Sex differences in the pulmonary circulation: implications for pulmonary hypertension

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Martin YN, Pabelick CM. Sex differences in the pulmonary circulation: implications for pulmonary hypertension. Am J Physiol Heart Circ Physiol 306: H1253–H1264, 2014. First published March 7, 2014; doi:10.1152/ajpheart.00857.2013.—Pulmonary arterial hypertension (PAH), a form of pulmonary hypertension, is a complex disease of multifactorial origin. While new developments regarding pathophysiological features and therapeutic options in PAH are being reported, one important fact has emerged over the years: there is a sex difference in the incidence of this disease such that while there is a higher incidence in females, disease outcomes are much worse in males. Accordingly, recent attention has been focused on understanding the features of sex differences in the pulmonary circulation and the contributory mechanisms, particularly sex hormones and their role in the pathological and pathophysiological features of PAH. However, to date, there is no clear consensus whether sex hormones (particularly female sex steroids) are beneficial or detrimental in PAH. In this review, we highlight some of the most recent evidence regarding the influence of sex hormones (estrogen, testosterone, progesterone, dehydroepiandrosterone) and estrogen metabolites on key pathophysiological features of PAH such as proliferation, vascular remodeling, vasodilation/constriction, and inflammation, thus setting the stage for research avenues to identify novel therapeutic target for PAH as well as potentially other forms of pulmonary hypertension.

PULMONARY HYPERTENSION (PH) is a disease characterized by elevated pulmonary artery pressure, often resulting in right ventricular failure. The diagnosis of PH is made when the mean pulmonary artery pressure is ≥25 mmHg at rest (6). PH occurs in men and women of any race or age. PH was previously categorized as either primary or secondary, but this classification proved inadequate, leading to the revised World Health Organization classification system from the Fourth World Symposium on Pulmonary Hypertension (86, 115). The World Health Organization classification system includes five categories based on mechanism of disease (Table 1). Group I refers to pulmonary arterial hypertension (PAH) and encompasses idiopathic, drug-induced, heritable, and PH associated with other systemic diseases such as schistosomiasis. Of all the categories, it is within group I that the epidemiology is most revealing in regard to sex differences in the incidence and severity of PH that is leading to increasing interest in understanding what contributes to intrinsic sex differences versus the potential effects of sex steroids in PH. In this review, we highlight recent evidence regarding these important issues, focusing on PAH (group I), but also indicating evidence in other groups (broadly referred to by the term PH).

Epidemiology. Multiple registries have contributed to our current understanding of the epidemiology of PAH. The earliest identified modifier noted in all of the epidemiological studies of PAH is female sex, which increases the incidence of this disease 1.9- to 10-fold, depending on specific subtype and registry of patients (7, 22, 56). While PAH can develop at all ages, the mean age of patients with PAH depends on the subtype, with familial PH having the youngest age at diagnosis (37 ± 11 yr). The age at onset in the other subtypes of group I PAH is around the fifth decade of life (7, 56). Neither registry shows any difference in the age at onset for males versus females. In ethnic groups, there has been a consistently higher female predominance among blacks with earlier registries indicating a 4.3:1 ratio (104). More recent data show a 5.5:1 female-to-male ratio (38). Explanations for the ethnic differences are unclear at the present time. Nonetheless, these epidemiological data point to female sex as representing a higher risk for occurrence of PAH.

With regard to prognosis, the influence of sex is reversed. Both the French and the US-based registry (Registry to Eval-
H1254 PULMONARY HYPERTENSION AND SEX DIFFERENCES

Table 1. Classification of PH

<table>
<thead>
<tr>
<th>Group 1</th>
<th>Pulmonary arterial hypertension</th>
</tr>
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<tr>
<td>Pulmonary arterial hypertension</td>
<td></td>
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<tr>
<td>Idiopathic</td>
<td></td>
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<tr>
<td>Heritable (BMPR2)</td>
<td></td>
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<tr>
<td>Drug-and toxin-induced</td>
<td></td>
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<tr>
<td>Disease associated</td>
<td></td>
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<tr>
<td>Persistent PH of the newborn</td>
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<tr>
<td>Pulmonary venoocclusive disease and/or pulmonary capillary hemangiomatosis</td>
<td></td>
</tr>
</tbody>
</table>

| Group 2                        | PH owing to left heart disease |

| Group 3                        | PH owing to lung diseases and/or hypoxia |

| Group 4                        | Chronic thromboembolic PH |

| Group 5                        | PH with unclear multifactorial mechanisms |

PH, pulmonary hypertension; BMPR2, bone morphogenetic protein receptor type 2. See Simonneau et al. (115).

Role of Estrogen

Estrogen paradox. Epidemiology confirms that sex differences exist in clinical forms of PAH (7, 56). However, in various animal models, female animals have less evidence of PH compared with males (102). In these animal studies, sex differences are attributed to protective effects of estrogen, since estrogen and its metabolites have been shown to prevent and rescue experimental PH (34, 127, 128, 132). Therein is the crux of the so-called “estrogen paradox”: experimentally, estrogens are beneficial in the pulmonary vasculature, whereas, clinically, women are more likely to have the disease. Although this paradox is acknowledged, there still remain ample areas for further investigation on the implication of sex hormones and PAH, which may ultimately reveal the true nature of the disease pathophysiology.

Estrogens and the estrogen receptors. Of the three main estrogens, 17β-estradiol, estrone, and estriol, the primary circulating estrogen is 17β-estradiol. Figure 1 summarizes the synthesis and metabolism of the different sex hormones. In females, fluctuating estrogen levels depending on age, menstrual status, and pregnancy can further contribute to the complexity of estrogen signaling. Indeed, estrogen signaling can be relevant in males as well, particularly with aging, where increased testosterone to estradiol conversion via aromatase can occur.

Estrogen synthesis. While circulating estrogens likely play a role in the development and modulation of PAH, there is emerging evidence that sex hormone synthesis takes place in peripheral tissues such as the brain and heart (97). Within the lung expression of some of the enzymes involved in estrogen synthesis and inactivation has been reported (20). Thus, the lung is not exclusively and passively exposed to circulating sex hormones but has the capability to synthesize and inactivate them, thus modulating sex steroid action at the cellular level. However, it is currently unknown whether local synthesis occurs in the pulmonary artery and, if so, to what extent. The involvement of pulmonary arterial smooth muscle cells (PASMCs) or pulmonary arterial endothelial cells (PAECs) in such synthesis and signaling is also unknown.

Among the enzymes involved in sex steroid metabolism (Fig. 1), aromatase cytochrome P-450, family 19, subfamily A, polypeptide 1 gene (CYP19A1) is at the forefront since it is the principal enzyme responsible for generation of estrogen from testosterone. The importance of aromatase expression and activity is not only related to its role in the synthesis and maintenance of estrogens but in testosterone metabolism. A recent study by Roberts et al. (105) demonstrated that genetic variation in the aromatase gene was associated with an increased risk of developing portopulmonary hypertension. Also, two aromatase variants found in the 5’ region upstream of the aromatase gene were associated with increased circulating estrogen levels, demonstrating the contribution of estrogens to the sex bias in PAH (105). Aromatase expression is present in the vasculature and specifically the pulmonary artery (50). Thus the pulmonary artery possesses the required machinery to synthesize estrogen from testosterone via aromatase. Local regulation of aromatase expression will influence estrogen levels in the pulmonary artery.

Subsequent metabolism of estrogen is regulated by several P450 enzymes: CYP1A1, CYP1B1, CYP3A4, and catechol-O-methyltransferase (Fig. 1). Among those, CYP1B1 has been

![Fig. 1. Overview of the metabolism of sex hormones. ER, estrogen receptor; 3β-HSD, 3β-hydroxysteroid dehydrogenase; GPR, G protein-coupled receptor; CYP1A1, cytochrome P-450, family 19, subfamily A, polypeptide 1 gene; COMT, catechol-O-methyltransferase.](http://ajpheart.physiology.org/doi/10.1152/ajpheart.00857.2013)
examined the most. CYP1B1 is expressed in the lung and is responsible for generating the 4-hydroxy-, 2-hydroxy-, and 16-hydroxyestrogens from estrogen (5, 48). Several studies implicate a role for CYP1B1 in the development of PAH. In a gene expression array study using lymphoblastoid cells, patients with familial PAH carrying a bone morphogenetic protein receptor type 2 (BMPR2) mutation—a known genetic marker predisposing to PAH discussed later in this review—showed significantly decreased CYP1B1 expression compared with those unaffected. This decrease was most striking in females carrying the BMPR2 mutation with disease compared with male carriers with disease (135). Also, in patients with familial PAH, a common polymorphism in the CYP1B1 gene Asn453Ser was suggested to modify familial PAH risk (2) since the wild-type genotype was more frequently found in female affected carriers of the BMPR2 mutation. None of the aforementioned studies have examined expression of CYP1B1 in the pulmonary artery per se, thus limiting our ability to conclude that tissue-specific changes in estrogen metabolism contribute to familial PAH development. However, a recent study in patients with idiopathic PAH was the first to show increased CYP1B1 expression by immunohistochemistry within the pulmonary artery (138). Functionally, manipulation of CYP1B1 by either genetic knockout or pharmacological inhibition of CYP1B1 protected against the development of PH in mouse models of PH involving hypoxia only or the addition of a VEGF inhibitor SU5416 (138). The proposed mechanism by which CYP1B1 contributes to PAH development is through the increased production of the CYP1B1 metabolite 16α-hydroxyestrone, a potent inducer of proliferation.

Further support for CYP1B1 in PAH development has been demonstrated in dexfenfluramine-induced PAH. During the 1960s and 1980s, dexfenfluramine was widely used as an anorexigen but was removed from the market because of reported associations with PAH among female users of this drug (1, 64). The proposed mechanism by which dexfenfluramine induces PAH is thought to be increased serum concentration of serotonin via the serotonin transporter (SERT) (108). For example, microarray analyses reveal increased expression of CYP1B1 in SERT+ mice, serotonin-exposed human PASMCs and those from patients with idiopathic PAH (139). In addition, Dempsie et al. (26) exposed female mice to dexfenfluramine and observed increases in physiological markers of PAH (systolic right ventricular pressure and pulmonary vascular remodeling), as well as increased whole lung expression of CYP1B1, effects absent in female CYP1B1−/− mice.

Taken together, these studies provide evidence that CYP1B1 may have a broader role in group I PAH. No studies to date have demonstrated that alterations in the remaining metabolizing enzymes affect the pulmonary artery or PAH.

**Estrogen receptors.** The action of estrogen or its derivatives is mediated through estrogen receptors (ERs), primarily ERα and ERβ, and the novel G protein-coupled receptor GPR30. The specific cellular effects of estrogen are dictated by the relative expression and different actions of ERα versus ERβ versus GPR30, suggesting the potential for complex estrogen signaling within the pulmonary vasculature. For example, it is thought to be the activation of ERα that leads to aberrant proliferation (79, 123), whereas activation of ERβ mediates antiproliferative effects, thereby counteracting ERα effects (66). Such differential effects of ER subtypes may explain the protective effect of estrogens in systemic vasculature (e.g., the well-recognized effects in the coronary vasculature) but the higher incidence of PAH in young women. Although both ERα and ERβ are expressed in the pulmonary artery, reports suggest that ERβ may be the predominant receptor mediating protective effects of estrogen, at least in hypoxic PH (65). Given that insults such as hypoxia are risk factors in certain forms of PH, these differential roles for ERα and ERβ may be relevant to sex differences in this disease. In regard to the novel receptor GPR30, its expression and function in vascular biology has been studied in the endothelium and smooth muscle of aortic, carotid, and cerebral vasculature from mouse and rat tissue, human internal mammary arteries, and cultured human internal mammary artery smooth muscle cells [reviewed in (74)]. Low-level expression of GPR30 has been found in whole lung of mice and is similar between males and females (83). However, further studies are needed to investigate the presence and effects of GPR30 specifically in the pulmonary vasculature.

**Effects of Other Sex Hormones and Estrogen Metabolites on Pulmonary Circulation**

Although estrogen is at the forefront of studies related to sex differences in PAH, there could plausibly be a role for testosterone and progesterone in regard to sex differences in PAH. Specifically, the interplay of these three hormones may be of significance in the pulmonary artery. Here we review the known effects to date of these hormones in the pulmonary circulation (outlined in Table 2).

**Testosterone.** Testosterone levels are greater in males than females. However, females still have measureable testosterone levels (118). Testosterone has been demonstrated to induce vasodilation in isolated human and rat pulmonary arteries (33, 59, 109) and therefore has the potential to improve hemodynamics in patients with PH. This is in contrast to animal models of PH. Male rats have been noted to have more severe disease than females in the hypoxia models of PH (99), and ovariecetomized female rats have more severe disease compared with those who retained their ovaries (128). Therefore, testosterone per se does not seem to explain the female predominance of PAH.

**Progesterone.** The role of progesterone in the pulmonary circulation has received little attention. Tofovic et al. (125) showed in the monocrotaline (MCT) rat model of PH that a continual delivery of progesterone for 30 days attenuated the disease by decreasing right ventricular end-diastolic pressure and that mortality was decreased in the MCT with progesterone group. To date, progesterone has not shown any sex differences in regard to effects on human pulmonary vessels (33).

**2-Methoxyestradiol.** 2-Methoxyestradiol (2ME) is a well-studied biologically active metabolite of estrogen. It has antiproliferative properties that are not mediated through ERs (90). Its therapeutic potential in pulmonary vascular disease has been investigated in MCT-induced PH in rats (127, 128). Treatment with 2ME was found to provide a reduction in disease progression (126). However, so far, 2ME levels have not been compared in patients with and without PAH.

**Dehydroepiandrosterone.** Dehydroepiandrosterone (DHEA) is a steroid hormone synthesized by the adrenal gland. In humans, DHEA exhibits sex differences in circulating levels, with men having consistently higher serum levels (63).
Table 2. Summary of the effects of sex hormones on the pulmonary vasculature

<table>
<thead>
<tr>
<th>Sex Steroid</th>
<th>Known Effect(s)</th>
<th>Potential Mechanisms and Evidence</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estrogen</td>
<td>Prevention and reversal of PH</td>
<td>ERβ mediated (rat model)</td>
<td>(132)</td>
</tr>
<tr>
<td></td>
<td>PA Vasodilation</td>
<td>Stimulation of NOS activity</td>
<td>(69, 94)</td>
</tr>
<tr>
<td></td>
<td>Antiproliferative</td>
<td>Inhibition of Ca²⁺ influx via T-type VDCC (group III PH)</td>
<td>(65)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Inhibition of PAEC proliferation via ERβ and decreased ERK1/2 activation (rat)</td>
<td></td>
</tr>
<tr>
<td>16α-Hydroxyestrone</td>
<td>Proliferative</td>
<td>Increased PASMC proliferation</td>
<td>(137)</td>
</tr>
<tr>
<td></td>
<td>Anti-inflammatory</td>
<td>Limited monocyte infiltration via ERβ</td>
<td>(8, 70, 132)</td>
</tr>
<tr>
<td>2-Methoxyestradiol</td>
<td></td>
<td>Increased PASMC proliferation via unknown mechanism</td>
<td>(138)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Unknown mechanism, not mediated through ER</td>
<td>(127, 128)</td>
</tr>
<tr>
<td>Testosterone</td>
<td>PA Vasodilation</td>
<td>Inhibition of Ca²⁺ influx via T-type VDCC (group III PH)</td>
<td>(33, 59, 109)</td>
</tr>
<tr>
<td></td>
<td>Anti-inflammatory</td>
<td>Suppression of proinflammatory cytokines</td>
<td>(52)</td>
</tr>
<tr>
<td>Dehydroepiandrosterone</td>
<td>Prevention and reversal of PH</td>
<td>Activation of K⁺ channel (rat)</td>
<td>(13, 47, 93)</td>
</tr>
<tr>
<td></td>
<td>PA Vasodilation</td>
<td>Inhibition of Ca²⁺ influx via T-type VDCC</td>
<td>(19, 93)</td>
</tr>
<tr>
<td>Progesterone</td>
<td></td>
<td>Increased soluble guanylate cyclase</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Antiproliferative</td>
<td>Via Src/STAT3 pathway (human PASMC)</td>
<td>(96)</td>
</tr>
<tr>
<td></td>
<td>PA Vasodilation</td>
<td>Increased NO and cGMP</td>
<td>(33, 72, 125)</td>
</tr>
</tbody>
</table>

ER, estrogen receptor; NOS, nitric oxide (NO) synthase; PA, pulmonary artery; PAEC, pulmonary arterial endothelial cell; PASMC, pulmonary arterial smooth muscle cell; VDCC, voltage-dependent Ca²⁺ channel.

DHEA can be converted into testosterone and estrogen. In animal models of PH, DHEA treatment prevents and reverses PH (13, 47, 93). In humans with group III PH, treatment with DHEA showed improvement in pulmonary hemodynamics (31). Although smaller studies have implicated DHEA as having beneficial effects in PH, the true efficacy and feasibility of DHEA as a therapeutic option for PH remain to be established.

Pathophysiological Features of PAH Influenced by Sex Hormones

PAH is a proliferative vasculopathy, characterized by vasocnstriction, cell proliferation, and fibrosis. Pathological findings of the small pulmonary arteries include medial hypertrophy, intimal hyperplasia, fibrosis, and development of plexiform lesions (87, 98, 130). Cellular components most involved in the dysfunction of the pulmonary artery are PAECs and PASMCs (87) (Fig. 2). The pathological appearance of medial hypertrophy, which results from proliferation of smooth muscle cells, and intimal fibrosis seen in the small pulmonary arteries is similar across patients with group I PAH (46). Given the myriad of known pathophysiological features of PAH, estrogens have the potential to influence several of these features. Here we will discuss the effects of estrogens on the more commonly known pathophysiological features of PAH: balance between vasoconstriction and vasodilation, increased cell proliferation, and inflammation (schematically shown in Fig. 2).

Vasodilation/nitric oxide pathway. Endothelial nitric oxide (NO) synthase (eNOS) is the main enzyme responsible for production of NO in the pulmonary vasculature. NO signals via cGMP to the vascular smooth muscle resulting in vasodilation (110). NO levels have been found to be lower in human lungs from PAH patients compared with normal subjects, suggesting NO deficiency in the pathogenesis of PAH (60). Multiple reports have focused on altered pulmonary NO synthase (NOS) expression and function as a principal mechanism for NO deficiency, and earlier studies showed decreased expression of NOS (42, 141). However, recent studies in human lung and animal models of PH show unchanged or even increased NO expression, but with uncoupled NOS activity, resulting in a shift toward production to superoxide generation rather than NO synthetase (84, 148). While it is unclear whether increased superoxide itself has detrimental effects, the deficiency in NO results in incomplete vasodilation by adjacent smooth muscle cells (141).

Drawing from the well-documented effects of estrogens on the circulation, endothelial NO synthesis has been recognized as one pathway by which estrogen modulates the systemic vasculature. Estrogen targets the endothelial NO pathway by modulating NOS. Estrogen activation of the Akt pathway via ERα can rapidly induce endothelial NO generation. This non-genomic process leads to phosphorylation of eNOS, augmenting its ability to generate NO (17, 53). Additionally, estrogen activation of ERα leads to upregulation of eNOS protein, thereby increasing NO generating capacity (107, 122). Specific to the pulmonary artery, increasing evidence indicates that estrogen enhances vasodilation via increased NO production in PAECs and upregulates NOS expression via gene transcription (17, 117). The net effect of increased NO bioavailability is improved pulmonary hemodynamics and vascular remodeling (68, 95). Whether estrogens have any effects on superoxide generation may be an area for future investigation.

Progesterone has been shown to be the most efficacious vasodilator in isolated rat pulmonary arteries compared with testosterone and 17β-estradiol (33). However, the proposed mechanisms of progesterone-mediated vasodilation have been sparsely studied. Li et al. (72) demonstrated that progesterone induces vasodilation via NO and cGMP. However, it remains to be demonstrated whether progesterone effects in terms of NO are different (or deficient) in PAH, or specifically in women with this disease. Although testosterone induces vasodilation (109, 116), the mechanism is not thought to be through the NO pathway (59). Recent studies demonstrate that testosterone causes vasodilation by inhibiting calcium influx via voltage-dependent calcium channels (59) as discussed further below. It is currently unknown whether other estrogen metabolites affect the NO pathway.
Calcium regulation: vasoconstriction and proliferation. Enhanced pulmonary arterial vasoconstriction and increased cell proliferation are considered to be two mechanisms responsible for the structural and functional changes that occur in PAH, leading to elevated mean pulmonary artery pressure. Vasoconstriction of PASMCs and proliferation of PASMCs and PAECs are all stimulated by increases in intracellular Ca\(^{2+}\) concentrations ([Ca\(^{2+}\)]\(_i\)). It is known that resting [Ca\(^{2+}\)]\(_i\) values are increased in PASMCs of patients with idiopathic PAH (146). Therefore, increased proliferation and vasoconstriction in PAH could result from increased [Ca\(^{2+}\)]\(_i\). However, the efficacy of inhibiting influx alone in abrogating structural or functional changes in PAH is relatively limited, suggesting more complex regulatory pathways being at play. Nonetheless, modulating Ca\(^{2+}\) regulatory pathways may represent at least a partial approach to alleviating PH. Accordingly, understanding sex differences or the roles of sex steroids in terms of Ca\(^{2+}\) regulation is relevant.

Calcium influx via voltage-dependent calcium channels (147), transient receptor potential (TRP) channels (44), and calcium release from intracellular stores (129) are known mechanisms available for elevating [Ca\(^{2+}\)]\(_i\) in PASMCs (Fig. 2). Elevations in [Ca\(^{2+}\)]\(_i\) occur following activation of L- and T-type voltage-dependent calcium channels, both of which are found in PASMCs (35). The effects of estrogen and other sex steroids on calcium influx via voltage-dependent channels have been predominantly studied in the context of hypoxia-induced PH (group III). Both group I and group III PH share the common feature of increased [Ca\(^{2+}\)]\(_i\) and sustained vasoconstriction. Oral DHEA treatment of rats exposed to chronic hypoxia restores [Ca\(^{2+}\)]\(_i\) levels to normal (13). In vitro, hypoxia-induced increases in [Ca\(^{2+}\)]\(_i\) in rat PASMCs are also attenuated by DHEA (13). More recently, Chevalier et al. (19) showed that DHEA inhibits calcium influx by inhibition of T-type calcium channels, suggesting this as a mechanism by which DHEA normalizes and maintains PASMC [Ca\(^{2+}\)]\(_i\) levels in the setting of hypoxia. These emerging studies emphasize the importance of DHEA, at least in group III PH. Whether estrogen or progesterone per se are equally effective (or work through specific mechanisms) is not clear.

The TRP channels belong to a superfamily of proteins that allow entry of Ca\(^{2+}\) to varying degrees of specificity. The canonical TRP (TRPC) channel is a known regulator of Ca\(^{2+}\) influx, and there are at least six isozymes recognized (29). Yu et al. (144) found that TRPC6 is the predominant isoform in PASMCs and is significantly upregulated at the protein and mRNA levels in PASMCs in PAH, compared with normals. Increased expression of TRPC6 leads to enhanced proliferation of PASMCs in PAH (121, 144). TRPC6 has also been found to contribute to enhanced Ca\(^{2+}\) entry in this setting (44, 145). However, the modulating effect of sex hormones on TRPC6 in the context of PH or PAH has not been examined.

A more recently described regulator of [Ca\(^{2+}\)]\(_i\) also found in PASMCs is the Ca\(^{2+}\)-sensing receptor (CaSR). CaSR is a G

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Fig. 2. Schematic summarizing the effects of sex hormones on pulmonary arterial endothelial cells (PAECs) and smooth muscle cells. While the effects of sex hormones on the pulmonary endothelium alleviate pulmonary arterial hypertension, the effects on the smooth muscle are mixed. NO, nitric oxide; DHEA, dehydroepiandrosterone; VDCC, voltage-dependent calcium channel; TRPC, transient receptor potential channel; CaSR, calcium sensing receptor; PASMCs, pulmonary artery smooth muscle cells; [Ca\(^{2+}\)]\(_i\), intracellular Ca\(^{2+}\) concentration.
protein-coupled receptor activated by extracellular calcium and results in increased \([\text{Ca}^{2+}]\), via calcium release from intracellular stores [reviewed in (54, 81)]. Yamamura and colleagues (142) studied human PAH PASMCs and noted elevated CaSR expression and function in terms of enhanced Ca\(^{2+}\) signaling and augmented proliferation, compared with normal PASMCs. CaSR has been studied in hormone-responsive diseases such as prostate and breast cancers, and so there is a framework for future study with CaSR and sex hormones in the pulmonary vasculature.

**Proliferation/remodeling.** Remodeling of the pulmonary vasculature is a result of PASMC proliferation leading to obliteration of the vascular lumen and resulting in increased pulmonary vascular resistance. While much research and therapy have focused on the vasoconstriction/vasodilation aspect of PAH, proliferation and remodeling in PAH are more recent areas of active investigation (51, 87, 111, 112). It is known that estrogen inhibits proliferation and migration in human systemic vascular cells (25). However, in vitro studies investigating the effects of estrogens on proliferation in the pulmonary artery are conflicting, particularly when comparing cell types (PASMCs vs. PAECs) and models (human vs. animal models). One study reported estrogen-induced inhibition of PAEC growth (65), whereas another study showed estrogen-induced PASMC proliferation (137). When estrogen attenuates proliferation, as demonstrated under hypoxic conditions, it is mediated via ERs (65). However, estrogen-induced proliferation has been shown to occur via upregulation of the serotonin signaling system (27, 137).

Of the other sex hormones and estrogen metabolites, DHEA has been studied and shown to inhibit cellular proliferation potentially through the Akt pathway (57). 16α-Hydroxyestrone, a metabolite of CYP1B1 activity, promotes pulmonary vascular remodeling (2, 138), whereas progesterone has been shown to attenuate hypertrophy in MCT rat model, although no mechanism for this reduction has been elucidated (125).

**Immunomodulation/inflammation.** It has been proposed that inflammation plays a pivotal role in the progression of experimental and clinical forms of PAH (30). When characterizing a large cohort of PH samples from the Pulmonary Hypertension Breakthrough Initiative, Stacher and others (120) found significant perivascular inflammation in numerous PAH lungs.

Estrogen has shown anti-inflammatory properties in a variety of tissues including the lung (119). In animal models of PH, estrogen significantly reduces pulmonary inflammation by limiting pulmonary monocyte/macrophage infiltration associated with PH, thereby alleviating PH (132). These estrogen-induced effects appear to be mediated via ER\(\beta\) (8, 32, 70). The anti-inflammatory effect of testosterone is mediated via suppression of proinflammatory cytokines TNF-\(\alpha\), IL-1, and IL-6, at least in vitro (52). Testosterone-induced increase in the anti-inflammatory cytokine IL-10 has been shown in macrophages and monocytes (62, 82) but not in pulmonary vasculature.

Age-Related Effects of Estrogen on the Pulmonary Vasculature

**Pediatric PH.** The estimated prevalence of PAH is 2–16 cases per million children (49). In the past, studies in adults with PH have been used to make assumptions regarding the disease in children, but this approach has been recognized as inadequate. A recent, comprehensive analysis of pediatric registries has outlined epidemiology, clinical practice, and outcome of PAH in children (11, 49). It appears important to recognize the fetal origins and developmental and adaptive processes of pulmonary vascular disease in children (see below), along with the role of congenital heart disease.

**Role of estrogens during the development of the pulmonary circulation.** Pathological events during the fetal period predispose to systemic cardiovascular disease later in life. However, there is little known regarding the involvement of the pulmonary circulation in these events (9). The pulmonary circulation is particularly vulnerable during the late fetal and perinatal period because of significant structural and functional changes that occur in preparation for the transition from gas exchange via the placenta to postnatal pulmonary gas exchange following rapid and sustained fall in pulmonary vascular resistance. Ventilation, increased oxygenation, shear stress, and changes in vasoactive factor expression (e.g., NO, prostacyclin, and vascular endothelial growth factor) all seem to play an important role in this normal transition (12, 71). In contrast, lack of change in vasoactive factors contributes to persistent PH in the newborn (24). Stimuli such as acetylcholine, bradykinin, ADP, and shear stress are dependent, at least in part, on NO release, thereby contributing to the decrease in pulmonary pressures during the perinatal period (80, 100). \(\alpha_2\)-Adrenoreceptor activation has also been shown to mediate pulmonary vasodilation in fetal life. This effect is partly related to NO production (80). In this regard, estrogens themselves play a role in the perinatal cardiopulmonary transition (18, 68). 17\(\beta\)-Estradiol stimulates NO production in ovine PAECs through nongenomic mechanisms that are calcium- and tyrosine kinase-mitogen-activated protein dependent. In addition, 17\(\beta\)-estradiol stimulates prostacyclin synthesis in ovine fetal PAECs. This effect is calcium dependent but tyrosine kinase-mitogen-activated protein kinase independent (114).

It is known that maternal factors affect the well-being of the fetus and newborn. For example, maternal preeclampsia and transient perinatal hypoxia predispose the newborn to increased hypoxic pulmonary vasoconstriction (58). Maternal undernutrition increases oxidative stress within the placenta, thereby affecting the circulatory system in humans and animal models (14, 103, 106). Whether altered estrogen effects on the pulmonary vasculature in the context of perinatal transition contribute to PH in the neonatal and pediatric population has not been examined. In animal models, there is an age-related effect of the NO system on the pulmonary vasculature promoting pulmonary vascular relaxation (134). In regard to the effects of estrogens on the pulmonary system, it has been demonstrated in ovine fetal PAECs that eNOS is upregulated through activation of PAEC ERs (68, 78). This upregulation may occur during late gestation to optimize the capacity for NO-mediated pulmonary vasodilation at birth. Parker et al. demonstrated in fetal lambs that a brief administration of estrogen (1, 10, and 100 \(\mu\)g) directly into the pulmonary artery did not affect baseline pulmonary hemodynamics. However, prolonged estrogen infusions (2–8 days) increased pulmonary blood flow (95). Administration of a NOS inhibitor reversed this finding, demonstrating the importance of estrogens as pulmonary vasodilators at birth and their effect via the NO pathway. On a different study, Parker et al. (94) used their fetal...
sheep model to induce PH by ligation of the ductus arteriosus and compared the changes within the pulmonary system with versus without estrogen infusion. The study demonstrated that chronic (8 days) estrogen treatment attenuated the pulmonary and histological changes seen after ductus arteriosus ligation. Overall, these preclinical studies suggest a potential beneficial role for estrogens in maintaining pulmonary vascular health in the perinatal period. The relevance of such effects (and greater examination of the underlying mechanisms) lies in exploration of whether and how estrogens may be beneficial in the prematurely born infant, or one with persistent newborn PH.

Role of estrogens during puberty and its implications in the pulmonary circulation. As mentioned before, adult females have a higher incidence of PAH. It has been reported in prepubertal children with PAH that the sex ratio is nearly even (49). However, the Tracking Outcomes and Practice in Pediatric Pulmonary Hypertension (TOPP) registry noted that the female preponderance was 1.4 to 1 in children with PAH age < 18 yr (11). Unfortunately, this study did not differentiate between prepubertal and postpubertal age.

In regard to sex differences in puberty, there is evidence that female airways and lung parenchyma grow proportionally, whereas in males airway growth lags behind growth of the lung (16). However, to our knowledge there is nothing known specifically regarding the effects of puberty on the development of the pulmonary circulation and estrogen-specific effects. This may be partly due to the fact that it is very difficult to enroll sufficient children with PH into clinical trials given ethical implications. In addition, pediatric PH is a rare disease with a complex pathophysiology that makes its investigation even more difficult. At least, the development of registries for children with pediatric PH will allow us in the future to improve our understanding of this complex disease in the pediatric population.

Genetic Predisposition and Estrogens: Sex Hormones

There is a well-documented genetic predisposition to pulmonary vascular disease (76). To date, mutations or variation in the following genes has been demonstrated to be associated with different group I PAH subsets.

\textbf{BMPR2 mutations.} BMPR2 is a member of the TGF-\(\beta\) family involved in cellular proliferation and differentiation. Downstream signaling of the BMPR2 occurs through multiple pathways including SMAD1/5/8, pERK, INK, and Akt/phosphatidylinositol 3-kinase (45, 124). Reduction of BMPR2 signaling as a result of mutations results in abnormal pathway activation, permitting excess smooth muscle cell growth and proliferation (88, 143). Genetic mutations in BMPR2 were the first to be associated with heritable forms of PAH (28, 67). Several mutations have been documented and appear to play an important role in the pathogenesis of group I PAH, accounting for \(\sim 25\%\) of patients with idiopathic PH (92). Recent investigations demonstrated a potential overlap between the BMPR2 signaling pathway and estrogens (2, 3). Austin et al. reported dysregulated CYP1B1 gene expression in B lymphocytes cultured from female PAH patients with a BMPR2 mutation (2). More recently, the same authors looked at ER and BMPR2 mutations and concluded that ER stimulation may decrease BMPR2 expression, thereby increasing the risk of developing PAH (3).

\textbf{Caveolin-1 mutations.} Caveolin-1 (Cav1) is a plasma membrane-associated scaffolding protein involved in a number of cellular processes including Ca\(^{2+}\) regulation, cell-cycle progression, and cell proliferation (23). Mutations within this gene have been described in familial and idiopathic PAH (4). In breast cancer, where Cav1 expression has been found to be altered, Cav1 mutations correlate with ER\(\alpha\) levels (73) and contribute to enhanced growth of breast epithelial cells. The interrelationship between Cav1 and ERs in PAH has not been addressed but may be an interesting direction to further elucidate the differential incidence of PAH in females versus males.

\textbf{SMAD9.} Gene array studies show a differential expression in the TGF-\(\beta\) signaling pathway between PAH patients and controls (101). Of the many proteins involved in TGF-\(\beta\) signaling, SMAD9 is an important intracellular signaling molecule downstream of the TGF-\(\beta\) receptor. Rare mutations of SMAD9 have been found in patients with idiopathic PAH (91). No studies to date have investigated the roles of sex hormones or receptors and SMAD9 in PAH. However, there is evidence for cross talk between the TGF-\(\beta\) and estrogen pathway via interaction of SMAD with the ER (85).

\textbf{K\(^+\) channel subfamily K member 3.} K\(^+\) channel subfamily K member 3 (KCNK3) is a recently discovered gene which encodes a novel potassium channel. Mutations in KCNK3 were identified in familial and idiopathic PAH (77) and can be altered by pharmacological manipulation. Given the already known interaction of estrogen and sex hormones on ion channels in pulmonary vascular smooth muscle, future studies may include interactions of this potassium channel with estrogens and estrogen signaling.

\textbf{Therapeutic options for PH: should there be a sex bias?} Patients with PAH have been shown to have increased endothelin levels, decreased NO levels and/or decreased prostacyclin levels (15, 42, 43, 131). Current therapies targeting PAH focus on inhibiting endothelin, replacing prostacyclin, and targeting NO pathways (133). Among the known therapies, newer studies suggest some sex differences in therapy efficacy and outcome.

\textbf{Endothelin receptor antagonists.} Endothelin-1 is a potent vasoconstrictor and smooth muscle mitogen. Endothelin receptor antagonism emerged as therapy for group I PAH since high concentrations of endothelin-1 were recorded in the lungs of patients with PAH as well as animal models of PH (37, 43). To our knowledge, there is currently one published study demonstrating that women with PAH have greater benefits from endothelin receptor antagonist therapy than men (39). The authors speculate that estrogen may play a role; however, no mechanistic insight is provided as to how this may occur. Nonetheless, given the efficacy of endothelin receptor antagonists in PH, a synergistic role for ER agonist (or antagonist depending on whether estrogens are beneficial or not) could be explored.

\textbf{Prostanoids.} Prostacyclin is a potent vasodilator that has antiproliferative and anti-inflammatory activities in patients with PH (21, 43, 89). None of the three available prostacyclin formulations, epoprostenol, treprostinil, and iloprost, has been studied for differential effects in males versus females.

\textbf{Phosphodiesterase-5 inhibitors.} Phosphodiesterases (PDEs) are a family of enzymes that hydrolyze cyclic nucleotides and are upregulated in the pulmonary vessels of PAH patients (36, 136). The isoenzyme PDE5 regulates cGMP-dependent NO
signaling, thus highlighting this enzyme in the treatment of PH (140). In PASMCS, NO binds to soluble guanylyl cyclase, which catalyzes the production of cGMP. cGMP induces relaxation of PASMCs, and its action is terminated by PDE5. Thus PDE5 inhibitors act by regulating the degradation of cGMP, thereby increasing cGMP availability, which promotes vasodilation. Food and Drug Administration-approved agents sildenafil, tadalafil, and vardenafil are orally administered PDE5 inhibitors used to treat PH (40, 41).

There is recent evidence in a mouse model of PH that sildenafil may be more effective in females compared with males (61). By measuring cGMP levels after PDE5 inhibition with sildenafil, the authors found increased cGMP levels only in female mice compared with male mice. Thus sildenafil may play a role in differential treatment efficacies between males and females (61). As with other limited studies, it is currently unknown whether estrogen signaling can interact with PDE5 to modulate the extent of PH.

Conclusions

The increased prevalence of PAH among women has persisted over decades and even worsened among some ethnic groups. This supports the continued need for research in the area of sex hormones and PAH. The vast majority of the studies geared toward understanding sex differences in the pulmonary circulation have focused on two main areas: 1) sex hormone signaling and synthesis in the pulmonary circulation and 2) influence of sex hormones on pathophysiological features of PAH.

Studies dedicated to understanding differences in the sex hormone signaling mechanisms within the pulmonary circulation have identified a protective role of ERβ. Newer studies on sex hormone synthesis implicate aromatase and CYP1B1 as modulators or predictors for disease. Estrogen has been the most widely investigated sex steroid on the pulmonary circulation, which is appropriate given its higher levels in females. While the current literature does not support a role for testosterone and progesterone in PAH prevalence, studies with estrogen metabolites such as 2ME and 16alpha-hydroxyestrone have shown promise in regard to protection and worsening disease, respectively. Although there is continued areas for further investigation, this focus on sex hormone signaling and synthesis in the pulmonary circulation has broadened our perspective on the potential functionality of sex steroids in PH biology beyond just estrogen. Continued focus on key estrogen-metabolizing enzymes aromatase and CYP1B1 in the pulmonary artery has the potential to identify novel targets to predict, diagnose, or treat PAH.

Studies addressing mechanisms by which sex hormones influence pathophysiological features of PAH have supported both protective and deleterious effect of sex steroids. The most detailed studies with sex steroids have been focused on NO-induced vasodilation, Ca2+-mediated vasoconstriction, and proliferation. As novel mechanisms of the pathophysiological features of PH are uncovered, evidence for the effects of sex steroids on those mechanisms will be germane to a better understanding of sex differences in PH.

The use of genomic technology has also contributed to the understanding of sex differences in PH. Genomic association studies have identified variants in the sex steroid pathway as well as other genes such as BMPR2, Cav1, and the novel potassium channel KCNK3. Interestingly, the cross talk between sex steroid signaling and genomic variants (such as ER stimulation and BMPR2 mutations) has established new mechanisms by which sex steroids play a role in PAH.

In conclusion, undoubtedly PAH is a devastating disease. Fortunately, the emphasis on sex hormone effects on the underlying pathological and pathophysiological features has contributed insight into potential mechanisms and opened the door for development of novel therapies.

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DISCLOSURES

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AUTHOR CONTRIBUTIONS

Y.N.M. and C.M.P. prepared figures; Y.N.M. and C.M.P. drafted manuscript; Y.N.M. and C.M.P. edited and revised manuscript; Y.N.M. and C.M.P. approved final version of manuscript.

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