Kannel et al., 1976). Hormone replacement therapy (HRT) is presently considered to bestow cardioprotective effects in perimenopausal women, pointing to the importance of sex hormones in the maintenance of health in women (Hodis et al., 2016; Lobo, 2017; Pereira et al., 2015). Estrogen specifically has been the most popular target hormone of study, dominating the literature on cardiovascular health in comparison to the effects of progesterone. However, progesterone is equally as important of a hormone, considering the fact that common contraindications for the use of estrogen, such as history (or family history) of CVD and endometrial cancer have necessitated the prescription of several types of progestin- or progesterone-only therapies (e.g., progesterone-only oral contraceptive pills and certain intrauterine devices) (Pereira et al., 2015; Valdes & Bajaj, 2021). Despite these developments, much is still not

known about how progesterone avoids the cardiovascular side effects that accompany estrogen therapies or how it may bestow cardioprotective effects.

Arteries are made up of several layers; the innermost endothelium, the outermost tunica adventitia, and the smooth muscle that resides between these layers (Fung & Liu, 1995). Changes in vascular smooth muscle tone are responsible for the maintenance of artery diameter, and factors released from the endothelium, such as nitric oxide, stimulate these changes in smooth muscle tone (Ignarro et al., 1987; Vane et al., 1990). Endothelial cells themselves maintain vascular health by repairing endothelial wounds, regulating fibrinolysis, and playing a major role in the early development of atherosclerotic lesions (Cushing et al., 1990; Itoh et al., 2010; Marui et al., 1993). Thus, endothelial cells such as human vein umbilical endothelial cells (HUVECs) or human aortic endothelial cells are often used *in vitro* to model the effects that certain chemicals or compounds may have on endothelial and vascular function.

The hypothesized effects of progesterone on the vasculature remain contentious. The actions of progesterone vary depending on the type of cell the hormone is applied to; for example, progesterone has been shown to counteract the vasoprotective effect of estrogen on murine vascular smooth muscle cells by abrogating the attenuation of reactive oxygen species production that is seen in the presence of estrogen alone (Wassmann et al., 2005), while progesterone is thought to enable and amplify the vasodilatory actions of estrogen on endothelial cells (Pang & Thomas, 2017). Contradicting findings also exist between studies of progesterone's effects within the same cell type. Progesterone has been reported to increase

endothelial nitric oxide synthase (eNOS) activity, expression, and migration in endothelial cells (Simoncini et al., 2004a; You et al., 2020; Zheng et al., 2012) but inhibit endothelial cell proliferation (Hsu et al., 2008). However, a perusal of the literature shows that more studies exist that support the theory that progesterone elicits vasodilatory and proangiogenic responses from HUVECs than studies that refute this theory. Therefore, the aim of this review is to summarize and clarify existing evidence on the effects and mechanistic actions of progesterone on endothelial cells.

Chapter 2: Literature Review

Current knowledge of progesterone's effects on the vasculature

Circulating progesterone concentrations fluctuate *in vivo* within healthy premenopausal women. There are two major phases within the menstrual cycle, luteal and follicular, that are characterized by the relative concentrations of progesterone in each (Wenner & Stachenfeld, 2020). The follicular phase, which includes menses and the thickening of the endometrial wall, is accompanied by low (~5.5 nmol/L, or nanomoles per liter) serum progesterone concentrations, while in the luteal phase an average concentration of about 32.8 nmol/L is seen in women who have an ovulatory cycle (Wathen et al., 1984). While many studies have not observed changes in vascular reactivity according to circulating hormone concentrations in healthy premenopausal women, authors acknowledge difficulties in controlling for factors such as aerobic fitness which are known to have significant effects on vascular reactivity (Shenouda et al., 2018; Williams et al., 2001). On a cellular level, progesterone is understood to be a vasodilatory hormone, acting through endothelial eNOS to produce endothelium-dependent vasodilation (Rupnow et al., 2001).

One vasoactive property of progesterone, the upregulation of eNOS expression in endothelial cells, has been observed in some, but not all, studies. Differences in findings have been attributed to the duration of exposure of endothelial cells to progesterone, among other factors. Those that apply progesterone to endothelial cells for a short length of time (30-60 minutes) acknowledge that whatever changes are seen in the cells are not genomic, as the cells are not given enough time to complete the process of transcription and translation. In contrast,

studies that apply progesterone to endothelial cells for a longer period (24-48 hours) have reported genomic changes. Shorter-term nongenomic effects of progesterone are thought to be mediated by membrane progesterone receptors, while longer-term genomic effects of progesterone are thought to be mediated by nuclear progesterone receptors. However, very few studies, if any, have assessed the combined contributions of each progesterone receptor subtype to the response of endothelial cells to progesterone.

Characteristics of nuclear progesterone receptors

In humans, nPRs are known as class I nuclear receptor subfamily members and are intracellular proteins activated when bound to ligands (Evans, 1988). Ligand-induced activation causes nPRs to act within the nucleus and bind to progesterone response elements (PREs) on promoter regions of target genes, a function that also classifies these receptors as transcription factors. Three known nPR isoforms are known to exist: PR-A; PR-B; and PR-C. PR-A and PR-B are the most well-researched isoforms and structurally differ by the presence of a B-upstream segment present on PR-B, but not PR-A (Zhang et al., 1994). In female PR-A knockout mice, the absence of PR-A upregulated the expression of histidine decarboxylase, supporting the idea that PR-A and PR-B are also functionally distinct (Mulac-Jericevic et al., 2000). PR-C mRNA has been shown to be abundantly expressed in human T47D breast cancer cells (Wei et al., 1990) and was reported to translate into a 60 kDa protein that may enhance the transcriptional activity of PR-A and PR-B (Wei et al., 1996). However, PR-C itself is unable to initiate transcription and thus is not

considered a transcription factor. To date, the actions of PR-C in endothelial cells have not been characterized.

PR-A is known to be expressed in human endothelial cells and in HUVECs specifically, moderately when unstimulated and more abundantly when stimulated by the presence of progesterone (Toth et al., 2008). However, the function of PR-A differs depending on what tissue expresses the receptors. For example, PR-A is known to repress the effects of progesterone and the "cardioprotective" actions of PR-B in human myometrium (Karteris et al., 2006; Merlino et al., 2007; Vegeto et al., 1993) and increase ROS production in murine vascular smooth muscle cells (Wassmann et al., 2005). In contrast, PR-A, but not PR-B, was shown to increase eNOS expression and NO production in female HUVECs and increase endothelium-dependent vasodilation of arterial rings isolated from female rats (You et al., 2020). In human T47D breast cancer cells, PR-B was found to dominate the mediation of progesterone-induced production of vascular endothelial growth factor (VEGF) (Wu et al., 2004). In breast cancer cells, PR-B was implicated in anti-inflammatory functions such as inhibiting proinflammatory gene expression and reducing the cells' sensitivity to inflammatory stimuli (Tan et al., 2012). Furthermore, PR-B expression increased in this environment, while PR-A expression dropped dramatically as stronger inflammatory stimuli were introduced. Other studies assessing the importance of the PR-A to PR-B ratio show that cells that have been in the presence of progesterone for a prolonged period show upregulation of PR-A (Merlino et al., 2007; Mesiano et al., 2002; Pieber et al., 2001). In the presence of high concentrations of progesterone, the actions of PR-B were suppressed, most likely by

the increased expression of PR-A. This information suggests that PR-A and PR-B may have contrasting functions, with PR-A counteracting the more negative effects of PR-B.

The function of nPRs is also closely tied to the localization of the receptors within a cell. nPRs were initially thought to reside almost exclusively in the nucleus in breast cancer, myometrial, and vascular smooth muscle cells (W.-S. Lee et al., 1997; Merlino et al., 2007; Reiner et al., 1990). However, these studies quantified PR after staining biopsied tissue from live subjects with undetermined concentrations of circulating progesterone. A study observing PR-A/B distribution in breast cancer cells *in vitro* noted that unliganded PR-A resides primarily in the nucleus, while unliganded PR-B is distributed across both the nucleus and the cytoplasm (Lim et al., 1999). Taken together, findings from both *ex vivo* and *in vitro* studies suggest that PR-B proteins most likely translocalize from the cytoplasm to the nucleus when activated by progesterone. It should be noted that nuclear progesterone receptors are also involved in extranuclear progesterone signaling; in breast cancer cells, PR-B has been shown to activate intermediaries such as src and MAPK. This is most likely due to the receptors' partial extranuclear localization, suggesting that progesterone receptors outside of the nucleus mediate cell signaling in response to progesterone (Boonyaratanakornkit et al., 2007).

In summary, nPRs have been shown to enact many effects of progesterone on a wide range of cell types. These effects are considered genomic due to the identity of the steroid receptor as a transcription factor. However, progesterone was observed to affect cells in seconds, a timeframe that does not allow for the relatively slow

processes of transcription and translation (Blackmore et al., 1991); additionally, receptors on the membrane of reproductive cells that bind progesterone in a manner distinct from classical (nuclear) PRs have been identified since the 1970s (Godeau et al., 1978). It was thus concluded and confirmed that a different class of nonclassical PR receptors, termed membrane progesterone receptors (mPRs), were also directly involved in enacting the effects of progesterone (Zhu, Bond, et al., 2003; Zhu, Rice, et al., 2003).

Characteristics of membrane progesterone receptors

mPRs are members of the progestin and adipoQ receptor family (Lyons et al., 2004; Tang et al., 2005). The existence of five mPRs are currently known: mPRα, mPRβ, mPRδ, mPRγ, and mPRε. All five mPR isoforms have been found in human tissues (Pang et al., 2013; Zhu, Bond, et al., 2003). While mPRδ and mPRε are known to be abundantly distributed in neuronal tissues (Pang et al., 2013), mPRα, mPRβ, and mPRγ have been most heavily implicated in numerous functions in both reproductive and other non-reproductive tissues (Fernandes et al., n.d.; Zhu, Bond, et al., 2003).

The ratio of expression between the three most abundant mPR isoforms is known to be modified by changing hormone concentrations. For example, mRNA expression of mPRα in endometrial tissue has been shown to peak in the luteal phase of the menstrual cycle when circulating sex hormone concentrations are relatively high, while mPRγ mRNA expression was found to peak in the follicular phase of the menstrual cycle when circulating sex hormone concentrations are relatively low

(Fernandes et al., n.d.). In a more targeted study, mPRα mRNA in myometrial cells was shown to increase after 4 hours of exposure to both progesterone and estrogen, while mPRβ mRNA only increased after 8 hours of incubation with estrogen alone (Karteris et al., 2006).

mPRs mediate several signaling pathways in the tissues they reside. mPRs, especially mPRα and mPRβ, have been shown to activate G proteins in a manner similar to G protein-coupled receptors (GPCRs) (Thomas et al., 2007). However, mPRs lack the structure and signaling pattern of typical GPCRs (Kasubuchi et al., 2017). For human mPRs, G proteins are only one intermediate between initiation of signaling by progesterone and the hormone's resultant effects (Smith et al., 2008). In the oocytes of zebrafish where mPRα proteins dominate the PR ratio, Hanna & Zhu (2011) established a generalized signaling pathway for cells containing both mPRα and nPRs. When mPRα is activated by a ligand, it activates an inhibitory G protein which then allows eNOS activity to increase via the MAPK pathway (Cutini et al., 2014; Pang et al., 2015; Pang & Thomas, 2017). mPRβ has also been implicated in the modification of eNOS activity (Karteris et al., 2006); however, mPRα appears to have a more significant functional role in mediating the effects of progesterone (Pang & Thomas, 2018). Interestingly, the actions of mPRs do not appear to stand alone; mPR-activated G proteins have been shown to act on PR-A in breast cancer cells and transactivate PR-B in human myometrium (Fu et al., 2008; Karteris et al., 2006). These results imply that mPRs may mediate the actions of nPRs and contribute to the wide range of cell-specific effects of progesterone. These functions have not been established in endothelial cells; however, mPRs, especially mPRα, are known to

mediate the rapid, nongenomic effects of progesterone in these cells (Pang et al., 2015).

Nuclear progesterone receptor-mediated effects of progesterone in endothelial cells

The actions of nuclear PRs have been defined in HUVECs, albeit with few findings accounting for the sex of HUVECs studied. Nuclear translocation of PR-A, but not PR-B, within pooled HUVECs increases in the presence of at least 500nM (nanomolar) progesterone (Hsu et al., 2015). The importance of the transcriptional actions of PR-A were further shown by studies employing immunoprecipitation assays that assayed the increased relative amounts of PR-A that were forming complexes with other transcription factors such as NF-kB and SP-1 when in the presence of 500nM progesterone (Hsu et al., 2015; You et al., 2020). Thus, in HUVECs, as opposed to most other cell types, PR-A, not PR-B, seems to be the nuclear PR with the more significant role in enacting the pro-angiogenic and vasodilatory effects of progesterone.

Furthermore, modulation of nuclear PR expression in HUVECs is likely dependent on the sex of the studied HUVECs. Expression of PR-A in pooled HUVECs is increased by the presence of at least 100nM progesterone for a prolonged exposure time of 24 and 72 hours (Goddard et al., 2014; Toth et al., 2009); in contrast, neither PR-A nor PR-B expression increased in female HUVECs stimulated with 500nM progesterone for 24 hours (You et al., 2020). While it is known that HUVECs have distinct sex-based differences, differences in receptor response to sex steroids between male and female HUVECs have not been characterized and estrogen and androgen receptor expression has been shown on multiple occasions not to

significantly differ between the sexes (Addis et al., 2014; Annibalini et al., 2014). HUVECs express both PR-A and PR-B mRNA (Oishi et al., 2004; Tatsumi et al., 2002; Toth et al., 2008). However, one study in pooled HUVECs failed to show evidence of PR-B expression via immunocytochemical staining (Toth et al., 2008), while more recent studies show the presence of the B isoform via Western blot (Hsu & Lee, 2011; You et al., 2020). Thus, I will be assuming the presence of PR-B protein in HUVECs when summarizing all subsequent studies.

Many pro-angiogenic, vasoactive, and anti-atherosclerotic long-term effects of progesterone on HUVECs have been reported. Firstly, progesterone increases eNOS expression and NO release in HUVECs (Simoncini et al., 2004a). Furthermore, progesterone has been shown to prevent the expression of adhesion molecules on HUVECs stimulated by specific inflammatory cytokines (Aziz & Wakefield, 1996; Otsuki et al., 2001). Progesterone has also displayed anti-inflammatory effects on HUVECs. When serum from pre-eclamptic women combined with progesterone was used to treat HUVECs for 48 hours, concentrations of released endothelin-1 (ET-1) were significantly lower than concentrations seen in HUVECs treated only with serum from pre-eclamptic women (Kiprono et al., 2013). Additionally, 100nM of progesterone decreased the amount of inflammatory cytokines released by HUVECs both when stimulated with lipopolysaccharide (LPS) and when not in the presence of an inflammatory stimulus (Goddard et al., 2013).

Findings from functional assays involving HUVECs are contentious. While HUVEC migration was enhanced by the presence of 5-100nM progesterone for 48 hours (Fu et al., 2008; Zheng et al., 2012), multiple studies have demonstrated

putatively anti-angiogenic and inflammatory effects of progesterone on HUVECs. Studies applying a range of 5-8000nM progesterone to a 3-hour capillary tube-like formation assay, 6-hour migration assay, 48-hour proliferation assay, and 48-hour apoptosis assay showed increased apoptosis and decreased angiogenic, migratory, and proliferative capacity of HUVECs (Hsu et al., 2008; T.-S. Lee et al., 2015; Powazniak et al., 2009). It should be noted, however, that many of these studies isolated their own HUVECs from donor umbilical cords without providing HUVEC characteristics such as sex.

Membrane progesterone receptor-mediated effects of progesterone in endothelial cells

Progesterone also enacts rapid effects that cannot be attributed to genomic changes within HUVECs. The most robust example of this is the increase in NO production in response to progesterone; when progesterone is administered to cells for a prolonged period of time (around 6 or more hours), eNOS expression increases likely because cells have enough time to transcribe and translate DNA to eNOS mRNA, then finally eNOS protein (You et al., 2020). However, when progesterone is administered to cells for shorter periods of time, such as 30-60 minutes, increases in eNOS expression are not seen. Instead, eNOS phosphorylation increases, showing that progesterone increases the activity rather than the expression of eNOS (Oishi et al., 2004; Pang & Thomas, 2017). Furthermore, NO increases are seen when progesterone is administered to HUVECs for both short and long periods of time regardless of eNOS protein concentrations (Pang et al., 2015; Simoncini et al., 2004a).

As previously mentioned, mPRα has been identified as the most important mediator of the rapid, nongenomic effects of progesterone. This finding has been confirmed by multiple studies employing small-interfering RNA (siRNA) coded to cleave mPRα mRNA. When the expression of mPRα is prevented, the increase in NO production stimulated by the presence of progesterone is not seen (Pang et al., 2015). Furthermore, this result is not seen when the expression of nPR is prevented with siRNAs (Pang et al., 2015). While knockdown of mPRα alone in HUVECs has been shown to almost entirely abolish short-term upregulation of eNOS activity seen in the presence of progesterone, contributions of mPRγ and mPRβ have not been ruled out, especially considering the fact that mPRβ is frequently coexpressed alongside mPRα in immune cells (Dosiou et al., 2007; Pang et al., 2015). Overall, mPRα signaling has consistently been shown to lead to increases in NO production, HUVEC migration, and eNOS phosphorylation, as described above (Pang et al., 2015; Pang & Thomas, 2017; Zheng et al., 2012).

Conclusions

Each isoform of progesterone receptor residing in a cell has a distinct function compared to the other progesterone receptor isoforms. However, these functions are highly cell-specific. Nuclear PRs are putatively responsible for "long-term" or genomic effects involving transcription and translation of DNA and mRNA, while membrane PRs are thought to be responsible for "short-term" or nongenomic effects involving post-translational modification of proteins and intracellular signaling. Nuclear isoforms PR-A and PR-B are functionally and structurally distinct nuclear

transcription factors; PR-C, while also considered a nuclear progesterone receptor, is a smaller protein that modulates but does not initiate the transcription activity of target genes. In many cell types, PR-A and PR-B have opposing functions, with PR-A repressing the activity of PR-B when progesterone is in abundance. However, this does not seem to be the case in HUVECs, where PR-A is most active in forming complexes with other transcription factors to initiate transcription in the production of eNOS proteins. In contrast, when membrane PRs are activated by a ligand, signaling occurs through several intermediaries to increase the phosphorylation of eNOS and the production of NO.

Questions remain regarding the interactions between membrane and nuclear PRs in many cell types, including HUVECs. While there is abundant information about the characteristics of nPRs, mPRs have been studied far less and by a limited number of research groups. Furthermore, contradicting studies in all cell types should be addressed by standardizing the techniques used to study the effects of progesterone. For example, the sex of assessed HUVECs should be noted, and progesterone should be used in physiological concentrations to increase the applicability of studies. This thesis is designed to address these demands by addressing the aims listed in the following chapter.

Chapter 3: Rationale and Aims

Progesterone, a prominent sex steroid, is often associated with increased vasodilatory capacity and vasoactive effects that may not be accompanied by the harmful side effects seen in treatments involving 17β-estradiol. Furthermore, individuals who cannot be prescribed 17β-estradiol due to existing contraindications may rely on progesterone-only treatments. The cardioprotective effects of sex steroids that can be seen on an epidemiological level when comparing CVD risk between males and females imply significant cardiovascular benefits of progesterone; however, there is no clear consensus on how progesterone affects endothelial cells. Endothelial cells themselves are an early site of dysfunction in the development of CVD, making them an important mediator of cardiovascular health. Progesterone is not unanimously thought to improve the function of endothelial cells, as findings both supporting and refuting this theory have been published. The mechanisms that underlie the actions of progesterone on endothelial cells have also not been fully elucidated. The majority of studies involving the effects of progesterone on endothelial cells focus on the mechanistic actions of either membrane or nuclear PRs, leaving potential additive or unique effects of the receptor subtypes unobserved. The few studies demonstrating this cooperation between membrane and nuclear PRs have not reported the outcomes of nuclear PR transactivation by intermediaries of membrane PR activation. This may be due in part to the fact that a limited number of studies have quantified the combined effects of progesterone on both nuclear and membrane PRs over shorter (30-60 minute) and longer (6-72 hour) incubation periods. In addition, endothelial cells have only recently been discovered to have sex-

specific characteristics (Addis et al., 2014), further complicating the interpretation of the existing body of literature surrounding the effects of progesterone. Lastly, studies often assess the effects of serum progesterone concentrations seen during pregnancy and in HRT, but very few, to our knowledge, have explicitly observed the effects of serum progesterone concentrations matching those seen in the follicular and luteal phases in healthy premenopausal women.

This study tested the hypothesis that physiologically relevant progesterone concentrations seen in the luteal phase of the menstrual cycle will increase endothelial cell function compared with progesterone concentrations seen in the follicular phase, and that these increases in function will be due to the actions of membrane PRs in the short-term and the combined effects of membrane and nuclear PRs in the longer-term. This study tested this hypothesis in female human umbilical vein endothelial cells (HUVECs) by addressing the following aims:

Aim 1: Determine the effects of different concentrations of progesterone and contributions of nuclear vs. membrane progesterone receptors on endothelial cell function.

A. Determine the effects of different physiological concentrations of progesterone on endothelial cell migration and proliferation.
B. Distinguish whether the effects of progesterone on endothelial cells are mediated by membrane and/or nuclear receptors using agonists for each progesterone receptor subtype.

Aim 2: Determine changes in eNOS expression and activation in endothelial cells in response to different concentrations of progesterone and assess the contributions of nuclear and membrane progesterone receptors to these changes.

A. Determine the effects of increasing concentrations of progesterone on eNOS protein expression and phospho-eNOS after 30 minutes (short-term exposure) and 24 hours (longer-term exposure).
B. Distinguish whether the effects of progesterone are mediated by membrane and/or nuclear receptors using agonists for each progesterone receptor subtype.

Chapter 4: Methods

Reagents and chemicals

Bioidentical progesterone (MilliporeSigma, Burlington, MA), 10-Vinyl-19-norprogesterone (Org OD 02-0) (Axon Medchem LLC, Reston, VA), and promegestone (R5020) (PerkinElmer, Waltham, MA) were used as progesterone receptor ligands in the present study. Primary antibodies used for western blotting were Recombinant Anti-eNOS antibody [EPR23750-3] (Abcam, Cambridge, United Kingdom), phospho-eNOS (Ser1177) monoclonal antibody (H.83.2) (Invitrogen, Waltham, MA), and B-actin (D6A8) Rabbit monoclonal antibody (Cell Signaling Technology, Danvers, MA). The secondary antibody used for detection of all targets was anti-rabbit IgG, HRP-linked antibody (Cell Signaling Technology, Danvers, MA).

Study design

HUVECs were obtained as primary cultures, then were cultured between passages 3-5. When at a usable passage, HUVECs were either used in migration or proliferation assays or underwent protein purification for multi-target Western blotting. HUVECs for migration and proliferation assays were incubated in the conditions listed in Table 1 and positive and negative controls for 24 hours, while HUVECs harvested for Western blotting were incubated in these same conditions for either 30 minutes or 24 hours. Four progesterone concentrations were applied to HUVECs: 0, 1, 20, and 500nM. PR agonists were applied on their own in 20nM concentrations to isolate the actions of each receptor subtype; agonists were also incubated in combination with each other or with progesterone to detect effects of progesterone not attributable to the actions of either agonist. Experiment flow is

demonstrated in figure 1; components of this diagram are explained in following sections.

Table 1

Steroid conditions to be applied to Human Umbilical Vein Endothelial Cells

Condition	Progesterone	R5020	Org OD 02-0
Condition 1	0nM		
Condition 2	1nM		
Condition 3	20nM		
Condition 4	500nM		
Condition 5		+	
Condition 6			+
Condition 7		+	+
Condition 8	+	+	
Condition 9	+		+
Condition 10	+	+	+

Note. Concentrations in nanomolar (nM) or plus signs (+) indicate the presence of steroid in each condition. + also indicates a concentration of 20nM of the indicated steroid in each respective condition. Twelve total conditions, including positive and negative controls (not shown), were applied to HUVECs for two separate incubation periods of 30 minutes and 24 hours. Promegestone (R5020) and 10-Vinyl-19-norprogesterone (Org OD 02-0) are agonists for nuclear and membrane progesterone receptors, respectively.

Figure 1.

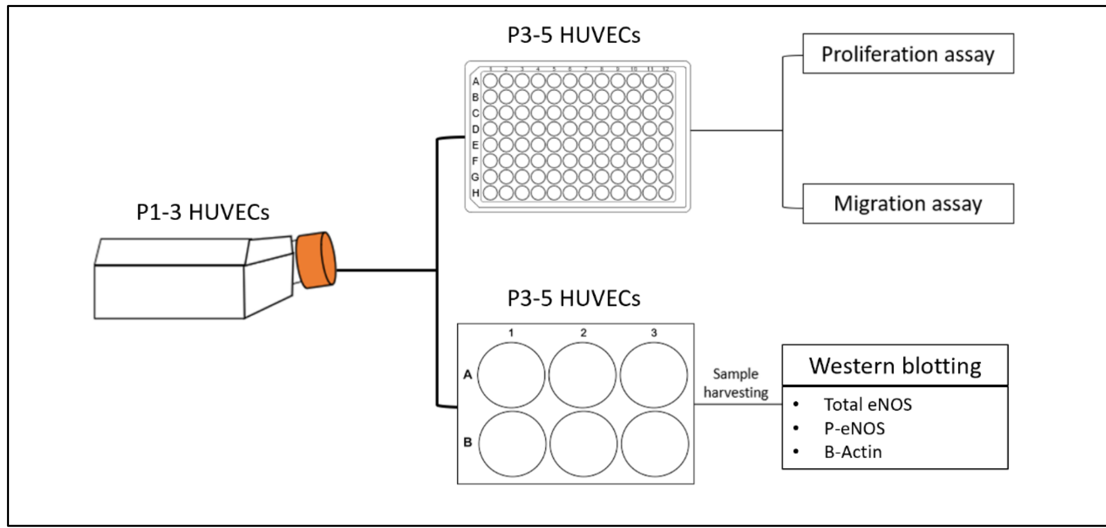

Figure 1. Study design. HUVECs were initially grown out in T75 flasks until passages 3-5. For functional assays, cells were passed to 96-well plates for proliferation and migration assays, while for Western blotting samples cells were passed to 6-well plates for Western blot sample harvest. Proteins targeted in the Western blot were eNOS and phosphorylated eNOS (phospho-eNOS); B-actin was used as the loading control.

Cell culture

All culture media contained 1X antibiotic-antimycotic (anti-anti) unless otherwise stated. Female HUVECs were obtained through Lonza (Bazel, Switzerland) at passage 1. Cells were then grown out in endothelial growth medium (EGM-2) (Lonza, Basel, Switzerland) and used in assays between passages 3-5. HUVECs were then passed or seeded into plates for each assay or harvesting for Western blotting. After HUVECs grew out to 80% confluence, conditions diluted in EGM-2 with 0.5% fetal bovine serum (FBS) containing 0, 1, 20, or 500nM progesterone and/or 20nM of other listed PR ligands were applied.

Protein purification and immunoblotting

HUVECs harvested for Western blotting samples were first seeded in 6-well plates at a density of 150,000 cells per well in EGM-2. After cells grew out to 80%

confluence, the cells were washed twice with 1X phosphate buffered saline (PBS) (Quality Biological, Gaithersburg, MD) and media in each well was replaced with each condition as described in the cell culture section. After either 30 minutes or 24 hours of incubation at 37°C at 5% CO_2, HUVECs were put on ice, media was removed, and the cells were washed twice with 1X PBS. 200uL of Halt™ Protease Inhibitor Cocktail (Thermo Fisher Scientific, Waltham, MA) containing 1X radioimmunoprecipitation assay buffer, 100X protease inhibitor, 100X phosphatase inhibitor, and 100X ethylenediaminetetraacetic acid were applied to the HUVECs for cell lysis. The monolayer of HUVECs in each well were detached with a rubber scraper, and the resulting solution containing cells and the previously described radioimmunoprecipitation assay buffer were transferred to Eppendorf tubes. These tubes were rotated at 4°C for 20 minutes, then homogenized at 4°C in a microcentrifuge set to 10,000xg for 20 minutes. The supernatant was then aliquoted and frozen at -80°C for immunoblotting. A Pierce™ bicinchoninic acid assay (Thermo Fisher Scientific, Waltham, MA) was run to quantify the protein yield from each well of HUVECs and standardize protein amounts in each well of the Western blot.

Protein harvested from HUVECs was diluted in a 1:4 ratio with deionized water and 4X Laemmli sample buffer (Bio-Rad, Hercules, CA) then boiled at 70°C for 5 minutes. Protein was then loaded onto mini-PROTEAN® pre-cast TGX™ gels formulated with 4-15% polyacrylamide (Bio-Rad, Hercules. CA), separated by polyacrylamide denaturing gel electrophoresis, then run for 1 hour at 110 volts. Proteins were then transferred to 0.2μm mini PVDF membranes contained in Trans-

Blot Turbo Transfer Packs (Bio-Rad, Hercules, CA), washed three times with 1X tris-buffered saline with 10% Tween® 20 (TBS-T) (Bio-Rad, Hercules, CA) at room temperature, and blocked with blocking buffer containing 5% bovine serum albumin (Sigma-Aldrich, St. Louis, MO) or non-fat dry milk (Nestle, Vevey, Switzerland) for 1.5 hours at room temperature. After blocking, primary antibodies for eNOS and phospho-eNOS were applied to membranes overnight at 4°C. Membranes were washed in 1X TBS-T for three 5-minute washes at room temperature. Membranes were incubated with HRP-conjugated secondary antibody diluted in blocking buffer for 1 hour at room temperature, then washed three more times with 1X TBS-T for 5 minutes at room temperature. After washes, membranes were briefly incubated in Clarity ECL Western blotting substrate. Images were acquired with the Bio-Rad ChemiDoc XRS+ System and analyzed in ImageJ for signal strength/protein density. B-actin was used as a loading control to normalize target protein signal to total protein in each well.

Functional endothelial cell assays

For both assays described below, positive and negative control conditions were also assayed in triplicate. The positive control condition was EGM-2 + 10% FBS, while the negative control condition was endothelial basal medium, which does not contain any growth factors and thus should not promote wound closure or cell replication.

Migration assay

HUVECs were seeded in 96-well plates at a density of 20,000 cells per well then incubated in EGM-2 at 37°C and 5% CO_2. When cells reached 80% confluence,

a scratch wound was created vertically down the middle of the HUVEC monolayer using a p1000 pipette tip. Progesterone and/or PR agonists conditions in EGM-2 with 0.5% FBS were applied to each well, with each condition plated in triplicate. Pictures of each well were taken at 0 hours, then every 4 hours until 24 hours passed. The 96-well plates were incubated at 37°C at 5% CO_2 between each picture-taking period. Percent closure of wounds in each well were quantified using ImageJ and plotted at each time point. The resultant area under the curve for each condition will then be averaged for analysis; percent closure of each wound after 24 hours was also averaged for each condition and used for analysis.

Proliferation assay

HUVECs were seeded in 96-well plates at a density of 5,000 cells per well, then incubated in EGM-2 at 37°C and 5% CO_2 for 8-10 hours to ensure adhesion of HUVECs to the plate. EGM-2 was then removed, and cells were washed twice in 1X PBS before conditions were added to wells in triplicate and HUVECs were incubated for another 24 hours. Afterwards, nuclear dye and lysis buffer provided in the Fluorometric Cell Proliferation Assay kit (BioVision, Milpitas, CA) were applied to each well to induce fluorescence and detect the number of cells contained in each well. Plates were then run in a microplate reader at an excitation wavelength of 480nm and an emission wavelength of 538nm; cell density in each well is represented in relative fluorescence units.

Statistical analysis

Effects of different concentrations of progesterone on HUVECs in each assay were analyzed using one-, two-, and three-way analysis of variances (ANOVA) to

assess main effects and interactions. Pairwise comparisons were then used to identify differences between each condition. A sample size was determined by a priori power analysis based on data published by Pang et al. (2015) and pilot data generated from migration and proliferation assays applying different concentrations of progesterone to pooled HUVECs.

Chapter 5: Results

Endothelial cell function

Proliferation

When testing the effects of different progesterone concentrations (0 – 500 nM) on HUVEC proliferation, there was no significant main effect of progesterone (Figure 2A, P = 0.746). However, when testing the effect of 20nM progesterone independently or with nuclear (R5020) or membrane (Org OD 02-0) receptor agonists, different trends were observed. Comparing the effects of 0nM or 20nM progesterone with or without 20nM Org OD 02-0 on HUVEC proliferation revealed a main effect of Org OD 02-0 (P = 0.034), but not progesterone (P = 0.094), to reduce HUVEC proliferation (Figure 2B). When comparing HUVEC proliferation under conditions of 0nM or 20nM progesterone with or without 20nM R5020, a main effect of progesterone was seen to decrease proliferation (Figure 2C, P = 0.034). Comparing the effects of 20nM progesterone, 20 nM Org OD 02-0, and 20 nM R5020 together on HUVEC proliferation in a three-way repeated measures ANOVA (Figure 2D) showed no 3-way interaction (P = 0.315) but did show 2-way interactions between progesterone and Org OD 02-0, and between R5020 and Org OD 02-0 (P = 0.036 and P = 0.044, respectively). Additionally, a main effect of Org OD 02-0 itself to decrease HUVEC proliferation was again found (P = 0.043). Post-hoc analysis of the main effect of Org OD 02-0 showed that 20nM Org OD 02-0 significantly reduced proliferation compared with the 0nM progesterone condition (P = 0.017). However,

post-hoc analysis of either two-way interaction did not result in any significant pairwise comparisons.

Figure 2.

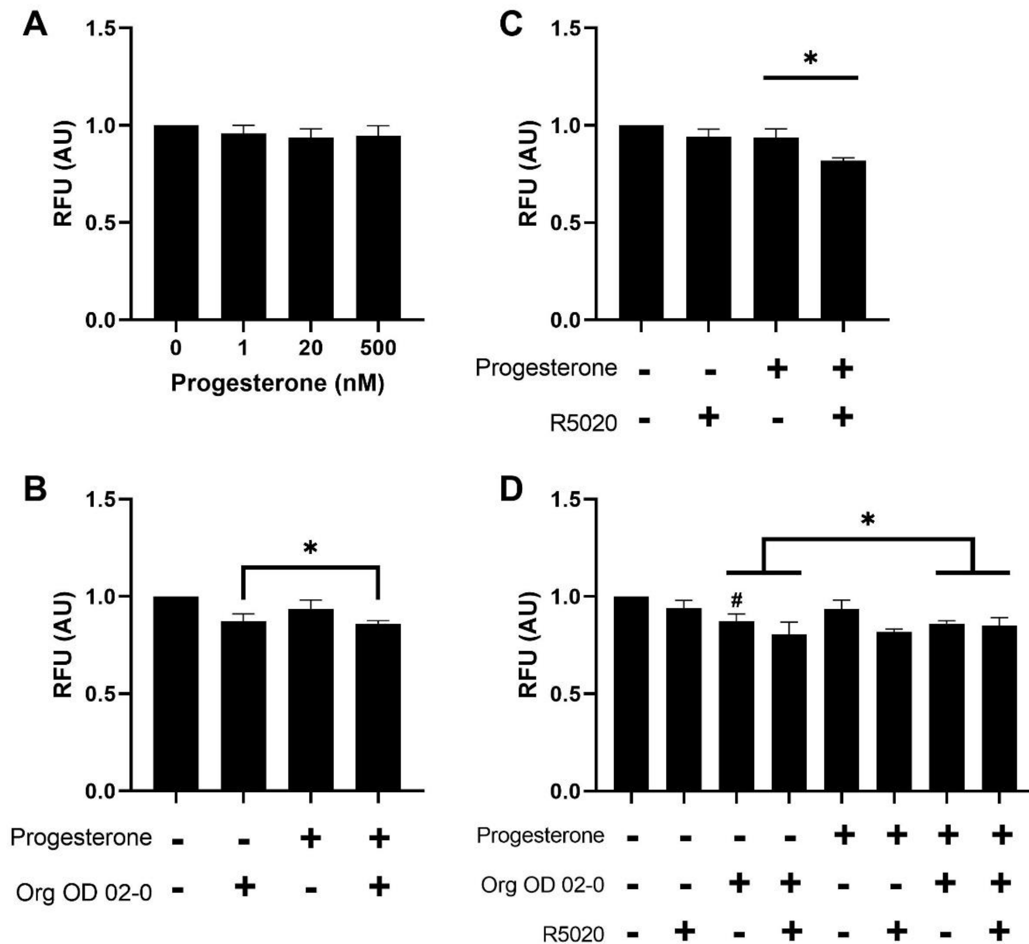

Figure 2. HUVEC proliferation [relative fluorescence units (RFU)] in the presence of progesterone and/or progesterone receptor agonists. (A) A RM ANOVA comparing concentrations of 0, 1, 20, and 500nM progesterone showed no main effect of progesterone. (B) A two-way RM ANOVA comparing progesterone and Org OD 02-0 showed a main effect of Org OD 02-0 ($P = 0.032$). (C) A two-way RM ANOVA comparing progesterone and R5020 showed a main effect of progesterone ($P = 0.034$). (D) A three-way ANOVA comparing progesterone, Org OD 02-0, and R5020 showed a main effect of Org OD 02-0 ($P = 0.043$) and interactions between progesterone*Org OD 02-0 ($P = 0.036$) and R5020*Org OD 02-0 ($P = 0.044$). Data are means ± SEM and are expressed relative to the 0nM progesterone condition. * $p<0.05$ main effect; # $p<0.05$ vs. 0nM progesterone. RFU: relative fluorescence units. AU: arbitrary units.

Migration

Percentage of scratch wound closure at 24 hours

When testing the effects of different progesterone concentrations (0 – 500 nM) on HUVEC migration, there was a main effect of progesterone to inhibit scratch wound closure after 24 hours of incubation (Figure 3A, P = 0.023). Post-hoc analysis further revealed that 500nM progesterone significantly inhibited scratch wound closure compared with both the 0nM and 1nM progesterone conditions (P = 0.013 and P = 0.023, respectively), but 1nM and 20nM progesterone elicited no significant differences when compared with lower concentrations of progesterone. Furthermore, analysis of the effects of 20nM progesterone alone or in combination with equal concentrations of either progesterone receptor agonist resulted in similar results for 20nM progesterone, but additional effects of the receptor agonists. Analysis of progesterone with or without Org OD 02-0 (Figure 3B) showed no interaction (P = 0.401) or main effect of progesterone (P = 0.119); however, a main effect of Org OD 02-0 to reduce scratch wound closure was seen (P = 0.006). When scratch wound closure in 0nM or 20nM progesterone with or without 20nM R5020 was assessed (Figure 3C), no significant interaction or main effects were found, but there was a tendency for a main effect of R5020 itself to inhibit scratch wound closure (P = 0.055). Analysis of scratch wound closure in HUVECs treated with combinations of 20nM progesterone, R5020, and/or Org OD 02-0 revealed an interaction between the two agonists (Figure 3D, P = 0.017) and a main effect of Org OD 02-0 (P = 0.021) to reduce closure. However, post-hoc analysis of this interaction or the main effect of

Org OD 02-0 did not reveal any differences in 24-hour scratch wound closure among individual conditions.

Figure 3.

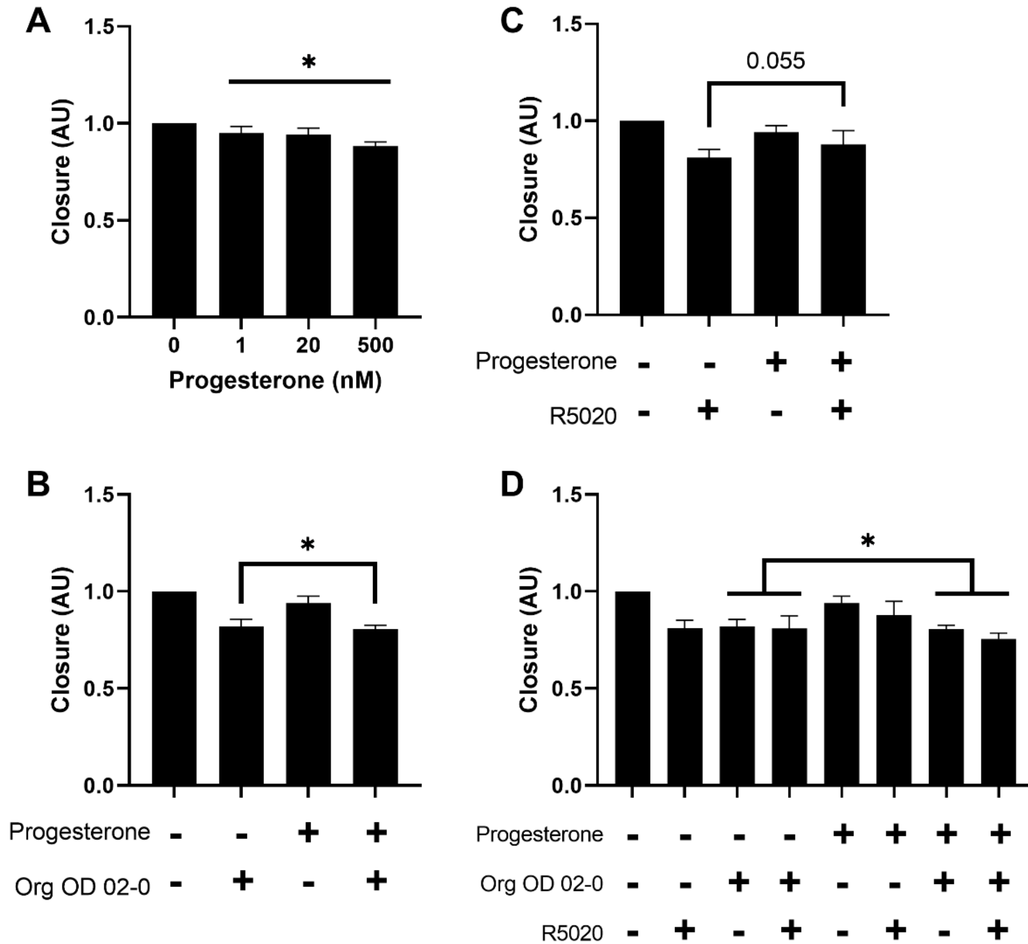

Figure 3. HUVEC scratch wound closure in the presence of progesterone and/or progesterone receptor agonists. (A) A RM ANOVA comparing concentrations of 0, 1, 20, and 500nM progesterone showed a main effect of progesterone (P = 0.023). (B) A two-way RM ANOVA comparing progesterone and Org OD 02-0 showed a main effect of Org OD 02-0 (P = 0.006). (C) A two-way RM ANOVA comparing progesterone and R5020 showed a tendency towards a main effect of progesterone (P = 0.055). (D) A three-way ANOVA comparing progesterone, Org OD 02-0, and R5020 showed a main effect of Org OD 02-0 (P = 0.021) and an interaction between R5020*Org OD 02-0 (P = 0.017). Data are mean percent closure ± SEM and are expressed relative to the 0nM progesterone condition. * p<0.05 main effect. AU: arbitrary units.

Total AUC of scratch wound closure over 24 hours

When scratch wound migration was expressed as total AUC, there was only a tendency for a main effect of increasing doses (0 – 500 nM) of progesterone (Figure 4A, P = 0.055). However, pairwise comparisons did show that 20nM progesterone elicited significantly lower total AUC compared to the 0nM condition (P = 0.004). In addition, 500nM progesterone significantly decreased total AUC compared to the 1nM condition (P = 0.043). Comparing conditions containing 20nM of progesterone, R5020, and Org OD 02-0 either separately or together in different combinations revealed patterns similar to those revealed from scratch wound closure data. Assessment of 0nM or 20nM progesterone with or without 20nM Org OD 02-0 (Figure 4B) showed a main effect of both progesterone (P = 0.026) and Org OD 02-0 (P = 0.014) to reduce total AUC, but did not reveal an interaction (P = 0.216). Post-hoc analysis showed that both progesterone and Org OD 02-0 independently decreased total AUC compared to the 0nM progesterone condition (P = 0.026 and P = 0.046, respectively). Furthermore, the combination of Org OD 02-0 and progesterone together significantly decreased total AUC compared to the 20nM progesterone condition (P = 0.003). Analysis of the independent or combined effects of 20nM progesterone and 20nM R5020 on total AUC (Figure 4C) did not reveal an interaction (P = 0.399) or a main effect of R5020 (P = 0.123) or progesterone (P = 0.064). A three-way repeated measures ANOVA comparing the effects of progesterone, R5020, and Org OD 02-0 on total AUC did not show any two- or three-way interaction effects but did reveal a main effect of Org OD 02-0 to reduce total AUC (Figure 4D, P = 0.023).

Figure 4.

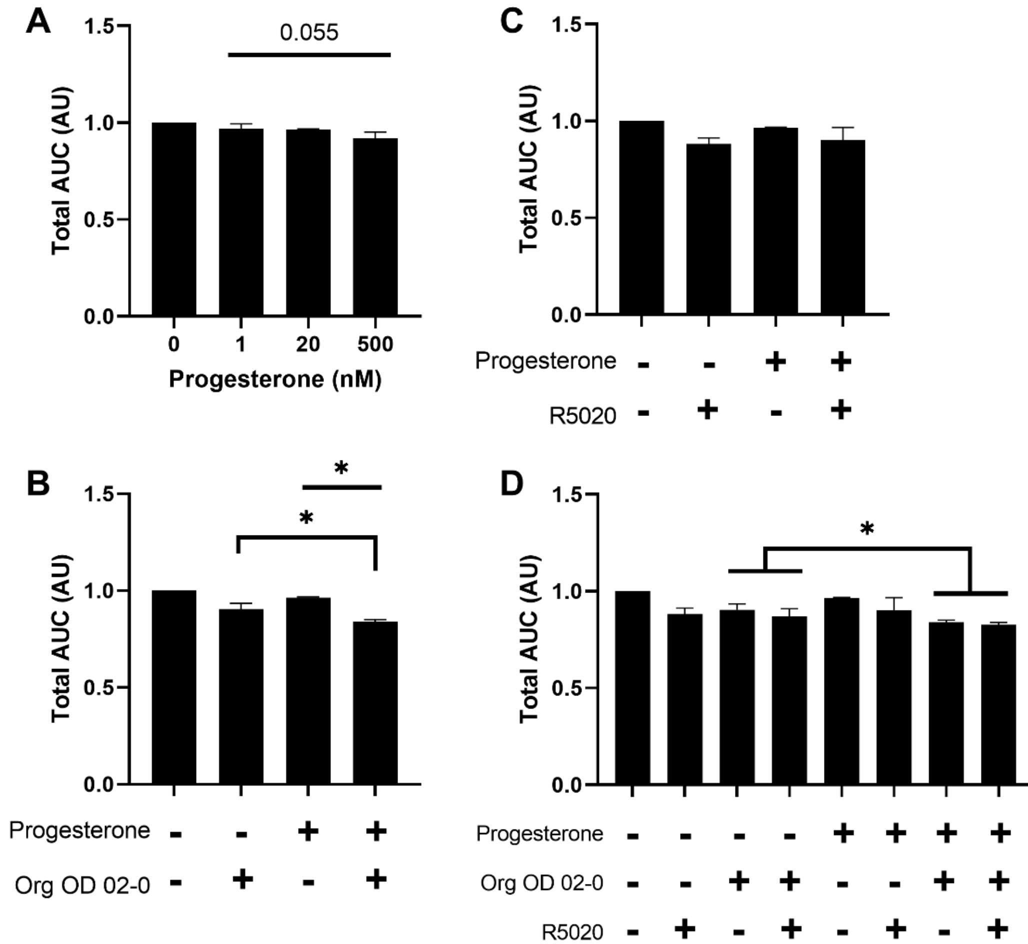

Figure 4. Total area under the curve (AUC) calculated over 24-hour period of scratch wound closure in HUVECs in the presence of progesterone and/or progesterone receptor agonists. (A) A RM ANOVA comparing concentrations of 0, 1, 20, and 500nM progesterone showed a tendency towards a main effect of progesterone (P = 0.055). (B) A two-way RM ANOVA comparing progesterone and Org OD 02-0 showed a main effect of both progesterone (P = 0.026) Org OD 02-0 (P = 0.046). (C) A two-way RM ANOVA comparing progesterone and R5020 showed no main effects or interaction. (D) A three-way ANOVA comparing progesterone, Org OD 02-0, and R5020 showed a main effect of Org OD 02-0 (P =0.023). Data are mean total AUC ± SEM and are expressed relative to the 0nM progesterone condition. * p<0.05 main effect. AU: arbitrary units.

eNOS expression and activity

Incubation of HUVECs in any condition included in this study for only 30 minutes did not result in any significant main effects or interactions of progesterone

or progesterone receptor agonists on total eNOS expression (Figure 5) or phosphorylation of eNOS (Figure 6).

Figure 5.

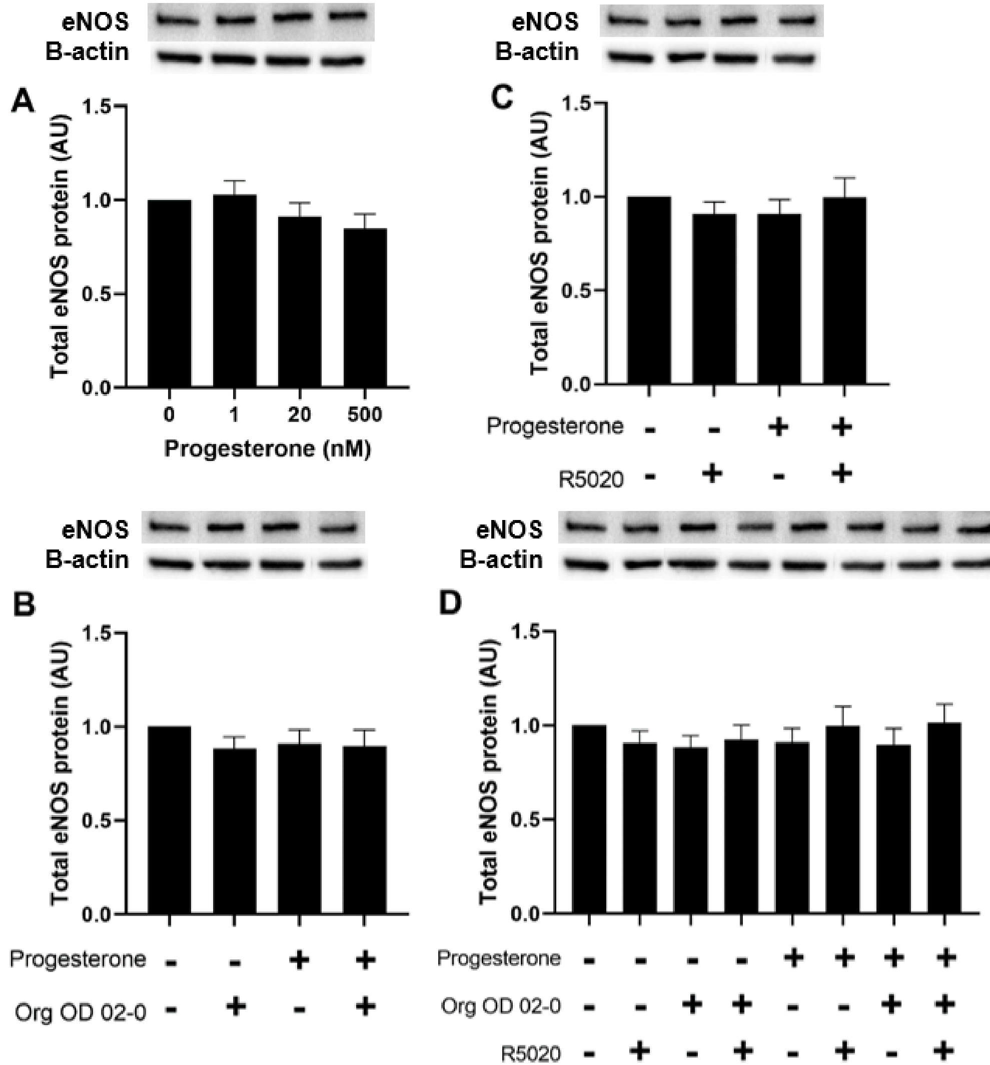

Figure 5. HUVEC total eNOS protein after 30 minutes of incubation with progesterone and/or progesterone receptor agonists. (A) A RM ANOVA comparing concentrations of 0, 1, 20, and 500nM progesterone showed no main effect of progesterone. (B) A two-way RM ANOVA comparing progesterone and Org OD 02-0 showed no interaction or main effects. (C) A two-way RM ANOVA comparing progesterone and R5020 showed no interaction or main effects. (D) A three-way ANOVA comparing progesterone, Org OD 02-0, and R5020 showed no interactions or main effects. Data are means ± SEM and are expressed relative to the 0nM progesterone condition. AU: arbitrary units.

Figure 6.

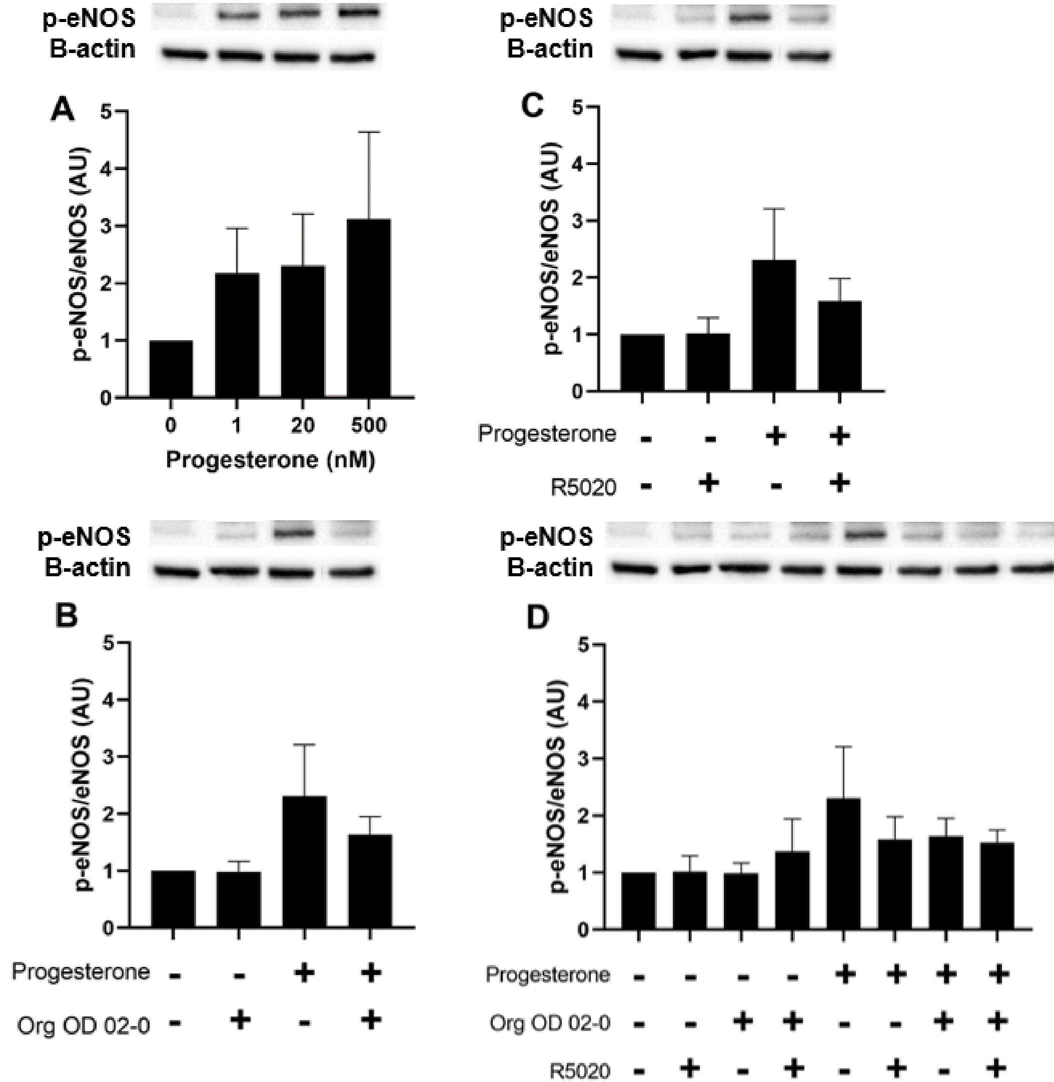

Figure 6. Phosphorylated eNOS to total eNOS protein ratio after 30 minutes of incubation with progesterone and/or progesterone receptor agonists. (A) A RM ANOVA comparing concentrations of 0, 1, 20, and 500nM progesterone showed no main effect of progesterone. (B) A two-way RM ANOVA comparing progesterone and Org OD 02-0 showed no interaction or main effects. (C) A two-way RM ANOVA comparing progesterone and R5020 showed no interaction or main effects. (D) A three-way ANOVA comparing progesterone, Org OD 02-0, and R5020 showed no interactions or main effects. Data are means ± SEM and are expressed relative to the 0nM progesterone condition. AU: arbitrary units.

After 24 hours, no differences in total eNOS protein were seen (Figure 7); however, differences in phosphorylated eNOS to total eNOS protein ratio were detected (Figure 8). Incubating HUVECs with increasing concentrations of

progesterone (0-500nM) for a period of 24 hours revealed a main effect of progesterone to increase eNOS phosphorylation (Figure 8A, ANOVA main effect $P = 0.016$). Post-hoc analysis showed that incubation of HUVECs with 500nM progesterone for 24 hours significantly increased the ratio of phosphorylated eNOS to total eNOS when compared with the 0nM condition ($P = 0.027$). Similar trends were observed when the effect of 20nM progesterone was compared with the effects of 20nM of either progesterone receptor agonist. To begin, comparison of eNOS phosphorylation values after 24 hours incubation with 0nM or 20nM progesterone with or without 20nM Org OD 02-0 (Figure 8B) revealed a main effect of progesterone to increase eNOS phosphorylation ($P = 0.009$), but no main effect of Org OD 02-0 ($P = 0.814$) or interaction ($P = 0.392$). Twenty-four hours of incubation with 0nM or 20nM progesterone with or without 20nM R5020 (Figure 8C) again revealed a main effect of progesterone ($P = 0.039$), but not R5020 ($P = 0.065$), to increase eNOS phosphorylation. Furthermore, there was no interaction between progesterone and R5020 ($P = 0.886$). Lastly, a three-way repeated measures ANOVA comparing the effects of equal concentrations of progesterone, R5020, and Org OD 02-0 showed no statistically significant two- or three-way interactions (Figure 8D); however, consistent with other analyses, a main effect of progesterone was detected ($P = 0.029$).

Figure 7.

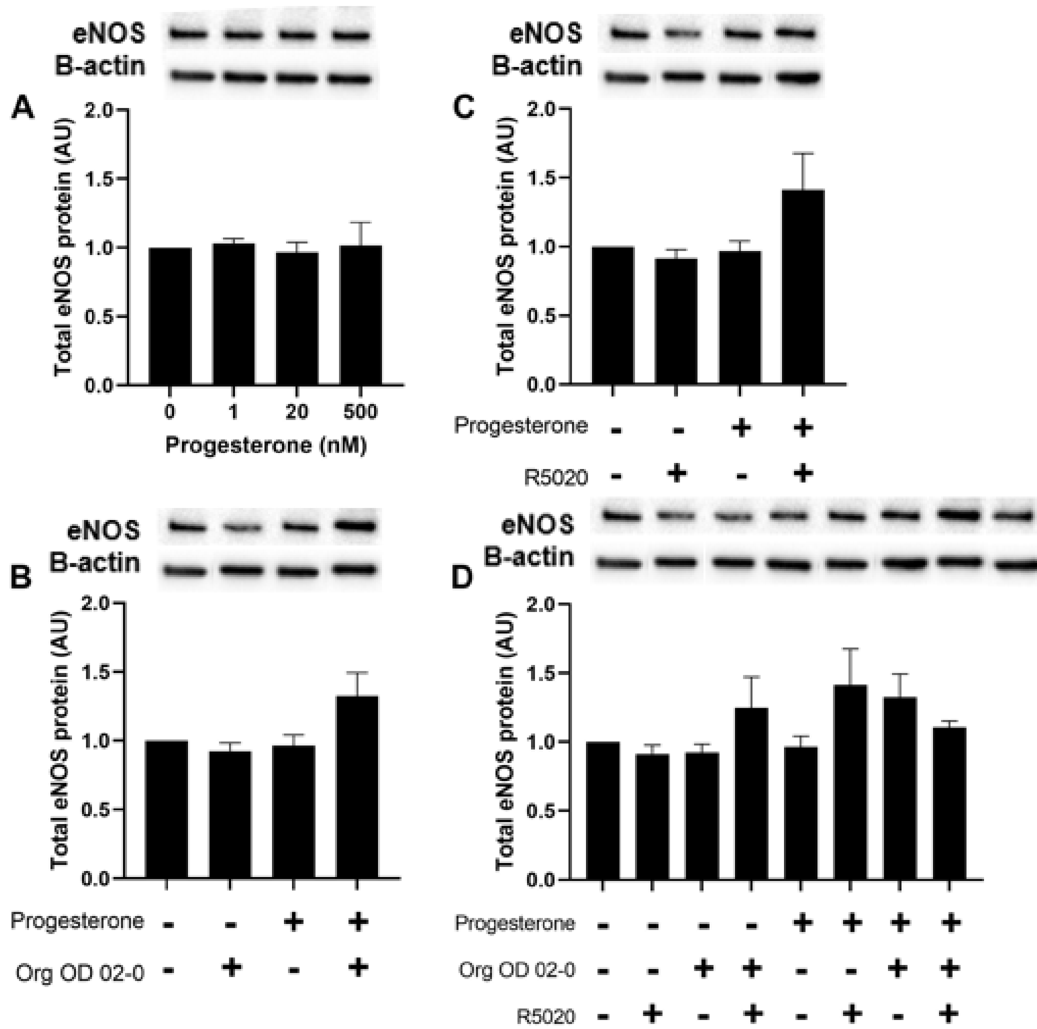

Figure 7. HUVEC total eNOS protein after 24 hours of incubation with progesterone and/or progesterone receptor agonists. (A) A RM ANOVA comparing concentrations of 0, 1, 20, and 500nM progesterone showed no main effect of progesterone. (B) A two-way RM ANOVA comparing progesterone and Org OD 02-0 showed no interaction or main effects. (C) A two-way RM ANOVA comparing progesterone and R5020 showed no interaction or main effects. (D) A three-way ANOVA comparing progesterone, Org OD 02-0, and R5020 showed no interactions or main effects. Data are means ± SEM and are expressed relative to the 0nM progesterone condition. AU: arbitrary units.

Figure 8.

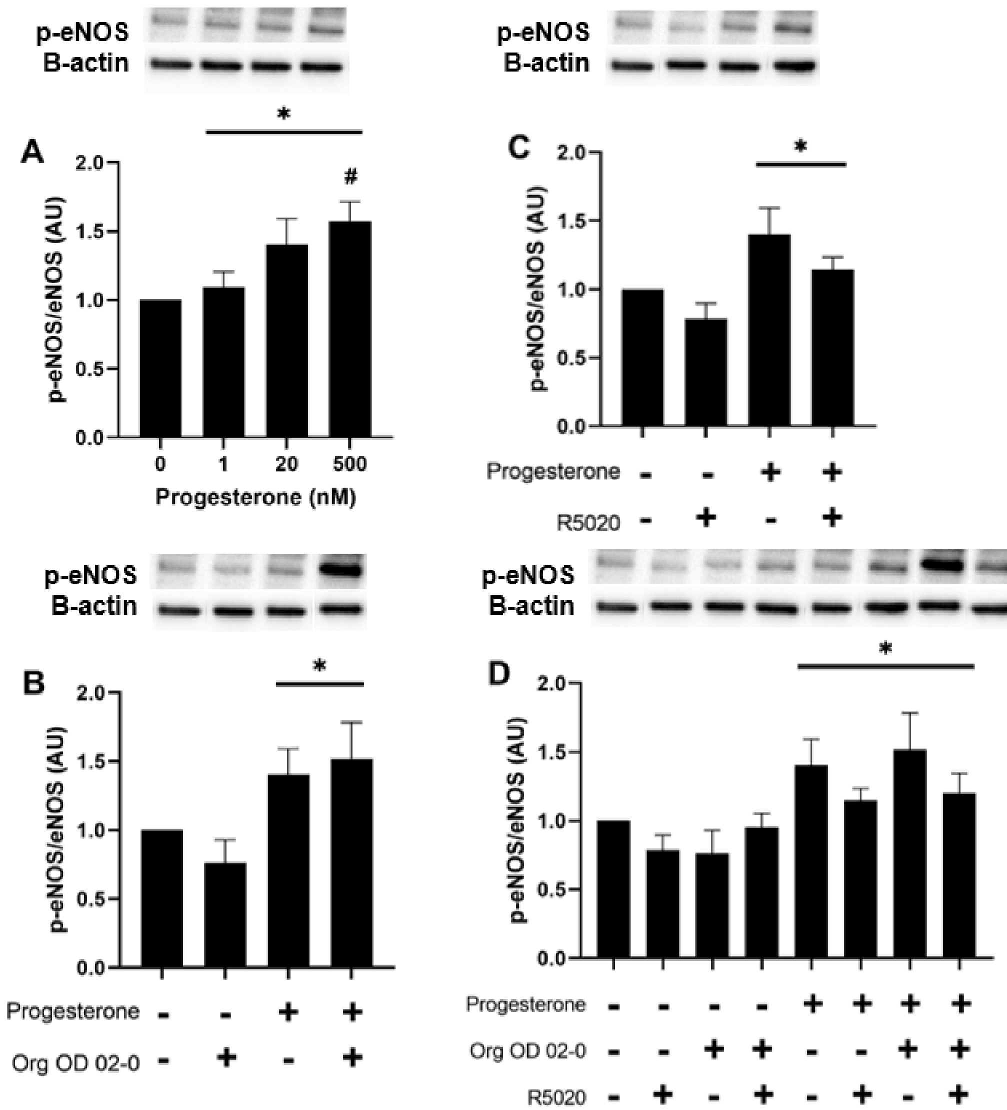

Figure 8. Phosphorylated eNOS/total eNOS protein ratio in HUVECs cultured in progesterone or progesterone receptor agonists for 24 hours. (A) A RM ANOVA comparing concentrations of 0, 1, 20, and 500nM progesterone showed a main effect of progesterone (P =0.016). (B) A two-way RM ANOVA comparing progesterone and Org OD 02-0 showed a main effect of progesterone (P = 0.009). (C) A two-way RM ANOVA comparing progesterone and R5020 showed a main effect of progesterone (P = 0.039). (D) A three-way ANOVA comparing progesterone, Org OD 02-0, and R5020 showed a main effect of progesterone (P = 0.029). Data are means ± SEM and are expressed relative to the 0nM progesterone condition. * p<0.05 main effect. AU: arbitrary units.

Chapter 6: Discussion

This study assessed the effects of progesterone and progesterone receptor agonists on endothelial cell functions related to angiogenesis and eNOS expression and activity. The findings of this study are as follows: (1) progesterone generally inhibited HUVEC functions related to angiogenesis, though results were not completely consistent across experiments; (2) the membrane progesterone receptor agonist Org OD 02-0 inhibited HUVEC migration and proliferation, whereas the nuclear receptor agonist generally did not; and (3) 24 hours of incubation with progesterone, but not the progesterone receptor agonists, increased phosphorylation of eNOS but not expression of eNOS in HUVECs. Thus, this study has identified a potential role of the membrane progesterone receptor in regulating HUVEC migration and proliferation – and a role of progesterone, irrespective of receptor activation, in regulating phosphorylation of eNOS in HUVECs. Altogether, these findings suggest that progesterone increases eNOS activity but may inhibit processes related to angiogenesis in HUVECs.

Endothelial cell angiogenic functions

The effects of progesterone on HUVEC function have been debated, with some studies indicating anti-angiogenic effects (Hsu et al., 2008; T.-S. Lee et al., 2015; Powazniak et al., 2009) and some describing pro-angiogenic properties (Fu et al., 2008; Zheng et al., 2012). However, these studies vary in the method of culturing HUVECs. Both referenced studies with pro-angiogenic findings (Fu et al., 2008; Zheng et al., 2012) used steroid-depleted FBS to culture HUVECs and do not indicate what passages of HUVECs were used in their assays. Furthermore, assays were not

consistent between publications: Razor-scrape migration assays were used in the "pro-angiogenic" studies, while a variety of functional HUVEC assays including capillary-like tube formation, trans-well or scratch wound migration, and cell proliferation assays were performed in the studies showing anti-angiogenic effects of progesterone (Hsu et al., 2008; T.-S. Lee et al., 2015; Powazniak et al., 2009). In the current study there was generally an effect of progesterone and Org OD 02-0 to decrease HUVEC migration and proliferation, but no significant effect of R5020 was detected. Therefore, the current results suggest that progesterone is not pro-angiogenic to HUVECs at the physiological concentrations used in this study and may even hinder angiogenesis under certain conditions. The potential effect of progesterone on HUVEC angiogenic functions may be mediated by the membrane progesterone receptor, as the membrane receptor agonist Org OD 02-0 consistently reduced HUVEC proliferation and migration, while the nuclear progesterone receptor agonist had no effect on these measures.

There were, however, interactions between a) R5020 and Org OD 02-0, and b) progesterone and Org OD 02-0 seen in the migration and proliferation assays to decrease HUVEC migration and proliferation when compared with equal concentrations of progesterone, indicating that the effects of the membrane progesterone receptor are, in part, affected by the actions of the nuclear progesterone receptor. While it does not appear that the nuclear progesterone receptor can inhibit angiogenic cell functions on its own, there is possibility for cross-talk between the membrane and nuclear progesterone receptors in that the actions of the membrane progesterone receptor could be amplified by the activation of the nuclear

progesterone receptor. Ultimately, progesterone and progesterone receptor activation in this study were shown to inhibit female HUVEC migration and proliferation. These findings indicate a potential role of the membrane progesterone receptor in inhibiting long-term angiogenic processes despite this receptor previously being considered to have primarily short-term effects in endothelial cells.

It seems counter-intuitive that the high concentration of progesterone usually only detected in circulation during pregnancy (500nM) had the most detrimental effect on HUVEC migration considering the need for increased vascularization in the uterus for fetal development. However, this may be explained by the tissue-specific actions of progesterone. For example, studies administering progesterone to ovariectomized mice found that progesterone increased endometrium endothelial cell proliferation (Heryanto & Rogers, 2002; Walter et al., 2005). Furthermore, progesterone alone has increased human endometrium endothelial cell proliferation in vitro (Kayisli et al., 2004). Additionally, endometrium endothelial cell proliferation in the presence of progesterone increased even when VEGF expression was reduced with VEGF anti-serum (Walter et al., 2005). This points to the possibility of a factor present in endometrium endothelial cells, but not HUVECs, that enacts progesterone's angiogenic effects.

eNOS expression and phosphorylation

In HUVECs, progesterone was expected to increase eNOS expression and phosphorylation. Previous studies showed that physiological doses of progesterone increased eNOS expression and activity and NO levels released by HUVECs in a dose-response pattern (Pang et al., 2015; Simoncini et al., 2004b). Furthermore, the

phosphorylation of eNOS by membrane progesterone receptors was expected to take effect in a short period of time, between 30 to 60 minutes (Pang et al., 2015; Simoncini et al., 2004b), while nuclear progesterone receptors were expected to take far longer to increase eNOS expression, around 24 hours (Goddard et al., 2014; Toth et al., 2009). The results of the current study do not completely agree with these previous findings. While there was no effect of progesterone on eNOS expression, 20nM progesterone significantly increased phosphorylation after 24 hours of incubation. It should be noted that a similar trend of increasing eNOS phosphorylation with progesterone was seen after 30 minutes of incubation; however, this was not statistically significant due to greater variability in eNOS phosphorylation among samples at this timepoint. The fact that eNOS expression did not increase at either timepoint implies that the transcriptional role of nuclear progesterone receptors on eNOS was not upregulated in the HUVECs cultured in this study.

While 20nM progesterone consistently increased eNOS phosphorylation at 24 hours, neither of the progesterone receptor agonists alone elicited changes in eNOS expression or phosphorylation at either timepoint. These findings are in opposition to previous reports of eNOS downregulation via incubation of HUVECs with a nuclear progesterone receptor antagonist (You et al., 2020) and increases in eNOS phosphorylation via incubations of HUVECs with Org OD 02-0 (Pang et al., 2015), though these disagreements may be explained by differences in HUVEC culture methods and nuclear progesterone receptor agonist vs. antagonist use. Regardless, the current study shows that progesterone increased eNOS phosphorylation after 24

hours, no increases in phosphorylation were seen in the presence of either agonist alone. This may imply that progesterone increases eNOS phosphorylation in ways not attributable to either its nuclear or membrane receptors. In other words, the actions of progesterone to phosphorylate eNOS in this study cannot be explained by the actions of either the nuclear or membrane progesterone receptors, which appear to inhibit eNOS phosphorylation when liganded in isolation.

While NO levels were not measured in the present study, it is possible that increased eNOS phosphorylation resulted in higher NO levels in the HUVECs. This may, in part, explain the observed down regulation of HUVEC proliferation and migration, as some studies show that NO may actually inhibit HUVEC migration (Kaur et al., 2010; Treggiari et al., 2018). For example, O2-(2,4-Dinitrophenyl) 1-[(4-ethoxycarbonyl)piperazin-1-yl]diazen-1-ium-1,2-diolate (JS-K), an NO donor, inhibited capillary-like tube formation and migration of HUVECs (Kaur et al., 2010). This anti-angiogenic effect is not wholly negative as NO bioavailability could prevent angiogenesis in non-specific sites such as tumor tissues by preventing the actions of VEGF in these tissues. Another study inducing inflammation in human lung microvascular endothelial cells showed that inflammation significantly stimulated eNOS expression and activity but blunted angiogenesis and wound-healing processes (Lowry et al., 2013), which is generally consistent with the findings of this study. Conversely, although significant increases in eNOS phosphorylation in HUVECs were observed at 24 hours incubation with progesterone, this may not indicate that NO bioavailability was increased in HUVECs. In a previous study, endothelial cells treated with AMPK inhibitor compound C significantly inhibited eNOS

phosphorylation at ser1177 while cytosolic NO levels in these same cells detected by real-time fluorescent imaging did not change (Eroglu et al., 2019). This imaging technique does not detect peroxynitrite, a reactive oxygen species that forms when NO scavenges superoxide (Radi et al., 1991). In contrast, NO assay kits used to quantify NO in samples in many of the studies referenced in the literature review infer NO presence in samples by quantifying byproducts (nitrate and nitrite ions) of this very reaction between NO and superoxide. Thus, studies using NO assay kits to report increases in NO stimulated by progesterone are not quantifying bioavailable NO in culture medium or HUVECs. It is possible that the increased eNOS activity in the presence of progesterone is concurrent with increases in superoxide production, meaning that while NO is upregulated, the NO may quickly react with superoxide and reduce bioavailable NO. In fact, progesterone has been shown to increase ROS generation and reduce superoxide dismutase (SOD) expression, further supporting the fact that progesterone may decrease bioavailable NO (Wassmann et al., 2005). Decreased NO bioavailability has been linked to endothelial and vascular dysfunction (Chen et al., 2018; Levine et al., 2012), which could provide an alternative explanation for the inhibition of HUVEC migration and proliferation brought about by progesterone and progesterone receptor agonists.

Limitations

One potential limitation of the present study is the use of HUVECs as a model of endothelial cells. While HUVECs are commonly used in in vitro experiments, they are not entirely representative of endothelial cells in the peripheral vasculature. Variability in gene expression and cell function exists among endothelial cell types

based on their location. For example, human aortic endothelial cells have previously exhibited higher angiogenic potential than HUVECs in a 3D culture system (Chi et al., 2003; Seo et al., 2016). HUVECs were also most likely exposed to different concentrations of sex hormones before isolation from donor umbilical cords; these differences could not be accounted for as the supplier did not provide umbilical cord blood hormone concentrations for each donor whose cells were included in each pooled female HUVEC lot. Another limitation was that HUVEC NO release in response to progesterone or progesterone receptor agonists was not quantified in this study. This limited the ability to confirm that progesterone-stimulated increases in eNOS phosphorylation led to changes in NO release in HUVECs. Another possible limitation is differential binding affinity of the agonists used in the study. It should be noted that Org OD 02-0 has a much higher binding affinity to membrane progesterone receptor alpha when compared with R5020's binding affinity to the nuclear progesterone receptors (Pang et al., 2015). These binding affinity characteristics may indicate that higher concentrations of R5020 would elicit differences as well, and that both progesterone receptors are involved enacting angiogenic processes. Unfortunately, we could not control for binding affinity as Org OD 02-0 is the only known agonist of membrane progesterone receptor alpha and was only synthesized recently. Furthermore, an antagonist of the membrane progesterone receptors does not currently exist, which is the reason for why only agonists were utilized in this thesis study. Lastly, despite power calculations having been run before performing the experiments in this study, the small sample size in the current study may have limited the ability to detect differences among conditions.

Future work/directions

To further describe the effects of membrane progesterone receptor activation on endothelial cell function, antagonists for both progesterone receptors or small interfering RNA (siRNA) to knockout receptor mRNA should be used in assays that quantify the angiogenic capacity of endothelial cells. Endothelial cells in these assays should be sourced from other locations than umbilical cords such as the aorta or endometrial blood vessels, and donor characteristics should be accounted for when analyzing data collected from these cells. The interactions among progesterone, estrogen, and other sex hormones (e.g., follicle stimulating hormone and luteinizing hormone) to affect endothelial cell function should also be explored, as these hormones are concurrently present in the circulation of premenopausal women and do not act in isolation. Additionally, NO should be quantified in future studies looking to observe the effects of female sex hormones in the vasculature, and the amount superoxide in the intra- and extracellular environment should also be recorded to confirm the nature of progesterone-stimulated nitric oxide increase.

Conclusions

In the present study, it appears that progesterone inhibits angiogenic processes in HUVECs, while at the same time increasing eNOS phosphorylation after 24 hours. Progesterone most likely acts through a mechanism separate from membrane or nuclear progesterone receptor activation to increase and maintain eNOS phosphorylation in HUVECs. For endothelial cell function, it appears that binding of the membrane progesterone receptor most likely inhibits migration and proliferation, but there may be some interaction with nuclear progesterone receptor activation.

Progesterone's lack of angiogenic effect in HUVECs compared with endometrium endothelial cells points to the possibility that HUVECs lack a factor that endometrium endothelial cells possess to promote angiogenesis in the presence of progesterone. Alternatively, progesterone may increase both superoxide and NO in the vascular environment, leading to reduced NO bioavailability and increases in peroxynitrite levels that both inhibit angiogenesis. The findings reported in this current study, taken together with previous findings in the literature, show that progesterone fails to bring about pro-angiogenic outcomes in HUVECs despite increases in eNOS phosphorylation. While eNOS activity is generally thought of as beneficial to endothelial cell function, progesterone may inhibit the angiogenic potential of HUVECs through phosphorylation of eNOS or other pathways.

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