Progress in solving the sex hormone paradox in pulmonary hypertension

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1Division of Pulmonary, Allergy, Critical Care, Occupational and Sleep Medicine, and Richard L. Roudebush VA Medical Center; Department of Medicine, Indiana University School of Medicine, Indianapolis, Indiana; and 2Program in Translational Lung Research, Division of Pulmonary Sciences and Critical Care Medicine, Department of Medicine, University of Colorado, School of Medicine, Denver, Colorado

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Lahm T, Tuder RM, Petrache I. Progress in solving the sex hormone paradox in pulmonary hypertension. Am J Physiol Lung Cell Mol Physiol 307: L7–L26, 2014. First published May 9, 2014; doi:10.1152/ajplung.00337.2013.—Pulmonary arterial hypertension (PAH) is a devastating and progressive disease with marked morbidity and mortality. Even though being female represents one of the most powerful risk factors for PAH, multiple questions about the underlying mechanisms remain, and two “estrogen paradoxes” in PAH exist. First, it is puzzling why estrogens have been found to be protective in various animal models of PAH, whereas PAH registries uniformly demonstrate a female susceptibility to the disease. Second, despite the pronounced tendency for the disease to develop in women, female PAH patients exhibit better survival than men. Recent mechanistic studies in classical and in novel animal models of PAH, as well as recent studies in PAH patients, have significantly advanced the field. In particular, it is now accepted that estrogen metabolism and receptor signaling, as well as estrogen interactions with key pathways in PAH development, appear to be potent disease modifiers. A better understanding of these interactions may lead to novel PAH therapies. It is the purpose of this review to 1) review sex hormone synthesis, metabolism, and receptor physiology; 2) assess the context in which sex hormones affect PAH pathogenesis; 3) provide a potential explanation for the observed estrogen paradoxes and gender differences in PAH; and 4) identify knowledge gaps and future research opportunities. Because the majority of published studies investigated 17β-estradiol and/or its metabolites, this review will primarily focus on pulmonary vascular and right ventricular effects of estrogens. Data for other sex hormones will be discussed very briefly.

PULMONARY HYPERTENSION (PH) encompasses a heterogeneous group of diseases that are characterized by elevated pulmonary artery pressures (147). The World Health Organization (WHO) differentiates five types of PH that differ in their etiologies and phenotypes (Table 1) (147). This review will focus on pulmonary arterial hypertension (PAH; WHO group 1 PH), a progressive and devastating disease of multifactorial etiology that results in significant hemodynamic alterations, severe pulmonary vascular remodeling, increased pulmonary vascular resistance (PVR), right ventricular (RV) failure, and death (129, 169, 170).

It has long been known that being female represents one of the most powerful risk factors for the development of PAH. However, only recently have we begun to better understand the relationship between sex, gender, sex hormones, and PAH. Despite an increase in publications and the intense scientific debate they generated, multiple questions remain, expressed by the recently coined concept of an “estrogen paradox” in PAH (23). In fact, this paradox is rooted on two main observations: First, there are differences between investigations in humans and data derived from animal studies. In the latter, estrogens have been found to be protective in various models of PAH, whereas PAH registries uniformly demonstrate a female susceptibility to the disease. The second observation is that, despite the pronounced tendency for the disease to develop in women, once affected by the disease female PAH patients exhibit better survival than men. The aggregate of animal and human studies investigating sex and gender differences and sex hormone effects in PAH has been reviewed recently in detail (6, 24, 87, 128, 157, 172). However, the rapidly evolving knowledge in this area and the overall progress in solving the estrogen puzzle necessitate a new assessment of the context in which sex hormones have a profound impact on PAH pathogenesis. For example, sex hormone effects may be beneficial or detrimental on the basis of the experimental system investigated. Furthermore, despite the awareness that RV function is a major determinant of survival in PAH (12, 66, 68, 173), existent literature includes reviews primarily focused on sex hormone effects on the pulmonary vasculature. The first objective of this review therefore is to dissect the currently available and rapidly advancing body of literature according to

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whether these studies suggest harm or benefit of sex hormones. Our second objective is to specifically discuss the effects of sex and sex hormones on RV function. The third objective is to interpret current study within the framework of resolving the sex paradoxes in PAH, identifying knowledge gaps needed to be addressed. According to the definitions published by the Institute of Medicine and embraced by the APS Journals (105, 188), we will use the term “sex” when biological concepts are described, but use the term “gender” when cultural or behavioral influences may play a role (e.g., in human studies). Given the paucity of data on sex hormones other than estrogens, we will primarily rely on published data on estrogens and their signaling. We refer the reader to Table 4 for an overview of the involvement of testosterone, progesterone, and dehydroepiandrosterone (DHEA) in PAH.

TABLE 1. WHO classification of PH (Nice, 2013)

<table>
<thead>
<tr>
<th>WHO Group</th>
<th>Disease Entities Included</th>
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<tbody>
<tr>
<td>1 — Pulmonary arterial hypertension (PAH)</td>
<td>Idiopathic PAH&lt;br&gt;Hereditary PAH&lt;br&gt;Drug and toxin induced PAH&lt;br&gt;PAH associated with&lt;br&gt;· Connective tissue disease&lt;br&gt;· HIV infection&lt;br&gt;· Portal hypertension&lt;br&gt;· Congenital heart disease&lt;br&gt;· Schistosomiasis</td>
</tr>
<tr>
<td>1’ — Pulmonary venoocclusive disease (PVOD) and pulmonary capillary hemangiomatosis (PCH)</td>
<td>Systolic heart failure&lt;br&gt;Diastolic heart failure&lt;br&gt;Valvular heart disease&lt;br&gt;Chronic obstructive and/or restrictive lung diseases&lt;br&gt;Sleep-disordered breathing&lt;br&gt;Chronic high altitude exposure</td>
</tr>
<tr>
<td>1” — Persistent PH of the newborn (PPHN)</td>
<td>Hematologic disorders&lt;br&gt;Systemic disorders&lt;br&gt;Metabolic disorders&lt;br&gt;Compression/obstruction of the pulmonary vasculature&lt;br&gt;Chronic renal failure</td>
</tr>
<tr>
<td>2 — PH due to left heart disease</td>
<td></td>
</tr>
<tr>
<td>3 — PH due to lung diseases and/or hypoxia</td>
<td></td>
</tr>
<tr>
<td>4 — Chronic thromboembolic PH (CTEPH)</td>
<td></td>
</tr>
<tr>
<td>5 — PH with unclear multifactorial mechanisms</td>
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</tbody>
</table>

Modified from Ref. 147.

PATHOGENESIS AND MANIFESTATIONS OF PAH

The hallmark of PAH is an abnormal and progressive hyperproliferative pulmonary vascular remodeling process that involves all cell types of the pulmonary artery (PA) wall (129, 169, 170). Initially thought to be a disease primarily characterized by vasoconstriction and abnormal shear stress, it is now established that the pathogenesis of PAH is much more complex and includes genetic abnormalities, epigenetic phenomena, altered microRNA function, enhanced extracellular matrix deposition and degradation, perivascular inflammation, mitochondrial dysfunction, and recruitment and activation of circulating and resident progenitor cells, leading to vascular obstruction with the formation of plexiform and occlusive lesions (129, 169, 170). These alterations work in concert to generate a profound pulmonary vascular remodeling process...
that shares similarities with malignant cell growth (129, 169, 170). Although it is generally accepted that vasoconstriction is important in the early stages of the disease, the major factor responsible for the high PVR in severe, established PAH is the formation of occlusive neointimal and plexiform lesions in small, peripheral PAs (129, 169, 170). Ultimately, the increase in PVR results in maladaptive RV remodeling and RV failure, characterized by systolic and diastolic dysfunction, alterations in mitochondrial bioenergetics, ischemia, inflammation, oxidative stress, proapoptotic signaling, and cardiomyocyte death (14, 54, 178). Consequently, the majority of deaths in PAH are due to RV failure (164).

THE EPIDEMIOLOGY AND CLINICAL FRAMEWORK OF SEX DIFFERENCES IN PAH

With the growing interest in PAH, multiple registries have been created over the last decades. Although these registries exhibit differences in inclusion criteria, enrollment periods, and data acquisition methods, and although they do not differentiate between sex and gender, they uniformly describe a predominance of female patients among the prevalent cases of PAH. For example, the NIH registry reported a 1.7:1 female-to-male ratio (133). More recent registries reported even more pronounced sex differences, with female-to-male ratios ranging from 1.4:1 to 4.1:1 (Table 2) (8, 38, 66, 67, 74, 96, 118, 125). The reason(s) for the marked 4.1:1 female predominance in the Registry to Evaluate Early and Long-term PAH Disease Management (REVEAL) (8) is/are unclear but may be based on different inclusion criteria, lifestyle factors, and/or drug exposure histories. Furthermore, cultural and gender differences may affect disease presentation and treatment and thus contribute to the observed differences in prevalence between registries.

On the other hand, data from these registries demonstrate that female PAH patients consistently have better survival than men. For example, in the French registry, the hazard ratio of death for women was 0.375 compared with men (66, 68). Similarly, male gender was a risk factor for death in the REVEAL registry, men older than 60 yr were at increased risk of dying (12). Although the exact reason for the female survival advantage is unclear, recent evidence suggests that women with PAH exhibit a more favorable hemodynamic profile (higher cardiac index and lower right atrial pressure, mean PA pressure, and PVR) (140, 177), better RV function (71, 81), and a more favorable time course for survival compared to men. For example, in the French registry, the hazard ratio of death for women was 0.375 compared with men (66, 68).

The gender differences in PAH are likely mediated at least in part by biologically relevant effects of sex hormones, evidenced by studies demonstrating a high prevalence of exposure to hormone replacement therapy (HRT) in women with PAH (154); genetic alterations in estrogen metabolism enzymes and estrogen receptors (ERs) in various forms of PAH (4, 131, 134); correlations of estrogen levels with RV function in healthy postmenopausal HRT users (176); and, finally, absence of hemodynamic differences between male and female PAH patients who are older than 45 yr (177). In addition, menopause represents a risk factor for PAH development in scleroderma patients, whereas HRT may attenuate this risk (13, 138). These data suggest that to understand sex and gender differences in incidence and clinical severity of PAH, it is critical to address the effects of sex hormones on the pulmonary vasculature and RV, in both health and disease.

NORMAL SEX HORMONE SYNTHESIS, METABOLISM, AND RECEPTOR PHYSIOLOGY

Overview of Sex Hormone Synthesis

A detailed description of sex hormone production, recently published elsewhere (124, 157), is beyond the scope of this review. In brief, conversion of cholesterol to pregnenolone represents the first step in sex hormone synthesis (Fig. 1). Downstream of pregnenolone, DHEA (a precursor for both male and female sex hormones) is further converted to testosterone (T) and estrone (E1). Conversion of testosterone to estradiol (E2) and dihydrotestosterone (DHT) is catalyzed by aromatase (CYP19A1). E1 and E2 are responsible for the high PVR in severe, established PAH. Indeed, recent studies have demonstrated that sex differences in the response of RV ejection fraction (RVEF) to initiation of medical therapy in idiopathic PAH (IPAH) explain a substantial portion of the worse survival seen in male IPAH patients (71). The molecular underpinnings of sex differences in RV performance will therefore be reviewed in detail later in this review.

Table 2. Female-to-male ratios in major PAH registries

<table>
<thead>
<tr>
<th>Registry</th>
<th>Reference</th>
<th>Cohort</th>
<th>Time Period</th>
<th>Patients Included</th>
<th>Female-To-Male Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>European (COMPERA)</td>
<td>118</td>
<td>IPAH, HPAH, APAH; mean age 68 yr</td>
<td>2007–2013</td>
<td>1283</td>
<td>1.8:1</td>
</tr>
<tr>
<td>UK/IRELAND</td>
<td>96</td>
<td>IPAH, HPAH, anorexigen-induced PAH; mean age 50 yr</td>
<td>2001–2009</td>
<td>493</td>
<td>1.4:1</td>
</tr>
<tr>
<td>Spanish</td>
<td>38</td>
<td>PAH, CTEPH; mean age 45 yr</td>
<td>2007–2008</td>
<td>866</td>
<td>2.4:1</td>
</tr>
<tr>
<td>REVEAL</td>
<td>8</td>
<td>IPAH, HPAH, APAH, anorexigen-induced PAH; mean age 53 yr</td>
<td>2006–2007</td>
<td>2525</td>
<td>4.1:1 APAH</td>
</tr>
<tr>
<td>Chinese</td>
<td>74</td>
<td>IPAH and HPAH; mean age 36 yr</td>
<td>1999–2004</td>
<td>72</td>
<td>2.4:1</td>
</tr>
<tr>
<td>French</td>
<td>67</td>
<td>IPAH, HPAH, APAH, anorexigen-induced PAH; mean age 50 yr</td>
<td>2002–2003</td>
<td>674</td>
<td>1.9:1</td>
</tr>
<tr>
<td>Scottish</td>
<td>125</td>
<td>IPAH, APAH; mean age 51 yr</td>
<td>1986–2001</td>
<td>374</td>
<td>2.3:1</td>
</tr>
<tr>
<td>NIH</td>
<td>133</td>
<td>“Primary PH” (IPAH and HPAH); mean age 36 yr</td>
<td>1981–1985</td>
<td>187</td>
<td>1.7:1</td>
</tr>
</tbody>
</table>

APAH, associated PAH; HPAH, hereditary PAH; IPAH, idiopathic PAH; NIH, National Institutes of Health.
Verted to each other via 17-ketoreductase (17β-hydroxysteroid dehydrogenase; 17β-HSD). In addition to production in the ovaries of women of reproductive age, E1 and E2 are also produced peripherally in classical (breast, uterus) and nonclassical estrogen target tissues [brain, skin, adipocytes, cardiac fibroblasts, vascular endothelial cells (ECs) and smooth muscle cells (SMCs)], all of which contain high amounts of aromatase and 17β-HSD (in both women and men) (58, 120). In addition, significant amounts of peripheral E2 may be produced in pathological conditions (e.g., breast cancer, endometriosis). The third major estrogen, estradiol (E3), is only produced during pregnancy.

**Estrogen Metabolites**

Although both female and male sex hormones may modulate PAH, an important concept is that several metabolites of sex hormones are biologically active and may thus be important modifiers of disease development (Fig. 1). In particular, there is a high activity of the cytochrome P-450 (CYP450) system in the lung (121). CYP1A1 and CYP1B1 oxidize estrogens at the C2, C4, or C16 position to produce 2-, 4-, or 16α-hydroxyestrogens (116, 168, 194). The 2- and 4-hydroxyestrogens are characterized by a short half-life and are quickly metabolized by catechol-O-methyl-transferase (COMT), with 2-methoxyestradiol (2-ME2) being the most relevant metabolite. 2-ME2 is characterized by ER-independent antiproliferative, proapoptotic, antiangiogenic, and anti-inflammatory effects (31, 116, 121, 168, 194). In contrast, 16α-hydroxy-estrogens such as 16α-hydroxy-estrone (16α-OHE1) and 16α-hydroxy-estradiol, as well as the 4-hydroxy-estrogens and their metabolites, promote proliferative and proinflammatory processes (31, 116, 121, 168, 194). In the case of 16α-hydroxy-estrogens, these effects are mediated at least in part by constitutive activation of the ER (45, 153). The 16α-hydroxy-estrogens seem to have a slightly greater affinity for ERβ over ERα (200). In addition to being more mitogenic, 16α-hydroxy-estrogens may also be genotoxic owing to induction of DNA damage (16, 135). Extrapolating data from breast and prostate cancer, it is tempting to speculate that individuals who produce more “16-estrogens” are at increased risk for developing diseases characterized by hyperproliferation and inflammation (11, 36, 76, 106, 113, 114, 153), whereas patients who produce more hydroxy- and methoxyestrogens may be protected (77, 103). This notion has led to a series of studies of estrogen metabolism in PAH that will be discussed later in this review.

**Estrogen Receptors**

ERs are members of the nuclear receptor superfamily and are major mediators of estrogenic effects. Two major ERs have been described: ERα and ERβ (reviewed in Ref. 59, 101, 102, 112, 146). In addition to these two classical ERs, an orphan G protein-coupled receptor (GPR30) can also bind estrogen and primarily mediate acute (nongenomic) estrogen effects (65) (Fig. 2A). Although ERα and ERβ exhibit structural homologies in their DNA- and ligand-binding domains, significant differences exist in their transcriptional control domains that interact with regulatory binding proteins. Both ERs were initially identified in the cytoplasm and nucleus; however, recent

![Fig. 1. Simplified overview of sex hormone synthesis and estrogen metabolism. 2-Hydroxyestradiol (2-OHE2) and 2-methoxyestradiol (2-ME2) exert estrogen activity in PAH.](http://ajplung.physiology.org/)
studies identified ERα and ERβ presence in the cell membrane, where they are involved in nongenomic estrogen signaling (145). ERs are expressed in multiple organ systems (e.g., reproductive, cardiovascular, respiratory, central nervous, immune, and skeletal systems); however, their function may differ on the basis of the ratio of ER subtypes, presence of coactivators and corepressors, cellular context, sequences of target genes, and cross talk with other transcription factors, phosphatases, and kinases (59). At a broader level, ER expression and activity are affected by multiple factors, e.g., sex, age, variations in endogenous sex hormone levels (e.g., menstrual cycle and menopause), and various disease states (reviewed in Ref. 112). A summary is provided in Fig. 2B. Additional layer of complexity is added by the presence of several truncated ER isoforms that are generated via alternative mRNA splicing (59). Even though these ER variants lack the functional domains found in wild-type ER, they may be biologically active and act as important regulators of wild-type ER expression and function. For example, alterations in quantity, tissue distribution, and function of ER splice variants have been implicated in the pathogenesis of cancer, autoimmune disease, and asthma (62).

**ER Signaling**

Two major ER signaling pathways are relevant to the pulmonary vascular system (reviewed in Refs. 59, 112): In the genomic pathway, E2 diffuses through the cell membrane, interacts with cytoplasmatic ERα or ERβ, dimerizes with another estrogen-ER complex, translocates to the nucleus, and binds to an estrogen responsive element (ERE), thus acting as a classical transcription factor, whose function is modified by coactivators and corepressors. Modifications of this process occur when the estrogen-ER-complex dimer, instead of di-

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**Fig. 2.** ER types, signaling, regulation, and localization in the pulmonary vasculature. The 2 major ER subtypes (ERα and ERβ), their splice variants, and G protein-coupled receptor (GPR) 30 are depicted in A. Three functional ERα and 5 ERβ variants have been demonstrated in humans. ERs mediate their effects through both genomic and nongenomic signaling pathways (the latter is also employed by GPR30). Factors affecting ER expression and activity are listed in B. Stimulators of ER expression/activity are marked in green; inhibitors are marked in red. Factors in black may either increase or decrease ER expression. The histology slide in C demonstrates positive staining for ERβ in a pulmonary artery of a patient with idiopathic pulmonary arterial hypertension (PAH). Note abundant ERβ staining in endothelial cells (arrow), smooth muscle cells (arrowhead), and perivascular and inflammatory cells (asterisk). Hematoxylin and eosin (H&E) counterstaining was performed. Size bar = 50 µm. Unstained slide provided by Drs. Serpil Erzurum and Suzy Comhair.
rectly binding to ERs, affects gene regulation more indirectly through binding to other transcription factors, or when nonestrogen ligands [e.g., epidermal growth factor (22)] trigger ER activation, dimerization, and nuclear translocation in the absence of E2 binding. In the nongenomic pathway, membrane-bound ERα, ERβ, or GPR30 can, upon E2 binding, directly activate kinases or second messengers, thus initiating rapid cellular effects [e.g., opening of ion channels, activation of endothelial nitric oxide synthase (eNOS) or prostacyclin synthase] without directly interacting with the genome (145). Nongenomic effects occur within seconds to minutes and play a prominent role in the cardiovascular and respiratory systems [e.g., in cellular responses to acute insults (7, 20, 21, 63, 141, 144, 152, 195)]. Activation of MAP kinases and other second messengers plays a central role in mediating nongenomic ER effects (141). Ultimately, however, nongenomic estrogen signaling may also culminate in changes in gene expression (59, 112). ER splice variants, although frequently lacking direct transcriptional activity, can act as dominant-negative inhibitors of other ERs and thus indirectly affect gene expression (62). ER isoforms may also activate nongenomic signaling pathways (62).

Having reviewed the role of normal sex hormone synthesis, metabolism, and receptor signaling, the remainder of this review will focus on the role of sex steroids in PAH.

SEX DIFFERENCES IN ANIMAL MODELS OF PAH

Overview of PAH Animal Models

To put the results obtained from various animal models of PAH into appropriate context, it is imperative to understand the strengths and limitations of these models. A detailed discussion is beyond the scope of this review and is provided in Refs. 150 and 50. In brief, the chronic hypoxia model and the monocrotaline model of PAH, although widely used, are characterized predominantly by media hypertrophy but lack the vasoocclusive and plexiform lesions that are the hallmark of human PAH. In addition, at least in the case of hypoxia-induced PH (HPH), the pulmonary vasculopathy is reversible upon reexposure to room air (150). Monocrotaline-induced PH (MCT-PH) is characterized by a prominent systemic inflammatory component (150). RV hypertrophy and remodeling occur in both models; however, frank RV failure and death typically are only seen in MCT-PH (150). To overcome the limitation of these "classical" PAH models, novel PAH models were recently developed that more closely replicate the human phenotype. As such, the sugen/hypoxia model of PAH (SuHx-PH) ensues the administration of the VEGF receptor 2 antagonist Su5416 (sugen), followed by exposure to chronic hypoxia (3–4 wk) and subsequent reexposure to room air (156). SuHx-PH is characterized by severe pulmonary vascular remodeling with vasoocclusion, RV failure, and death (15, 156). If the reexposure to room air is extended to 10–11 wk, plexiform lesions may be seen as well (1). Although these three models can be employed in rats and mice, the murine phenotype is usually less severe (50). Similarly, administration of the anorexigen dexfenfluramine to mice induces a mild-to-moderate PH phenotype (25). Transgenic mouse models of PAH, although excellent tools to study specific pathways or mediators, frequently are limited by relatively mild hemodynamic alterations and less robust pulmonary vascular remodeling (10, 50). Although mutation, deletion, or overexpression of a single gene [e.g., S100A4/Mts1 or bone morphogenetic protein 2 (BMPR2)] may lead to PH in absence of further modifications, other transgenic mouse models [e.g., serotonin transporter overexpression (SERT+] +) frequently are combined with a second hit (e.g., chronic hypoxia) to provoke a robust PH phenotype (26, 50, 185).

Sex Differences in Classical PAH Models

Early observational studies noting sex differences in classic animal models of PAH were performed in swine and rats and demonstrated that females exhibit less frequent and severe HPH than males (100, 130). Such findings were corroborated in hypoxic chicken and sheep, where female animals develop less hypoxic pulmonary vasoconstriction (HPV) (18, 183, 184). Subsequent studies identified estrogens as protective factors on pulmonary vascular tone. For example, HPV is attenuated in pregnancy (48, 107), and later studies demonstrated protective effects of both endogenous and exogenous estrogens on HPV and HPH (34, 86, 88, 132, 184). In particular, ovariectomized rats develop more severe HPH compared with intact females (34, 132), E2 supplementation attenuates HPH in ovariectomized females (34, 132), and isolated pulmonary arteries from female rats in the proestrus phase of the estrous cycle (characterized by high endogenous estrogen levels) exhibit less HPV than vessels from estrus or diestrus females or males (88). E2 and female sex were also found to be protective in MCT-PH (42, 161, 171). One limitation of the studies reporting sex differences in this model, however, is that the attenuated PH phenotype in females may be due at least in part to decreased bioactivation of MCT by CYP450 3A in the liver (80).

Sex Differences in SuHx-PH

No published comparisons between males and females exist in SuHx-PH. A recent study in female SuHx rats reported hemodynamic alterations that were less pronounced than what other studies reported in males (136); our own observations, however, indicated equal or even more severe pulmonary vascular disease, including higher PA pressures, in female vs. male Sprague-Dawley rats (T. Lahm, R. M. Tuder, unpublished observations). In SuHx-PH, more data exist for DHEA, which was shown to attenuate PH end points and RV dysfunction in male rats (2). Sex Differences in Transgenic and Anorexigen-Induced Mouse Models

Although the observational studies in classical models of PAH demonstrate favorable effects of female sex and/or estrogens on pulmonary vascular tone and on hypoxia- or MCT-induced PA remodeling, studies in transgenic PAH models reveal more complex results (Table 3). As mentioned above, like hypoxia and MCT models of PAH, some of these models are limited by incomplete recapitulation of the human phenotype (10, 50, 150). Keeping these limitations in mind, it is important to note that several models are characterized by female susceptibility to the disease (thus mimicking the female predisposition to PAH observed in humans), whereas others demonstrate female sex to be a protective factor. For example, in normoxic and hypoxic SERT+ + mice (185, 187), in S100A4/Mts1-overexpressing mice (26), and in dexfenfluramine-induced PH (25), female animals are more prone to developing...
CONTROVERSIES IN MECHANISTIC STUDIES OF ESTROGENS IN PAH

The data reviewed thus far strongly implicate sex and sex hormones in the modulation of the risk and pathogenesis of PAH but also indicate a complex interplay, the details of which have not been fully elucidated. However, many of the pieces of puzzle are being put into place, through recent patient-based studies and through preclinical mechanistic investigations (Tables 4 and 5). We will review recent studies that detail downstream effects of E1 and E2 in general, through active metabolites, and through ER-mediated signaling pathways. Each section will be separated into studies showing protective effects of estrogens in PAH and studies suggesting that estrogens contribute to disease pathogenesis. In contrast to E1 and E2, the role of E3 in PAH pathogenesis is poorly investigated.

Do Endogenous and Exogenous Estrogens Alleviate or Worsen PAH?

Although several studies primarily focus on estrogen metabolites and/or ER signaling (reviewed under Are Estrogen Metabolites Helpful or Harmful in PAH? and Do Estrogen Receptors Exert Adaptive or Detrimental Effects in PAH?), multiple studies exist that investigated sex hormone effects in PAH without specifically investigating the involvement of estrogen metabolism or ER signaling. Several of these studies (performed in classical PAH models) demonstrated beneficial effects of E2; others (performed in transgenic mouse models), however, implied E2 as a disease mediator.

E2-mediated protection. Studies by our group identified both endogenous (88) as well as exogenous (85, 86) estrogens as inhibitors of HPV and pharmacologically induced vasoconstriction in isolated pulmonary arteries. We further demonstrated that E2 attenuates acute HPV and drug-induced vasoconstriction by a rapid, nongenomic mechanism (86). Protective E2 effects were also demonstrated in the setting of chronic hypoxia exposure, where E2 attenuated hemodynamic alterations, RV hypertrophy, and pulmonary vascular remodeling (34, 84, 132, 192). The mechanisms underlying these effects include inhibition of hypoxia-induced activation of ET-1 (purportedly by competing with HIF-1α for its coactivator CBP/p300) (34), attenuation of hypoxic induction of erythropoietin (111), enhancement of hypoxia-induced proproliferative ERK1/2 (84), Akt and Skp-2 activation (192), as well as stabilization of the cell cycle inhibitor p27Kip1 (84, 192) and autophagy marker LC3-B (84). Interestingly, on the basis of the data from Resta and colleagues (111, 132), the major mechanism of action of E2 in PH does not appear to involve increased eNOS expression. Beneficial E2 effects in PH were noted when E2 was given to males as well as ovariectomized females (34, 84, 111, 132, 192).

E2 also attenuates PH end points in the MCT model (42, 196). Here, the compound was shown to suppress pulmonary artery smooth muscle cell (PASMC) proliferation and macrophage infiltration and to enhance apoptosis in females; these processes were accompanied by increases in NO and prosta-cyclin concentrations, reductions in ET-1 levels, and attenuation of PI3K and Akt phosphorylation (196). When the hormonal milieu was investigated, an “estrogen-deficient state” was noted, characterized by decreased E2 plasma levels and decreased lung aromatase and ERα (196).

E2-mediated promotogenic signaling. Studies of transgenic mice, however, reported opposite E2 effects compared with what was seen in rats. In particular, E2 was identified as a disease mediator in normoxic or hypoxic SERT+ mice (185) as well as in mice overexpressing S100A4/Mts1 (26), where female animals are more prone to PH than males. E2 involvement was demonstrated by ovariectomy studies as well as by experiments in cultured human PASMCs, where E2 stimulated proliferation and upregulated tryptophan hydroxylase (TPH)-1, serotonin (5-HT) receptor 1B, and SERT [all of which have been implicated in PAH pathogenesis (35, 108, 109, 185)]. Proproporative E2 effects were inhibited by both a TPH inhibitor as well as a 5-HT(1B) receptor antagonist (185). Similarly, E2 increased Mts1 expression in human PASMCs, and E2’s mitogenic effects in these cells were attenuated by soluble RAGE (an antagonist of the membrane-bound Mts1 receptor RAGE) (26). Microarray analysis of lungs from SERT+ mice revealed a unique expression pattern in hypoxic female SERT+ mice only that included increased expression of the mitogenic transcription factors CEBPβ and FOS, as well as upregulated CYP1B1 (187). E2 increased expression of these three mediators in human PASMCs (187).

Are Estrogen Metabolites Helpful or Harmful in PAH?

“Good” and “bad” estrogen metabolites. Because of their distinct effects on proliferation, apoptosis, angiogenesis and

Table 3. Overview of sex differences in animal models of PH

<table>
<thead>
<tr>
<th>Species</th>
<th>Female Sex Protective Reference</th>
<th>Female Sex Permissive Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat</td>
<td>Monocrotaline-induced PH 161, 171, 196</td>
<td>n/a</td>
</tr>
<tr>
<td>Rat</td>
<td>Hypoxia-induced PH/HPV 34, 84, 130, 132</td>
<td>n/a</td>
</tr>
<tr>
<td>Swine</td>
<td>Hypoxia-induced PH 100</td>
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<td>Chicken</td>
<td>Hypoxia-induced PH 18</td>
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<td>Mouse</td>
<td>eNOS null 104</td>
<td>SERT+ 185, 187</td>
</tr>
<tr>
<td>Mouse</td>
<td>VIP null 137</td>
<td>S100A4/Mts1+ 26</td>
</tr>
<tr>
<td>Mouse</td>
<td>ApoE null + high-fat diet 57</td>
<td>Dexfenfluramine-induced PH 25</td>
</tr>
</tbody>
</table>

ApoE, apolipoprotein E; eNOS, endothelial nitric oxide synthase; HPV, hypoxic pulmonary vasoconstriction; n/a, no studies available; SERT+, serotonin transporter overexpression; S100A4/Mts1+, S100A4/Mts1 overexpression; VIP, vasoactive intestinal peptide.

disease whereas ovariectomy (at 8–10 wk of age) attenuates PH, implicating E2 as a disease mediator. On the other hand, in mice lacking VIP (137), eNOS (104), or apoE and fed a high-fat diet (57), female animals develop less severe PH than their male counterparts. These data demonstrate that an estrogen paradox is also present in animal models. The described sex differences could therefore be exploited to further understand the mechanisms of this paradox.

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inflammation, and because of their known roles in systemic vascular disease, breast cancer, and prostate cancer (29, 31, 116, 121, 158, 168, 194, 197, 198), there has been significant interest in the role of estrogen metabolites in PAH (Fig. 1). The antiproliferative and proapoptotic properties of hydroxy- and methoxyestradiols would suggest a beneficial effect in the hyperproliferative and proinflammatory milieu of PAH. In fact, administration of 2-ME2 is currently being evaluated in clinical studies as an antiproliferative strategy in various types of cancer (110). On the other hand, 16α-OHE1’s proliferative,

### Table 4. Summary of key findings from published animal studies investigating sex hormones and/or sex differences in PH models

<table>
<thead>
<tr>
<th>Model</th>
<th>Species</th>
<th>Major Findings</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isolated lungs or isolated pulmonary arteries (FAs)</td>
<td>Rat, sheep</td>
<td>Female sex, high estrogen states (pregnancy, proestrus) or exogenous E2</td>
<td>48, 85, 86, 88, 132, 191</td>
</tr>
<tr>
<td></td>
<td>HPV and/or drug-induced PA vasoconstriction</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>Testosterone, progesterone, DHEA</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>↑ PA vasoconstriction</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypoxia-induced PH (HPH)</td>
<td>Rat, chicken, swine, mouse</td>
<td>Females protected; OVX</td>
<td>37, 43, 75, 90, 117, 148</td>
</tr>
<tr>
<td></td>
<td>↑ PH, E2 replacement in OVX</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>↓ PH in male rats</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>E2</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>↓ ET-1, ERK1/2, Akt, Skp2</td>
<td></td>
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<tr>
<td></td>
<td>E2</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>↑ p27Kip1, LC3B</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>ESRα and ESRβ</td>
<td>pro-proliferative signaling</td>
<td>84</td>
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<tr>
<td></td>
<td>ERα-mediated anti-proliferative E2 effects on PAECs</td>
<td></td>
<td>84</td>
</tr>
<tr>
<td></td>
<td>↓ proliferation in hypoxic PASMCs from proestrus rats</td>
<td></td>
<td>191</td>
</tr>
<tr>
<td></td>
<td>CYP1B1</td>
<td>↑ in male and female mice; inhibition protective; knock-out</td>
<td>186</td>
</tr>
<tr>
<td></td>
<td>↓ PH and PA remodeling in male mice only</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>16α-OHE1</td>
<td>↑ in HPH; treatment of HPH mice with 16α-OHE1</td>
<td>186</td>
</tr>
<tr>
<td></td>
<td>↓ PH</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>DHEA</td>
<td>protective; ↑ calcium-activated and voltage-gated potassium channels and sGC</td>
<td>17, 56</td>
</tr>
<tr>
<td></td>
<td>DHEA</td>
<td>↓ PH &amp; RV dysfunction and ↑ PGC1α, eNOS, CREB, sGC</td>
<td>32, 33, 117</td>
</tr>
<tr>
<td>Monocrotaline-induced PH (MCT-PH)</td>
<td>Rat</td>
<td>Females protected; OVX</td>
<td>161, 171, 196</td>
</tr>
<tr>
<td></td>
<td>↑ PH, 2ME2 replacement in OVX</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>↓ PH</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>E2 metabolites (2OHE2, 2ME2, 2EE2)</td>
<td>protective</td>
<td>159–161, 163</td>
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<tr>
<td></td>
<td>E2</td>
<td>pro-angiogenic and anti-inflammatory</td>
<td>171</td>
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<tr>
<td></td>
<td>ERβ-mediated protection</td>
<td>171</td>
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<td></td>
<td>MCT-PH “estrogen-deficient” state (↓ lung aromatase, lung ERα, plasma E2, ↑ CYP1B1)</td>
<td>196</td>
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<tr>
<td></td>
<td>E2</td>
<td>↓ ET-1, ↑ NO, ↑ PGI2</td>
<td>196</td>
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<tr>
<td></td>
<td>DHEA</td>
<td>protective; ↓ PM-NFATc2-survivin axis, ↑ BMPR2 and miR-204; ↑ PASMC apoptosis</td>
<td>123</td>
</tr>
<tr>
<td>MCT + PNX</td>
<td>Rat</td>
<td>DHEA</td>
<td>64</td>
</tr>
<tr>
<td>Sugen/hypoxia-induced PH</td>
<td>Rat, mouse</td>
<td>Only mild hemodynamic alterations in female rats</td>
<td>136</td>
</tr>
<tr>
<td>Serotonin transporter overexpression</td>
<td>Mouse</td>
<td>CYP1B1</td>
<td>↑ in male and female mice; CYP1B1 inhibition</td>
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<td></td>
<td>↓ PH</td>
<td></td>
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<tr>
<td></td>
<td>DHEA</td>
<td>↓ RV dysfunction (anti-oxidant and-anti-apoptotic effects via ↓ NADPH, RhoK, STAT3, NFATc3)</td>
<td>2</td>
</tr>
<tr>
<td>SERT+</td>
<td>Mouse</td>
<td>Testosterone</td>
<td>↑ RV function and ↓ RV remodeling</td>
</tr>
<tr>
<td></td>
<td>Female rats develop ↑ PA pressure at normoxia and ↓ PH during hypoxia exposure; OVX protective; E2 detrimental</td>
<td>185</td>
<td></td>
</tr>
<tr>
<td></td>
<td>E2</td>
<td>↑ proliferation and Tph-1, 5-HT receptor 1B, and SERT+ in human PASMCs</td>
<td>185</td>
</tr>
<tr>
<td></td>
<td>CEBPβ, FOS, CYP1B1</td>
<td>↑ in female hypoxic SERT+ mice; E2 ↑ these factors in human PASMCs</td>
<td>187</td>
</tr>
<tr>
<td>S100A4/Mts1 overexpression</td>
<td>Mouse</td>
<td>Female rats more susceptible to PH development; OVX</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td>protective</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>E2</td>
<td>↑ Mts1 in human PASMCs</td>
<td>26</td>
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<td></td>
<td>E2-induced PASMC proliferation ↓ after RAGE inhibition</td>
<td>26</td>
<td></td>
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<tr>
<td></td>
<td>Only female rats develop PH; OVX protective</td>
<td>25</td>
<td></td>
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<tr>
<td></td>
<td>CYP1B1</td>
<td>necessary for PAH development in Dfen-treated mice</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>Dfen and E2</td>
<td>↑ CYP1B1 and Tph1 expression in cultured PAH-PASMCs</td>
<td>25</td>
</tr>
<tr>
<td>BMPR2 mutation-induced PH</td>
<td>Mouse</td>
<td>16α-OHE1</td>
<td>↑ disease penetration and ↑ RV dysfunction</td>
</tr>
<tr>
<td></td>
<td>16α-OHE1</td>
<td>↓ BMPR signaling in control mice but not in BMPR2 mutants</td>
<td>44</td>
</tr>
<tr>
<td></td>
<td>16α-OHE1</td>
<td>↓ cytokine expression but ↑ alterations in genes related to platelet function, angiogenesis, Wnt pathway, and energy metabolism</td>
<td>44</td>
</tr>
<tr>
<td></td>
<td>Lack of protective effect of 2-ME2</td>
<td>44</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Altered intracellular localization of ERα in BMPR2 mutant pulmonary microvascular endothelial cells (associated with insensitivity to activation by E2)</td>
<td>44</td>
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</tbody>
</table>

CYP1B1, cytochrome P-450 1B1; DHEA, dehydrieproandrosterone; E2, 17β-estradiol; ER, estrogen receptor; ET-1, endothelin-1; HPV, hypoxic pulmonary vasoconstriction; OVX, ovariecomy; PASMC, pulmonary artery smooth muscle cell; PNX, pneumonecomy; RV, right ventricular; 2EE2, 2-ethoxyestradiol; 2OHE2, 2-hydroxyestradiol; 2ME2, 2-methoxyestradiol; 16α-OHE1, 16α-hydroxyestrone. ↑ indicates increase/worsening; ↓ indicates decrease/attenuation.
antiapoptotic, proinflammatory, and DNA-damaging properties would be expected to promote disease development and worsen PAH. This has been evaluated by several investigators (reviewed in the following sections).

**Studies suggesting salutary effects of estrogen metabolites in PAH.** Several preclinical studies by Tofovic et al. demonstrated protective effects of hydroxy- or methoxyestradiols or their derivates in experimental PAH. This was first demonstrated in male rats with MCT-PH, where both 2-hydroxyestradiol (2-OHE2) and 2-ME2 attenuate RV and PA remodeling as well as inflammatory responses (160). Furthermore, in MCT-PH as well as in bleomycin-induced pulmonary fibrosis with concomitant PH, ovariectomy exacerbates the disease, whereas treatment of ovariectomized PH animals with 2-ME2 attenuates RV hypertrophy, pulmonary vascular remodeling, lung inflammation, and fibrosis (161, 162). 2-ME2 synergizes with sildenafl and bosentan in attenuating PA remodeling and inflammatory changes in male rats with MCT-PH (159). Similar effects were observed with the synthetic 2-ME2 analog 2-ethoxyestradiol (163). These functional effects of 2-ME2 are relevant, since the compound decreased mortality in several studies (159, 161, 162). On a molecular level, compared with its parent compound E2, 2-ME2 exerts stronger inhibitory effects on ET-1 secretion and MAPK activity in vascular ECs, as well as stronger antimitotic effects on ECs (28, 30, 162). In the systemic vasculature, inhibition of vascular remodeling by 2-ME2 is associated with downregulation of Akt and ERK1/2 phosphorylation and upregulation of cyclooxygenase-2 and the cell cycle inhibitor p27 (9). The enzymes responsible for conversion of E2 to hydroxy- and methoxyestradiol (CYP1B1, CYP1B1, and COMT, respectively) are present in ECs and SMCs, and several studies indicate that protective E2 effects are mediated by conversion to these metabolites (28, 197, 198).

Interestingly, this conversion process may be altered in pathological conditions, and various disease states may lead to decreased production of hydroxy- and methoxyestradiols. For example, hypoxia and inflammation decrease the activity of CYP1B1, resulting in decreased 2-hydroxylation and decreased conversion of E2 to 2-OHE2 and 2-ME2 (47, 155). In addition, decreased COMT activity and decreased 2-ME2 production have been implicated in the pathogenesis of pre-eclampsia (77). On the other hand, induction of oxidative CYP450 metabolism with increased production of hydroxyestradiols appears to be protective and has been linked to the antiproliferative and anticarcinogenic effects of indole-3-carbinol, an ingredient of cruciferous vegetables (e.g., cabbage, broccoli, kale) (103).

Taken together, these data suggest that the beneficial cardiovascular effects of E2 may be mediated at least in part by conversion to its major nonestrogenic metabolite 2-ME2, which then exerts its effects in an ER-independent fashion (30, 197). Since such a paradigm has been established in the systemic vasculature (28, 30, 197, 198), there has been considerable interest in further investigating estrogen metabolism in PAH.

**Studies suggesting harmful effects of estrogen metabolites in PAH.** Although several studies suggest beneficial effects of hydroxy- or methoxyestradiols in experimental PH, recent data suggest a paradigm of altered estrogen metabolism and aberrant production of 16α-OHE1 as contributors to PAH development. This was first explored by West et al. (182) and Austin et al. (4), demonstrating that female BMPR2 carriers are more prone to developing hereditary PAH (HPAH) than male carriers, and that lymphocytes from affected female carriers exhibited 10-fold lower activities of CYP1B1 (the enzyme responsible for converting E2 to hydroxyestradiols) than those from unaffected female carriers. Specifically, the penetration of HPAH was fourfold higher among subjects homozygous for the wild-type genotype (N/N) of CYP1B1 Asn453Ser (N453S), whereas no such difference was detected among males (4). This CYP1B1 genotype was associated with lower urinary 2-OHE2/16α-OHE1 ratios in the affected mutation carriers, suggesting a shift in the balance toward the proproliferative, antiapoptotic metabolite. Interestingly, in breast cancer, the same CYP1B1 genotype was shown to be associated with alterations in in vitro CYP1B1 activity and increased cancer risk (51). The observation that PAH prevalence is dramatically increased in the context of decreased CYP1B1 activity suggests that altered estrogen metabolism may contribute to disease development by increasing the otherwise low disease penetrance in patients with BMPR2 mutations or other genetic alterations predisposing them to PAH development. Such a notion is supported by subsequent studies by the same authors,
demonstrating that both E2 (in high concentrations) and 16α-OHE1 reduce BMPR2 gene expression and/or signaling, and that in the case of E2, this occurs at least in part via ERα binding to the BMPR2 gene promoter (5, 6, 44). Furthermore, when 16α-OHE1 is administered to male BMPR2-mutant mice, PH penetrance increases, cardiac output worsens, and alterations occur in genes related to platelet adhesion, angiogenesis, and metabolism (44). In lung microvascular ECs, BMPR2 expression is also reduced by E3 (5).

Given the excitement that distinct active estrogen metabolites may hold the key to explaining the estrogen paradox, our laboratory evaluated in a rat model whether and which estrogen metabolites are implicated in the protective effects of E2 in HPH. Specifically, we sought to evaluate whether conversion to hydroxy- or methoxymetabolites was required for E2 to attenuate HPH development in male rats. Using pharmacological inhibitors of the CYP450 system as well as COMT-inhibitors administered in conjunction with E2, our study demonstrated that, unlike HPAH, such a conversion process is not required for E2 to exert protective effects in HPH (84).

White et al. (186) further investigated the role of CYP1B1 in PAH and demonstrated increased CYP1B1 expression in the pulmonary vasculature of animals with hypoxia- or SuHx-induced PH, as well as in pulmonary vessels of patients with IPAH or HPAH. Interestingly, CYP1B1 null mice were protected from HPH development, but only if they were male. On the other hand, administration of the CYP1B1 inhibitor 2,3′,4,5′-tetramethoxyxisthene (TMS) significantly attenuated PH development in female as well as male HPH and SuHx mice. Similarly, TMS attenuated proproliferative E2 effects on PASMCs from healthy humans or PAH patients. In chronically hypoxic mice, the observed increase in CYP1B1 expression was accompanied by elevated urinary levels of 16α-OHE1. Administration of 16α-OHE1 to human PASMCs or to chronically hypoxic mice enhanced PASMC proliferation and worsened HPH development, respectively.

Although there are conflicting results between the studies from the Austin et al. (4) and White et al. (186) with regards to the question whether CYP1B1 activity is decreased or increased in PAH, this discrepancy may be explained at least in part by the fact that the first study evaluated CYP1B1 in peripheral lymphocytes, whereas the latter measured CYP1B1 expression in the pulmonary vasculature. Despite these differences, data from both groups demonstrate absolute or relative increases in 16α-OHE1, thus suggesting a paradigm of excess 16α-OHE1 levels as a contributor to PAH development. The cellular targets of 16α-OHE1, however, have not yet been identified, and it remains unknown whether this occurs via ERα- and/or ERβ-mediated mechanisms or through an ER-independent effect.

Although estrogen metabolism may be genetically determined, literature exists suggesting that environmental factors may play a role as well. For example, a diet low in fat decreases 16α-OHE1 levels in healthy female volunteers (97). In light of findings that insulin resistance and lipid alterations are common in PAH (61, 199), it is conceivable that dietary factors may modify 16α-OHE1 levels and thus affect PAH susceptibility and/or severity. Furthermore, estrogen metabolism is modified by drugs, suggested by studies in female SERT+ mice, female mice with dexfenfluramine-induced PAH, and female MCT-PH rats, all of which demonstrated increased lung CYP1B1 expression (25, 187, 196). In fact, CYP1B1 is necessary for PH development in dexfenfluramine-treated mice, and both dexfenfluramine as well as E2 increase CYP1B1 expression in cultured PASMCs from PAH patients (25). Although the MCT rat study also noted increased CYP1A1 expression, this was accompanied by decreased aromatase expression and decreased plasma E2 levels, consistent with a state of “estrogen deficiency” (196). 16α-OHE1 or other estrogen metabolites were not quantified in this study.

Evidence of alterations in estrogen metabolic pathways exists in other forms of PAH as well. For example, in patients with advanced liver disease, Roberts et al. (134) identified two single nucleotide polymorphisms (SNPs) in the promoter region of the gene coding for aromatase (CYP19A1) as a risk factor for the development of portopulmonary hypertension. Interestingly, one of these SNPs (rs7175922) was functionally relevant and associated with higher plasma E2 levels.

Do Estrogen Receptors Exert Adaptive or Detrimental Effects in PAH?

**ERs and the vasculature.** ERs located in ECs and SMCs of the systemic vasculature mediate protective estrogen effects during health and disease (19, 53, 119, 165, 190). The critical role of ERs in the systemic circulation is exemplified by studies demonstrating that SNPs in the gene encoding ERα (ESR1, or estrogen receptor 1) are associated with the development of myocardial infarction, hypertension, and stroke (127, 142, 143). Similarly, in hypertensive women, SNPs in the ERβ gene (ESR2) have been associated with increased left ventricular mass and hypertrophy (126).

In the lung, ERα and ERβ are expressed in epithelial cells (55, 73), alveolar cells (122), alveolar macrophages (151, 174), pulmonary artery endothelial cells (PAECs) (55, 174, 175), and PASMCs (55, 79). Although the role and expression of ERα and ERβ in the pulmonary circulation are less well defined than in the systemic vasculature, the data reviewed in the following sections demonstrate that both ERα and ERβ are present and physiologically active in lung blood vessels. Such a notion is also supported by recent data indicating that women have a more distensible pulmonary circulation (3). As in other organ systems, it is likely that expression pattern and activity of ER subtypes are modified by sex, age, hormonal status, and environmental influences (112). For example, we found that hypoxia selectively increases ERβ in rat PAECs in vivo and in vitro, whereas no such increases were noted in ERα (84, 139).

**Studies demonstrating favorable effects of ER activation in the pulmonary vasculature.** Studies of lungs of normal rats and mice, respectively, demonstrated both ERα and ERβ to be present in PAECs (55, 174), and multiple studies suggest favorable effects of E2 on lung EC biology. Studies in cultured PAECs showed that these involve ERα- and ERβ-mediated increases in eNOS activity (20, 63, 89, 98), as well as ERβ-mediated induction of prostacyclin synthesis (144). In isolated rat PA rings, administration of the selective ERα-agonist propylpyrazole triol (PPT) attenuates phenylephrine-induced vasoconstriction, whereas administration of the selective ERβ-agonist diarylpropionitrile (DPN) decreases HPV in an endothelium- and NO-dependent manner (85). Activation of either ER subtype results in rapid vasoconstriction, thus suggesting that these effects are mediated via a nongenomic pathway.
Such findings of nongenomic and ER-mediated NO release have also been reported in the systemic vasculature (21).

Although ERs are also present in PASMCs (55, 79), studies investigating ER signaling in this cell type are rare. A recent study demonstrated inhibitory effects of E2 on hypoxia-induced PASMC proliferation, which could be attenuated by the selective ER modulator rolaxifene (191). Similarly, stimulatory nongenomic E2 effects on cAMP activation in isolated PASMCs were attenuated after administration of tamoxifen (41). Taken together, these data suggest that ERs also play a role in PASMCs. However, further studies are needed to decipher the molecular mechanisms and ER subtype contribution to these effects.

ER signaling has also been investigated in animal models of PAH. A recent study by Umar et al. (171) in a MCT model demonstrated that administration of E2 to male rats attenuated the disease via angiogenic and anti-inflammatory mechanisms. The authors suggested that E2 protection in their model was mediated by ERβ, as administration of the selective ERβ antagonist 4-[2-phenyl-5,7-bis(trifluoromethyl)pyrazolo[1,5-a]pyrimidin-3-yl]phenol (PHTPP) abolished protective E2 effects on the pulmonary vasculature and RV, whereas treatment with a selective ERβ agonist (DPN) replicated E2-mediated protection. On the other hand, a recent study in female MCT-PH rats demonstrated downregulation of lung ERα and no change in ERβ, suggesting that decreased ERα either contributes to disease development or is merely decreased as a consequence of the disease (196). These results underscore the potential stimulus-specific activation of distinct ER signaling and the need to avoid generalization of results to all types of PH. We investigated the role of ER in the protective effects of E2 in male rats with HPH (84). Here, E2, in an ER-dependent manner, attenuated hypoxia-induced erythrocytosis, hemodynamic alterations, and PA and RV remodeling, accompanied by ER-mediated decreases in lung and RV proliferative ERK1/2 activation. Complementary experiments in cultured primary PAECs demonstrated E2 to decrease ERK1/2 activation and increase expression of the cell cycle inhibitor p27Kip1 and the autophagy marker LC3-II in hypoxic, but not room air-exposed rat PAECs. These findings were accompanied by a hypoxia-specific decrease in PAEC secretion of vascular endothelial growth factor (VEGF) as well as hypoxia-specific inhibitory E2 effects on cell proliferation. Co-treatment of HPH rats with ERα antagonist methyl-piperidino-pyrazole (MPP) attenuated E2 effects on hemodynamics, RV mass, and cardiac output. On the other hand, E2 effects on PA muscularization and PAEC ERK1/2 activation were attenuated after either selective ERα- or ERβ blockade, suggesting that both ERs need to be functional for E2 to exert protective effects on these parameters. ERβ appears to be of particular relevance to E2 effects on hypoxia-induced pulmonary vascular remodeling, evidenced by follow-up studies from our laboratory in which ERβ knockout mice exhibited increased hypoxia-induced PA muscularization (139).

**GPR30 and the cardiovascular system.** Although the role of GPR30 in the pulmonary vasculature and/or RV has not yet been defined, studies from the systemic vasculature suggest an important role of this receptor in vascular and cardiac function. For example, GPR30 has been implicated as a mediator of protective E2 effects on renin-angiotensin-aldosterone system activation and subsequent development of systemic hypertension (93). Along those lines, GPR30 is present in ECs and SMCs of the systemic vasculature and mediates vasodilatory E2 effects in conduit and resistance vessels from female rats (92, 93). Alterations in GPR30 expression and signaling, on the other hand, have been implicated in the vascular dysfunction and higher blood pressure noted in aging females as well as males (94). GPR30-mediated protection is due at least in part to antioxidant effects (95). Beneficial effects of GPR30 were also noted in the left ventricle (LV), where selective activation with its agonist G-1 attenuated negative effects of ovarectomy on remodeling and diastolic dysfunction (179). Protective G-1 effects were also demonstrated in intact females (72). Underlying mechanisms include antibioptic effects, enhanced cardiac contractility, and favorable effects on β-adrenergic receptor expression (78). Given this large body of literature suggesting beneficial effects of GPR30 in the systemic vasculature, it is plausible that the principles described here may also apply to the pulmonary vasculature and/or RV; however, no such investigations have been performed to date.

**Studies linking ERs to disease development in PAH.** Although the studies reviewed above reported beneficial effects of ER signaling, a recent study suggested that, in the setting of hypoxia, E2-induced ER signaling switches from augmenting BMPR2 signaling in human PAECs to inhibiting it (69), thus implying E2 as a potential contributor to PAH development. The mechanisms of this switch were not fully characterized; the authors suggested that HIF-1α may be involved. Data on pulmonary vascular ER signaling in humans are sparse. In a study of genomewide RNA expression profiling in lungs of patients with IPAH or PH secondary to idiopathic pulmonary fibrosis (IPF-PH) (131), upregulation of ESR1 was demonstrated in the PAH cohort relative to normal controls and IPF-PH. Both male and female IPAH patients exhibited a higher expression of ESR1 than female or male controls. ESR1 abnormalities were also associated with PH development in patients with advanced liver disease, where SNPs in the ESR1 gene were associated with an elevated risk of developing portopulmonary hypertension (134). However, it is important to emphasize that although these studies suggest an association between ESR1 and IPAH and/or portopulmonary hypertension development, they do not allow for conclusions regarding cause and effect, and it is not known whether these changes represent a pathogenic mechanism of disease development or merely a consequence of the disease (e.g., a compensatory mechanism for impaired ERα or ERβ signaling, or for other abnormalities in E2 signaling). The role of ERβ in PAH has not been studied in detail; exploratory studies in our laboratory found ERβ more abundant in pulmonary arteries from PAH patients than ERα (T. Lahn, unpublished data).

In summary, conflicting data exist on the role of ERs in the development of PAH. Although several cell culture and animal studies in MCT-PH and HPH suggest potential protective effects of ERα and/or ERβ, human studies suggest a possible association between ESR1 and the development of PAH. It is conceivable, though, that, similar to studies in the systemic vasculature, quantitative or qualitative defects in otherwise protective ER signaling may contribute to disease development. Such a notion is supported by studies in BMPR2 mutant pulmonary microvascular ECs; these cells exhibit aberrant intracellular localization of ERα and insensitivity to genomic E2 signaling (44).
ESTROGEN EFFECTS ON THE RV

RV function is a major determinant of functional status and survival in PAH (12, 66, 68, 173). A detailed understanding of the effects of sex hormones on RV function is therefore critical in deciphering functional and molecular correlates of sex differences in PAH. Unlike the studies in the pulmonary vasculature, studies focusing on E2 effects in the RV are more consistent.

Studies in various forms of acute or chronic left ventricular injury have demonstrated ERα- or ERβ-mediated protective effects of E2 on functional parameters and markers of myocardial metabolism, inflammation, fibrosis, and apoptosis (46, 91, 180, 181). These effects likely occur through E2 action on myocytes, ECs, and fibrocytes, as well as progenitor cells and inflammatory cells (reviewed in Ref. 112). Considering that RV systolic function is superior relative to men in both healthy women as well as in women with PAH, and given the fact that RVEF correlates with E2 levels in healthy postmenopausal hormone replacement users (81, 82, 176), it is conceivable that E2 also has direct, ER-mediated RV cardioprotective effects. Indirect evidence for protective RV effects of estrogens stems from studies reporting that the phystoestrogen genistein attenuates RV dysfunction in MCT-PH (99) and that pregnancy is associated with decreased oxidative stress in the heart (70). More direct evidence comes from studies by Umar et al. (171), our group (84), and Nadadur et al. (115), all of which demonstrated favorable effects of E2 on RV function. The study by Umar et al. (171) (using a MCT model) demonstrated that E2 administration in male mice was associated with increased RV capillary density. Subsequent experiments using the ERβ agonist DPN and investigating functional end points suggested an ERβ-mediated mechanism of action. Involvement of ERs in our recent studies (84) (performed in an HPH model), however, was more complex. Although we demonstrated E2-mediated increases in cardiac output that were attenuated after nonselective ER blockade as well as selective ERα blockade, E2 effects on RV capillarization as well as ERK1/2 activation were only attenuated after nonselective ER blockade, but not after ERα- or ERβ-selective blockade. This may suggest potential redundant functions of each ER subtype in mediating E2 effects on these end points. Lastly, Nadadur et al. (115) demonstrated antifibrotic effects of E2 in the RV of MCT-PH rats. In particular, E2, in an ERβ-dependent manner, reversed PH-induced RV fibrosis and upregulation of the extracellular matrix-degrading enzymes ADAM15 and ADAM17 and the remodeling mediator osteopontin. Experiments with ovariectomized females as well as E2-treated males demonstrated that both endogenous as well as exogenously administered E2 are involved in these protective effects. RV fibroblasts appear to be a direct target of E2, since fibrosis markers and metalloproteinases were decreased by E2 in cultured cardiac fibroblasts. We have recently demonstrated protective effects of E2 on RV function and exercise capacity in SuHx-PH (189). In particular, E2, when given to male animals, improves echocardiographic parameters of RV function and exercise capacity [determined by maximal oxygen consumption (\(\dot{V}O_2\text{max}\)), and attenuates increases in RV wall thickness. On a molecular level, this is accompanied by decreased RV proapoptotic signaling, GLUT1 expression (indicating improved mitochondrial function) and neurohormonal activation (measured by atrial natriuretic peptide mRNA). Although these studies clearly demonstrate direct RV-protective properties of E2, it is noteworthy that even in studies suggesting that E2 worsens proliferative processes in the pulmonary vasculature, RV-protective properties were observed. For example, in the study by White et al. (185), improved RV hypertrophy was observed in E2-treated animals. In contrast to E2, 16α-OHE1 appears to be detrimental for RV function, as BMPR2 mutant mice receiving 16α-OHE1 exhibit worsening cardiac output (44).

The role of the ER in the PAH-RV in humans has not yet been investigated. Considering that RV function in healthy individuals correlates with E2 levels (176), and considering the currently existing animal studies of ER-mediated E2 action in both the LV and RV (reviewed above), it is tempting to speculate that ERs are involved mediating potentially significant E2 effects on the human RV in PAH.

POTENTIAL EXPLANATIONS FOR THE OBSERVED ESTROGEN PARADOXES IN PAH

Although DHEA appears to be uniformly protective in PAH (see Table 4), the aggregate of studies reviewed in this manuscript demonstrates complex and disparate effects of estrogens and other sex hormones in PAH. In general, these effects are comparable to what is seen with other major disease modifiers in experimental or human PAH (6, 66, 81, 131, 134, 182), thus suggesting that sex hormones have a major impact on PAH development and/or severity. Although epidemiological studies in humans as well as mechanistic studies in animals reveal protective as well as detrimental effects of estrogens with regard to PAH development (Tables 4 and 5), several themes and concepts can be identified.

Altered Estrogen Metabolism in PAH

The studies by Tofovic et al. (159–162) suggest protective effects of hydroxy- and methoxyestradiols in PAH. On the other hand, the studies by the Vanderbilt (4) and the Glasgow groups (186) suggest that an excess production of 16α-OHE1 contributes to PAH development. Although data are conflicting as to whether CYP1B1 is down- or upregulated, several studies demonstrate increased expression in the lung (25, 186, 187, 196), thus supporting the latter. Since CYP1B1 is purported to oxidize E1 to 16α-OHE1, there may be a shift in PAH toward this pathway, with a subsequent increase in proproliferative signaling. Genetic predisposition [e.g., BMPR2 mutation (4, 6, 44)] or exposures [e.g., use of serotoninergic drugs (25, 187)] may create a permissive environment for such a shift in estrogen metabolism. In light of the multiple studies demonstrating beneficial effects of E2 on the pulmonary vasculature (e.g., Refs. 34, 84, 132, 171, 192, 196), it is therefore conceivable that otherwise vasoprotective estrogen signaling becomes maladaptive if it occurs in the context of a genetically or environmentally altered milieu.

Context-Specific E2 Effects

Along those lines, studies by others and us suggest that E2 effects may be highly context specific. In our studies in
PAECs, hypoxia induced a switch toward antiproliferative signaling that was not observed in normoxia (84). Such a paradigm would explain the consistently positive effects of E2 in the setting of HPV and HPH (34, 84, 85, 88, 132), whereas no such effects are seen in BMPR2 mutants or in systems characterized by serotonin upregulation (4, 6, 25, 187). In these contexts, proproliferative effects may predominate (Fig. 3).

MCT-PH, on the other hand, appears to be another context permissive for protective E2 signaling (possibly by inducing an “estrogen-depleted” state) (171, 196). Accordingly, 2-ME2 (protective in MCT-PH) failed to alleviate PH in BMPR2 mutants (44).

The “Timing Hypothesis”

The timing hypothesis of HRT and E2 administration builds on the concept of context-specific E2 effects. In particular, it implies that E2 exerts vasoprotective vasodilatory, anti-inflammatory, and antiproliferative effects in the setting of mild atherosclerotic changes but exerts opposite effects in the setting of more advanced atherosclerosis (101). This hypothesis is one of the explanations for the negative outcomes observed in the Women’s Health Initiative; it implies that HRT was administered to women that were too old and thus had atherosclerotic changes that were too advanced for HRT to exert protective effects. Extrapolating these observations to animal models of PAH, such a paradigm would explain the protective E2 effects observed in HPH and MCT-PH [models in which pulmonary vascular remodeling is relatively modest (150)]. The pulmonary vascular milieu in the setting of genetic predisposition or upregulated serotonin signaling, however, may promote maladaptive E2 signaling. Extrapolating the timing hypothesis to human PAH, it is conceivable that the lung vasculature of predisposed individuals represents an environment that is permissive for maladaptive E2 signaling. A thought-provoking finding in this context is that female PAH patients exhibit more pronounced pulmonary vascular remodeling than male patients (149).

**Age-Specific Effects**

Building upon the paradigm of the timing hypothesis and context-specific effects, the animal age at the time of estrogen replacement, estrogen withdrawal (ovariectomy), and end point assessment may contribute to sex differences in animal studies. For example, SERT+ does not increase RVSP or pulmonary vascular remodeling in young adult females, and female susceptibility to PH is not evident in this model until 5 mo of age (187). This suggests that age-related changes in the pulmonary vasculature may contribute to sex differences in pulmonary vascular disease. Furthermore, the stage of the estrus cycle at time of end point assessment may affect physiological parameters and thus experimental results (88, 191).

**Altered ER Signaling**

Multiple animal studies revealed protective ER signaling in PH. Viewed in light of studies demonstrating SNPs in genes...
encoding ERα or ERβ being linked to increased incidence of cardiovascular disease (126, 127, 142, 143), and considering that two studies of human PAH reported genetic alterations in the ERα gene (131, 134), these data support a paradigm of altered ER signaling as a potential predisposing factor to PAH. ER function may also be altered more indirectly as consequence of mutations in other genes. For example, BMPR2 regulates glucocorticoid receptor (GR) signaling (27), and ERα trafficking appears to be dysregulated in pulmonary microvascular ECs carrying a BMPR2 mutation (44). The observation that female PAH patients exhibit more insulin resistance than male patients (199) suggests a potential cross talk between impaired GR and ER signaling, which needs to be explored in future studies.

Estrogen Withdrawal

A more hypothetical thought is that it is estrogen withdrawal rather than estrogen excess that triggers vascular remodeling. Pulmonary vascular tone and diffusing capacity are affected by hormone levels and vary throughout the menstrual cycle (a finding also noted in asthma, where air flow varies according to the phase of the cycle) (39, 40, 88), and it is plausible that cyclic decreases in E2 levels promote vasoconstriction and vascular remodeling in otherwise predisposed individuals.

RV-Protective Effects of E2

Several studies revealed protective effects of E2 in the RV (84, 115, 171, 189), and beneficial effects of E2 on RV end points were seen even in studies showing PAH-promoting E2 effects in the pulmonary vasculature (187). An emerging concept thus would be that E2, even if it negatively affects the pulmonary vasculature via proproliferative effects, exerts beneficial prosurvival effects in the RV, thus allowing for better adaptation to increased RV afterload. Such a paradigm would be supported by human data, where E2 and/or female sex are associated with better RV function (71, 81, 82, 140, 176, 177).

Technical Issues

Although the sex of the experimental animals is usually reported in studies focusing on sex hormone effects and sex differences, the sex of cells used for in vitro studies is not always clear. Sex differences in cell origin may thus contribute to disparate effects between studies.

A schematic summarizing a potential explanation for the observed estrogen paradoxes and sex differences in PAH is depicted in Fig. 3.

KNOWLEDGE GAPS AND FUTURE RESEARCH DIRECTIONS

Despite significant advances in the study of sex and gender differences and sex hormone effects in PAH over the last decade, several knowledge gaps and open questions remain. These should be addressed through a combination of mechanistic animal studies and complementary investigations in PAH patients.

Optimization of Animal and In Vitro Studies

The effects of sex hormones, their receptors, and metabolites on lung vascular and RV end points need to be investigated in models that mimic human PAH more closely than HPH or MCT-PH. As such, the SuHx model of PAH offers many opportunities. In light of the female predominance in PAH, more investigations in female PAH animals are needed (105). To dissect direct RV effects from those secondary to changes in afterload (reflecting effects on the pulmonary vasculature), the effects of sex hormones on the RV should ideally be studied in models of isolated RV dysfunction, e.g., in a PA banding model. Adequate representation of both sexes in animal and cell culture studies is critical. The sex of cells used for in vitro studies should be reported.

End Points, Disease Modifiers, and Novel Pathways

Sex hormone effects on RV function need to be thoroughly characterized, by using pressure-volume loops and/or advanced imaging methods, ideally complemented by investigation of exercise capacity (ideally by V_{O2max} measurement) and survival. The effects of sex hormones on angiogenesis and RV capillary density have not been fully described, requiring more stringent approaches based on unbiased stereology. Given the complexity and different effects of E2 in distinct models of PH, effects of hypoxia, inflammation, and oxidative stress on ER expression and signaling require further investigations, as do interactions between sex hormones and inflammatory cells in the lung and RV. Furthermore, effects of external factors [e.g., diet (97, 103)] on estrogen metabolism and receptor expression in PAH should be investigated. Differential responses of males and females to therapeutic interventions need to be studied further. Paradigms derived from other lung or vascular diseases characterized by sex and gender differences in incidence and prevalence provide research opportunities in the pulmonary vasculature and RV; for example, pathways mediated by estrogens in asthma, lung injury resolution, left heart failure, or atherosclerosis may represent therapeutically treatable targets in PAH (52, 83, 101, 112, 166, 167).

Hormonal Milieu and Genetics

Estrogen metabolite levels in PAH patients need to be quantified with state-of-the-art methods [e.g., liquid chromatography-mass spectrometry (193)]. The role of ERs in PAH deserves further clarification. Genetic alterations in ER signaling in IPAH and characterization of the endogenous hormonal milieu of PAH patients are other areas that deserve further clarifications. This should include a characterization of the effects of menstrual cycle and menopause on PAH end points. Since sex differences may also be mediated in a sex hormone-independent manner via effects of genes located on sex chromosomes, such influences should be investigated as well.

Aromatase, GPR30, Progesterone, and Androgens

The role of aromatase in PAH requires further investigations, and an ongoing trial studies aromatase inhibition as a method to inhibit PAH development [inspired by the data from Roberts et al. (134) in portopulmonary hypertension; clinicaltrials.gov identifier NCT01545336]. Moreover, examination of the role of GPR30 in PAH and the fate of DHEA in PAH (conversion to androgens and estrogens vs. androgen- and estrogen-independent effects) are also warranted. Lastly, the roles of testosterone and progesterone as disease mediators in PAH deserve further clarification.
CONCLUSIONS

The effects of estrogens and other sex hormone effects on the pulmonary vasculature and RV are complex yet highly fascinating because of their direct relevance to clinical manifestation of various forms of PAH. Although both protective as well as detrimental E2 effects in PAH have been described, future studies will need to focus on animal models that more closely recapitulate the human phenotypes to understand the tissue compartment- and context-specific effects of sex hormones. These studies, combined with investigations in PAH patients, may help further elucidate the molecular mechanisms of sex differences in PAH and may facilitate the development of novel nonhormonal, targeted therapies for PAH patients afflicting both sexes.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

T.L., R.M.T., and I.P. conception and design of research; T.L. prepared figures; T.L., R.M.T., and I.P. drafted manuscript; T.L., R.M.T., and I.P. edited and revised manuscript; T.L., R.M.T., and I.P. approved final version of manuscript.

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Review


