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Estrogen and Cyp1b1 Regulate Pparγ in Pulmonary Hypertension Through a Ubiquitin-Dependent Mechanism

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ABSTRACT

Female sex increases risk of Group I pulmonary arterial hypertension by roughly threefold, but the mechanism is unclear. Low expression of Cyp1b1, an enzyme that metabolizes estrogens, is associated with disease penetrance, particularly in women. We previously found that lower Pparγ levels in murine PAH models, which may drive disease, are rescued by estrogen blockade. The goal of the current studies was to examine interaction of estrogen, Cyp1b1, and energy metabolism in cell culture and in knockout mice. We found that both estrogen and siRNA to Cyp1b1 resulted in reduction of Pparγ at a protein, but not transcript level, in addition to regulating Pparγ cofactors. siCyp1b1 reduced both basal and maximal respiration rates in a fatty acid oxidation Seahorse protocol. This Pparγ inhibition could be eliminated by blocking ubiquitination. RNA-seq suggested that Cyp1b1 may be having important pulmonary hypertension effects both in concert with and independently of its effect on estrogen. Cyp1b1 knockout mice have lower Pparγ levels than WT mice both in normoxia and hypoxia, and develop mild pulmonary hypertension on a high fat diet. RNA-seq on their lungs reflected similar pathways to those altered in endothelial cells alone – lipid metabolism, cytokines, and vasoreactivity-associated genes, among others, but added genes associated with circadian rhythm. These data suggest multiple potential points for intervention in estrogen and Cyp1b1 mediated etiology of PAH, in particular Pparγ ubiquitination, but also suggests that both the difference between E2 and 16aOHE and the impact of Cyp1b1 is more complex than simply "degree of estrogenicity".

1 | Introduction

Pulmonary arterial hypertension (PAH) is a disease which includes pulmonary vascular endothelial dysfunction, occlusion and dropout of the small and medium-sized pulmonary arteries, and hypertrophy and proliferation of smooth muscle and adventitial cells. This results in progressively worsening pulmonary vascular resistance and load stress on the right heart, eventually leading to right heart failure. No current therapy is curative.

Eric Austin and James West denotes equal contribution.

The heritable form of PAH is usually caused by mutations in the type 2 receptor for the BMP pathway, BMPR2 [1–4]. Moreover, even patients without BMPR2 mutations have decreased BMP pathway activity, making BMP central to development of PAH. BMPR2 mutations lead to defects in metabolism, inflammation, and proliferation, which feed back into each-other to drive the disease state. However, not all individuals carrying BMPR2 mutations develop disease.

The strongest risk factor for developing disease, with or without BMPR2 mutations, is female sex [5–8]. Women develop pulmonary arterial hypertension at roughly three times the rate of men, with increased estrogen the most likely reason. Close to half of women with BMPR2 mutation develop disease, as opposed to only 14% of men. We've previously published a striking finding that explains a large proportion of how penetrance is determined. Women who have high levels of the estrogen metabolite 16αOHE develop PAH; those with low levels do not [9–11]. Levels of 16αOHE are largely explained by expression of the cytochrome CYP1B1, which metabolizes estrogen. Women who developed PAH had nearly 10× lower expression of CYP1B1 than women who did not [9, 12]. In whole lung from PH patients of any etiology, CYP1B1 is decreased twofold (Figure S1, derived from uploaded data from Hong et al. [13])

Our previous research strongly suggested that the reason estrogen, particularly 16αOHE, increased PAH penetrance was through worsening the metabolic defects associated with BMPR2 mutation [14]. BMPR2 mutation resulted in reduced Pparγ protein and increased insulin resistance, particularly resistance in lipid metabolism. Estrogen made this worse; blocking estrogen made this better, in cells and in live mice. Although we think of Pparγ primarily as regulating metabolism [15], it also assists in maintaining endothelial homeostasis by promoting DNA repair [16]. Preventing Pparγ inhibition has thus been a primary goal in the treatment of pulmonary hypertension for nearly 20 years [17]. Interventions along the estrogen axis seem like a possible solution.

These differences in estrogens are likely causal, rather than consequence; adding 16aOHE to Bmpr2 mutant mice increased disease severity, and blocking it ameliorated disease [11]. Our success in reversing PAH in Bmpr2 mutant mice using estrogen blockade supported the rationale for current ongoing human trials, using tamoxifen in premenopausal women (NCT03528902), and anastrozole in postmenopausal women (NCT03229499). However, even if these trials work, blocking estrogen overall is likely to have numerous unfortunate side effects, and so it would be better if we could intervene only in the ways that estrogen exacerbates PAH, rather than blocking estrogen overall, which may be problematic especially for young women. The goal of these studies was to understand in more detail the interactions between estrogen, Cyp1b1, and endothelial metabolism, and in particular to determine mechanism by which estrogen was modulating Pparγ, as a potential point of intervention.

2 | Methods

2.1 | Cell Culture and Transfection

Wild-type (WT) and BMPR2 mutant (BMPR2R899X) mouse pulmonary microvascular endothelial cells (mPMVEC) were isolated from immortal mice which contain a transgenic insertion of the SV40 large T antigen, tsA58, under control of an interferon-gamma (IFN-γ) inducible promote. mPMVEC maintain the immortal phenotype with the presence of IFN-γ in 33°C and transit to a primary endothelial phenotype by removal of IFN-γand transition to 37°C. Before each experiment, mPMVEC were transit to 37°C for at least 72 h and 300 ng/mL doxycycline was added to induce the expression of the transgene to induce expression of the transgene (the construct being Rosa26-rtTA × TetO7-Bmpr2R899X). Human PMVEC was purchased from promocell (Heidelberg, Germany). Both PMVEC were cultured in EGM-2 culture medium (Lonza, USA) containing 5% fetal bovine serum (FBS) and then seeded into a 60 mm culture dish (1.6×10^6/well). Before transfection, the cells were pretreated with opti-MEM medium (Invitrogen, USA) for 2 h. For each dish, 10 nM of scramble or CYP1B1 siRNA (Invitrogen, USA) were used in transfection by Lipofectamine RNAimax Reagent. Twenty-4 h after transfection, the cells were then split into six well plates and cultured in EGM-2 complete culture medium containing E_2 or 16αOHE1 for further assay.

These mPMVEC were initially isolated based on flow sorting for Pecam1 and appeared well differentiated based on criteria used at the time [18]. We reassessed their differentiation state for this study using more recent marker sets developed using single cell RNA-seq from fresh lung. These mPVMEC still expressed a majority, but not all, endothelial specific markers [19], including caveoli and endothelial glycocalyx (Figure S2A), focal adhesion structures (Figure S2B), and adherens junction and regulators of endothelial migration (Figure S2C). However, they also showed moderate expression of genes related to endothelial-mesenchymal transition [20] (Figure S2D), and likely as a consequence of this transition, expressed several genes more associated with a mesenchymal state (Figure S2E). This loss of full differentiation of endothelial cells in culture is a common problem, and most cell culture results, including these, should be considered in light of the effect of culture on the cells.

2.2 | Measurement of Fatty Acid Metabolism of PMVEC

To assess fatty acid metabolism in PMVEC after CYP1B1 knockdown, WT and $BMPR2^{R899X}$ PMVEC were seeded and transfected with scramble or CYP1B1 siRNA. 24 h after transfection, mPMVEC were passed to a 96-well seahorse cell culture microplate at a seeding density of 20,000 cells per well. 2 h before assessment, the culture medium was changed to a limited substrate media consisting of limited DMEM (Agilent Technologies Inc. USA), 0.5 mM glucose, 1.0 mM GlutaMAX, and 1% fetal bovine serum. 1 h before the assay, the cells were incubated in Krebs-Henseleit buffer (111 mM NaCl, 4.7 mM KCl, 1.25 mM $CaCl_2$, 2.0 mM $MgSO_4$, 1.2 mM NaH_2PO_4, 5 mM HEPES). After 1-h incubation in KHB buffers, 25 μL FAO

substrate palmitate-BSA (102720-100, Agilent Technologies Inc. USA) were added and oxygen respiration, pH of each well were then measured before and after sequential administration of Oligomycin (2 mM), FCCP (2 mM), and Rotenone/Antimycin-A (0.5 mM) (Agilent Technologies Inc., USA) on a Seahorse XFe96 Analyzer.

2.3 | Quantitative PCR

mPMVEC were transfected with Scramble siRNA or CYP1B1 siRNA. After 24 h, cells were treated with 100 nM E2 or 16α-OHE1 for another 24 h. Cells were harvested using RNeasy kits (Qiagen, CA, USA) and converted to cDNA using QuantiTect Reverse Transcription Kit (Qiagen, CA, USA) following the manufacturers' instructions. Real-time PCR was performed in a 96-well format using Power SYBR green mastermix on the Applied Biosystems platform (Applied Biosystems Corporation, CA, USA). The expression of target gene transcripts was normalized against the internal control Hprt (hypoxanthine-guanine phosphoribosyltransferase) using the comparative ΔΔCT method ($2^{-\Delta\Delta Ct}$). Primer pairs used included CD36_mus_4F (GGCTGTG ATCGGAACTGTGGGC) with CD36_mus_98R (AGCATGTCTC CGACTGGCATGAG), Bmpr2_mus_2492F (CAGCTGGCCAGG CAGCCAAC) with Bmpr2_mus_2636R (TGGCCAGCCTGTTG CTCTCG), and Pparg_mus_1057F)AGC CTG CGG AAG CCC TTT GG) with Pparg_mus_1197R (CAG CAA GCC TGG GCG GTC TC).

2.4 | Western Blot

Cells were harvested and lysed in the RIPA buffer. Protein concentrations were determined using the BCA protein assay kit (ThermoFisher, USA). Forty μg proteins were separated by 10% SDS-PAGE and transferred onto a PVDF membrane. After blocking with TBST buffer containing 5% nonfat milk for 1 h at room temperature, the membranes were incubated with indicated primary antibodies overnight at 4°C. CD36 and PGC1α antibodies were purchased from NOVUS (NOVUS biologicals, USA). PPARγ was purchased from Abcam (Abcam 45036, USA). p-smad 1/5 (13820S), p-smad 2/3 (8828S), p-AKT (4060S), p-p44/42 (4376S), p-p38 (9211S), ubiquitin (3936S) were purchased from Cell Signaling Technology (CST, catalog numbers given above, USA). All antibodies were diluted following the manufacturers' instructions.

2.5 | Immunoprecipitation

mPMVEC were passed into 100 mm dish and transfected as usual. After 24 h for transfection, cells were added 1 μM MG132 to block the protein degradation. Cells were lysed in 1% NP40, after concentration measurement, 1000 μg protein per sample were used for reaction and pre-cleared. PPARγ antibodies were conjugated to the protein A/G beads following the manufacture's instruction. Beads and protein were incubated at 4°C overnight. After that, proteins were washed off and probed for anti-PPARγ or anti-ubiquitin.

2.6 | CYP1B1$^{-/-}$ Mouse Experiments

Cyp1b1 null mice on a pure C57BL/6J background were obtained from Dr. Frank J. Gonzalez [21] at the National Cancer Institute, Rockville, MD. All the mice utilized in this study were housed at Vanderbilt University Medical Center animal care facility, which is certified by the Association for Assessment and Accreditation of Laboratory Animal Care International. All animal protocols were approved by the Institutional Animal Care and Use Committee at Vanderbilt University Medical Center in compliance with National Institute of Health guidelines. Animals housed in the conventional cages were exposed to 12-h light and 12-h dark cycle with free access to food and water. The study was started by putting all animals on a Western diet (60% high-fat, high-calorie diet from Bio-Serv, Flemington, NJ, USA) this diet was continued until the termination of the study. After 6 weeks on the Western diet, some mice from both genotypes were placed in normobaric hypoxia (10% oxygen) for 4 weeks and some mice from both genotypes in normoxia (Room Air) for 4 weeks. All animals were kept on the Western diet until the end of the study.

Right ventricular systolic pressure (RVSP) was measured by introducing a 1.4F pressure transducer into the right ventricle by threading through the right jugular, with the chest closed and the mice spontaneously breathing, as previously described [22]. Surgical anesthesia was provided using tribromoethanol (avertin), because it is one of only a few anesthetics which preserve heart rate. After RVSP measurement, mice were killed, one lung inflated for histology, and the other collected for molecular measurements.

2.7 | RNA-Seq Experiments

For mouse, lung was flash frozen in liquid nitrogen immediately after sacrifice. RNA was isolated from whole lung using RNEasy kits (Qiagen), and delivered to Novogene (Sacramento, California) for paired end 150 sequencing on an Illumina platform. A nominal read depth of 30 million RNA (60 million ends) per animal was used, with actual falling between 26.4 and 36.4 million reads. These were uploaded to and analyzed on the Partek platform, using the STAR aligner to align to the mm39 reference mouse genome. An average of 96% of reads aligned to genome (94%–97%). Counts were normalized to Counts Per Million, and the results and raw data uploaded to GEO (number pending). Group differences were assessed using the ANOVA or Kruskal-Wallis (more appropriate) tests (similar results with both tests), and gene ontology determined using Partek's Gene Set Enrichment tool, or using Webgestalt. Data sets have been deposited in the GEO database at NCBI, as accession numbers (GSE166006(cells) and GSE225402 (mice)).

3 | Results

3.1 | Both 16aOHE and siRNA to Cyp1B1 Reduce Pparγ Protein Levels, But Not mRNA Levels

Mouse pulmonary microvascular endothelial cells (PMVEC) were treated with vehicle, estrogen (E2), or the highly estrogenic

metabolite 16αOHE, in the context of either scrambled siRNA or siRNA to Cyp1B1. Levels of mRNA for the metabolic regulator Pparγ, the fatty acid transporter CD36, and BMPR2 were measured by quantitative RT-PCR. Estrogens, either alone or in combination with siRNA to Cyp1B1, did not reduce Pparγ mRNA, and may have slightly increased them (Figure 1A). However, using protein levels as a readout shows that the highly estrogenic 16aOHE does reduce protein levels; this is eliminated by using ubiquitination inhibitors (Figure 1B).

siRNA against CYP1B1 also reduces Pparγ protein in murine (Figure 1C) and human (Figure 1D) PMVEC, compared to treatment with scrambled siRNA. Note that in this experiment, neither E2 nor 16aOHE dropped Pparγ protein levels; estrogen inhibition of Pparγ protein was reasonably reliable, but not universal, and seemed to be affected by both confluence and inflammatory state (even scrambled siRNA is inherently somewhat inflammatory). Note that we've previously published that in whole lung from live mice, estrogen signaling does drive down Pparγ protein [14]. PGC1a, a critical cofactor for Pparγ in regulating fatty acid oxidation, was also downregulated (Figure 1C), but the fatty acid transporter CD36 had unchanged protein levels. (Figure 1D).

3.2 | siRNA to Cyp1B1 Synergizes With BMPR2 Mutation to Reduce Fatty Acid Oxidation

Knockdown of Cyp1B1 reduces Smad1/5 phosphorylation, the canonical indicator of BMP signaling (Figures 1C and 2A). This was true either in WT cells, or in the context of Bmpr2 mutation, but did not block activation by adding BMP ligand (Figure 2A). Note that the R899X mutation for BMP represents the most common class of patient mutations, but is in the tail domain, and thus does not block Smad activation. Bmpr2 mutation reduces Pparγ protein levels, which is reduced further by siCYP1B1 (Figure 2B).

To determine whether this reduction in Pparγ was functionally meaningful, we performed a seahorse assay using a FAO protocol, and found that when selectively given long chain fatty acids (palmitate) for fuel, cells treated with siCyp1B1 had lower baseline and maximal respiration, which was pushed even lower in the context of Bmpr2 mutation (Figure 2C,D).

3.3 | siCyp1B1 Inhibition of Pparγ Protein Levels Is Through Ubiquitination, But siCyp1b1 May Also Regulate Activity Through Canonical Pparγ Regulatory Pathways

To determine how Pparγ protein levels were being reduced, we tested broad caspase inhibition (Figure 3A) and ubiquitin inhibition (Figure 3B). Blockade of ubiquitin (Figure 3B), but not caspase (Figure 3A), blocked siCyp1b1 reductions in Pparγ protein levels, in WT cells and in the context of Bmpr2 mutation. To confirm this, we conducted immunoprecipitation experiments; protein isolated from wild-type or Bmpr2 mutant murine PMVEC and precipitated with antibodies to ubiquitin showed a doubling in bound Pparγ with siCyp1b1 compared to scrambled controls (Figure 3C).

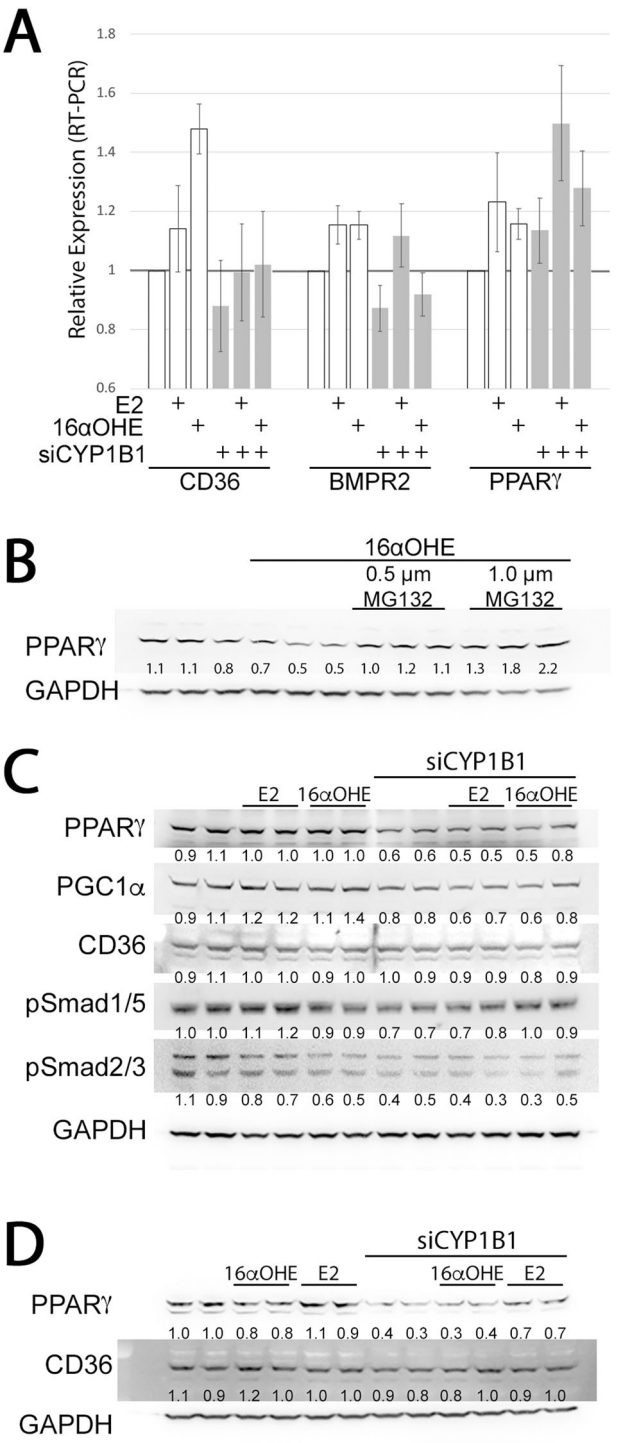

FIGURE 1 | Both 16αOHE and siRNA to CYP1B1 reduce Pparγ protein levels, but not mRNA levels. (A) Mouse pulmonary microvascular endothelial cells (mPMVEC) treated with 100 nM 16αOHE or 100 nM E2 for 24 h, 24 h after transfection with scrambled siRNA or siRNA to CYP1B1 lead to slight increases in PPARγ RNA expression, normalized to HPRT and to control. 16αOHE (B) mPMVEC with 24 h 100 nM 16aOHE treatment show reduction of PPARG protein of 30%–50%. This effect is abolished with 1 μM protease inhibitor MG132. (C) murine PMVEC treated with 100nm E2 or 16αOHE for 24 h, and scrambled siRNA or siRNA to CYP1B1, 24 h after transfection. (D) human primary PMVEC treated with 100 nM, E2 or 16αOHE for 24 h, and scrambled siRNA or siRNA to CYP1B1, 24 h after transfection.

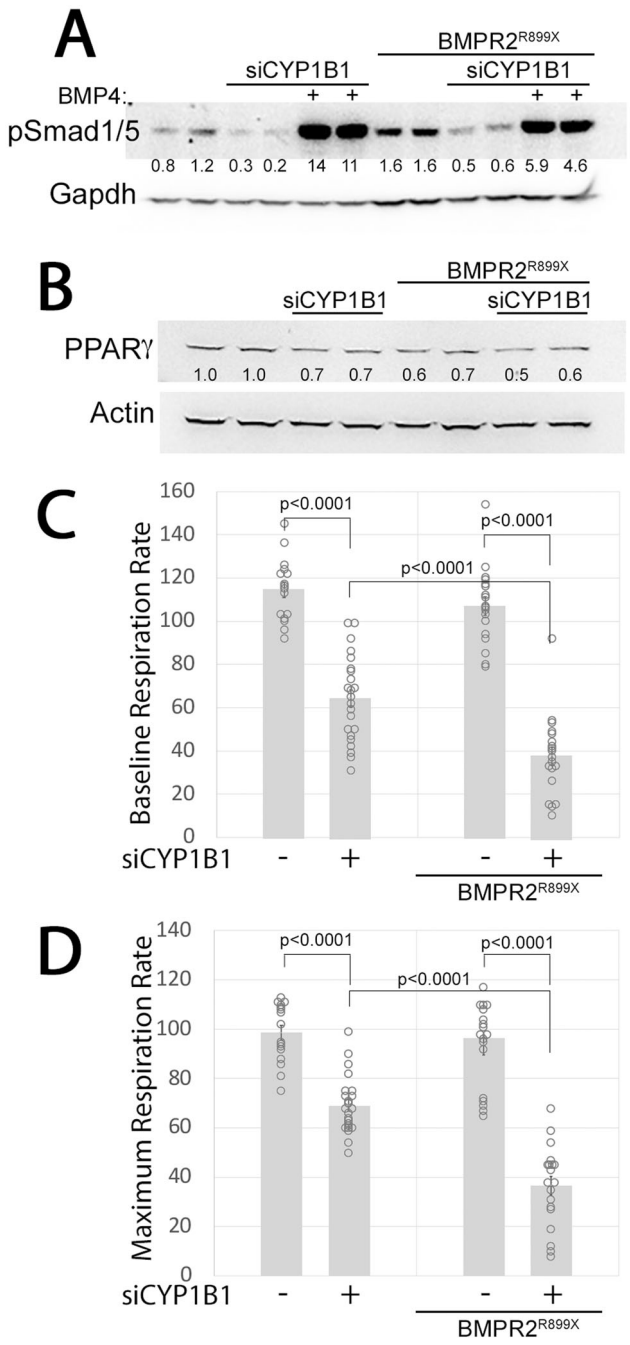

FIGURE 2 | siRNA to CYP1B1 synergizes with BMPR2 mutation to reduce fatty acid oxidation. (A) mPMVEC derived either from immortomice, or immortomice carrying the Bmpr2^{R899X} transgene, 24 h after transfection with either scrambled siRNA or siRNA to Cyp1B1, and treated with either vehicle or 10 ng/ml BMP4 for 24 h, and probed for phosphorylated Smad1/5, the marker of canonical BMP pathway activity. (B) mPMVEC as above, probed for PPARγ, show reductions in protein with either CYP1B1 or Bmpr2R899X mutation, and additional reduction with both. (C) Baseline respiration rate (oxygen consumption) by Seahorse Oxygraphy with mPMVEC treated as above, using the fatty acid oxidation substrate palmitate-BSA. (D) Maximum respiration rate in the above experiment. For both (C) and (D), each dot is an independent well. Error bars are standard error of mean, and statistical tests are two-way ANOVA with Tukey-Kramer HSD.

We also examined multiple pathways previously published to regulate Pparγ activity, including AKT [23], AMPK [24], JNK [25], p38 [25], and p42/44 [26], using both murine and human PMVEC, with scrambled or anti-Cyp1b1 siRNA (Figure 3D). We found strong concordance between murine and human PMVEC, with phosphorylation of AKT, p38, and p42/44 all upregulated in the context of siCyp1b1, AMPK unaffected, and JNK phosphorylation reduced (Figure 3D). Many of these are cofactors for particular transcriptional targets, implying that although Pparγ protein may be overall reduced, there is likely differential effect on downstream targets.

3.4 | Noncanonical Estrogen Effect on Pparγ Is Only Present in Confluent Cells, and Different in WT and Bmpr2 Mutant Cells

Because of the inconsistencies in estrogen effect we saw in previous experiments, we decided to look more closely at factors which may have led to it. We tested effect of BSA-conjugated estrogen on WT and Bmpr2 mutant murine PMVEC, subconfluent compared to confluent, and at various cycloheximide timepoints. BSA-conjugated estrogen cannot cross the membrane, which means it primarily interacts with noncanonical estrogen receptors–the canonical ER1 and ER2 receptors are cytoplasmic. Cycloheximide was used to determine differential effect on ubiquitination over time.

We found that noncanonical estrogen signaling did not impact Pparγ protein levels in subconfluent cells, but resulted in a large increase in WT and decrease in Bmpr2 mutant confluent PMVEC (Figure 4). Although overall protein levels decrease over time with cycloheximide, as expected, the ratio does not change in either WT or Bmpr2 mutant cells, likely indicating that the effect was early and not continuous.

3.5 | Impact of Estrogen and Cyp1b1 siRNA Are Distinct in Genes Affected, But Have Substantial Overlap in Pathways Affected

Murine pulmonary vascular endothelial cells were treated with either scrambled siRNA or siRNA to Cyp1b1, and then treated with equimolar quantities of estrogen, 16aOHE, or vehicle. Each condition was repeated in three experimental replicates, and RNA-Seq performed to assess impact on gene expression levels.

Principal components analysis using the complete data set has several surprising features. First, there is a very consistent trajectory caused by estrogens (Figure 5A), but equimolar 16aOHE causes lower change along this trajectory than E2. Second, the impact of siCyp1b1 is nearly perpendicular to the impact of estrogen, indicating that the effects of Cyp1b1 are very different than the effects of estrogen. Third, loss of Cyp1b1 doesn't meaningfully affect estrogen effect. All of these suggest that Cyp1b1 effect in cells is quite distinct from impact of estrogens. Examination of genes affected shows roughly a thousand genes affected at $p < 0.05$ and fold change > 1.2 by either estrogen or

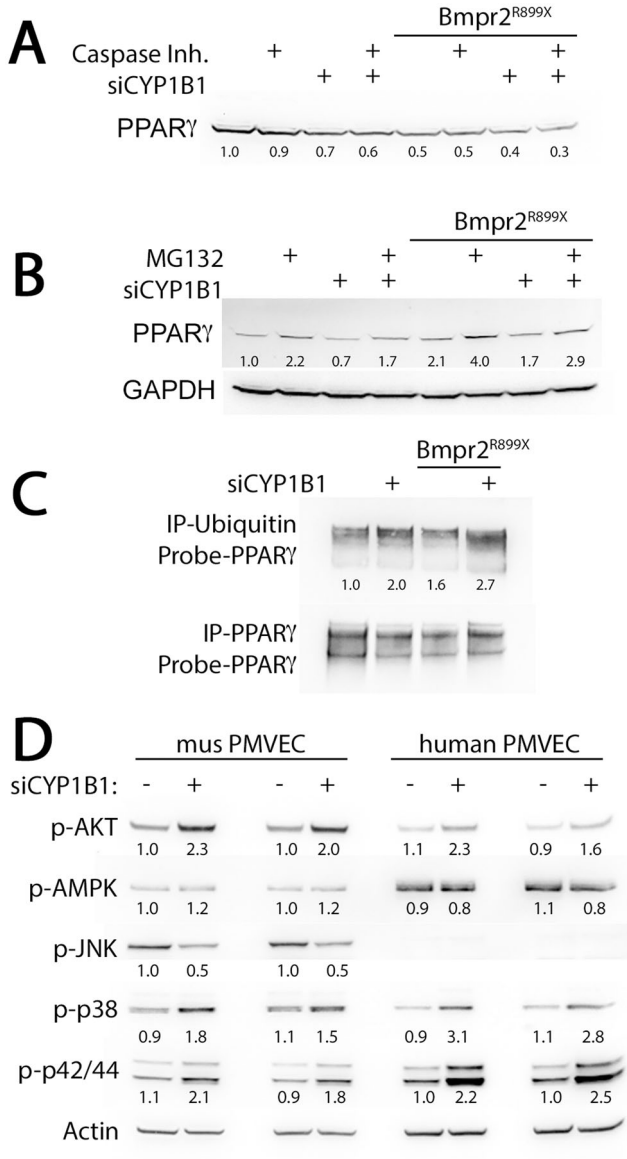

FIGURE 3 | siCyp1B1 inhibition of Pparγ protein levels is through ubiquitination, but siCyp1b1 may also regulate activity through canonical Pparγ regulatory pathways. (A) Caspase inhibition (10μM caspase inhibitor zVAD-FMK) in WT or Bmpr2 mutant PMVEC, with scrambled siRNA or siCyp1b1. siCyp1b1 reduces Pparγ levels, but this is not altered by caspase inhibition, in either WT or Bmpr2 mutant PVMEC. (B) Ubiquitin inhibition in WT or Bmpr2 mutant PMVEC, with scrambled siRNA or siCyp1b1. siCyp1b1 reduces Pparγ levels, but this is reversed with inhibition of ubiquitination. (C) immunoprecipitation with anti-ubiquitin antibodies shows roughly doubling of pulldown of Pparγ with siCyp1b1 compared to scrambled siRNA. siCyp1b1 has no impact on IP with PPARγ and probe with Pparγ. (D) Phosphorylation of proteins associated with activation of Pparγ signaling have high congruence between murine and human PMVEC, with some signaling partners showing increased phosphorylation, some decreased, and some unchanged. our p-JNK antibody didn't work for human samples. Two replicates are shown for each of human and mouse.

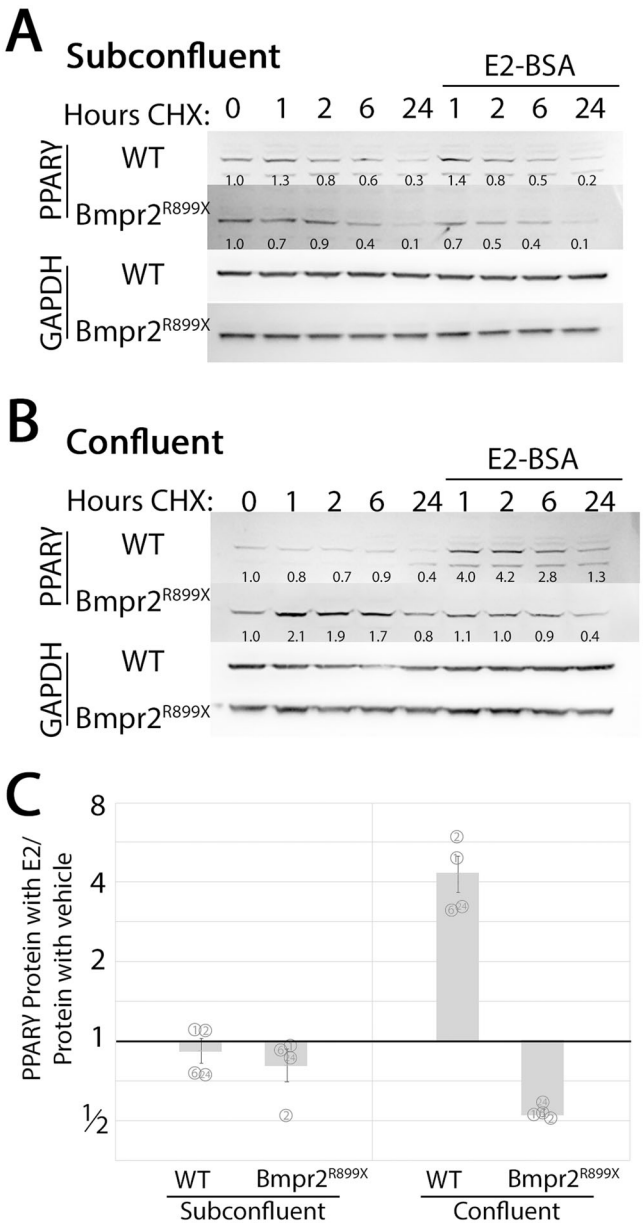

FIGURE 4 | Noncanonical estrogen signaling effect on Pparγ protein levels are dependent on confluence and Bmpr2 mutation status. (A) Wild type and Bmpr2 mutant murine PMVEC at approximately 70% confluence are treated with cycloheximide and either vehicle or E2 bound to BSA, which cannot cross the cell membrane, and thus activates primarily noncanonical estrogen signaling. Numbers are densitometry, normalized to GAPDH. (B) Similar to (A), except using cells at approximately 95% confluence (most cells are completely confluent). (C) Ratio of PPARγ levels in E2-BSA treated cells to that in controls, in (A) and (B). The numbers in the circles indicate which timepoint they correspond to; the bars indicate average and standard deviation.

Cyp1b1, and only about ¼ of these overlap (Figure 5B). Analysis of overrepresented gene ontology groups (using Webgestalt [27, 28]), however, found that despite rather different genes being altered, the gene ontology groups altered were very similar between siCyp1b1 and estrogen (Figure 5C,D). Both had significant changes (false discovery rate down to ~1×10^{-10}) in genes associated with adhesion, motility, morphogenesis, and angiogenesis, as well as carbohydrate, lipid, and small molecule metabolism. The signaling molecules were the most different category between estrogen and siCyp1b1. Looking at individual genes in these groups, there were indeed some heavily changed by estrogens but not siCyp1b1, such as angiopoietin 2 (Angpt2)

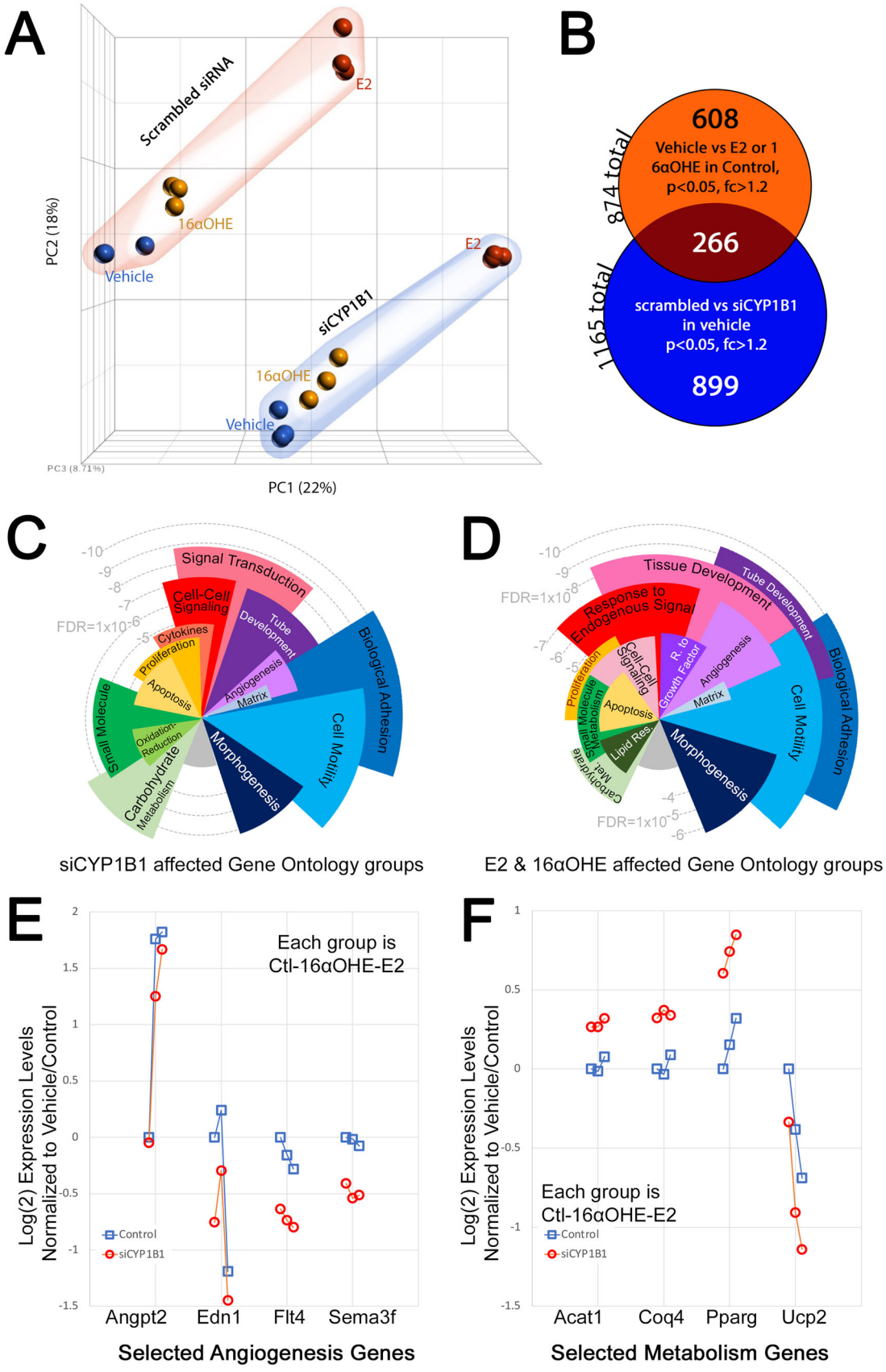

FIGURE 5 | Legend on next page.

(Figure 5E), but also some that were changed by both, such as Endothelin 1 (Edn1) and more subtly Flt4, and some such as Semaphorin 3 F that are only changed by siCyp1b1. Similar patterns are found in metabolic genes (Figure 5F). Pparγ is increased by both estrogens and Cyp1b1 in our RNASeq data, matching the results in Figure 1.

3.6 | Cyp1B1$^{-/-}$ Mice on a High Fat Diet Have Elevated RVSP and Clear Evidence of Metabolic Abnormalities

To determine if the changes seen in cell culture were reproduced in live mice, we examined Cyp1B1$^{-/-}$ mice. These mice, first created in 1999, do not have an obvious phenotype [29], and are primarily studied in the context of susceptibility to various toxins. In our hands, on normal mouse chow, they had normal right ventricular systolic pressure (RVSP), and no obvious health problems (not shown). However, when they were stressed with high fat diet, they developed more of an increase in RVSP than did control mice (Figure 6A), with matching increases in muscularized small vessels (Figure 6B). There was no genotype-dependent change in right ventricular mass (Figure 6C), although the pressures were low enough that one wouldn't necessarily expect there to be.

Although adult Cyp1B1$^{-/-}$ mice have the same weight as normoxic age and sex-matched mice (not shown), they gain weight much more slowly that wild type mice when fed a high fat diet (Figure 6D). They also have lower blood glucose than wild-type mice (Figure 6E) at close to the the same insulin level (Figure 6F). Previous studies have noted the lower weight gain with high fat diet in Cyp1B1$^{-/-}$ mice [30], but considered it a benefit. Regardless, it's clear that Cyp1B1$^{-/-}$ is causing alterations in how fats are being handled.

Malondialdehyde is only a three carbon chain, and so is generally a much shorter fatty acid product than is relevant to PAH, but was used here as a marker of lipid oxidation. We found an increase in a marker of lipid peroxidation malondialdehyde, although it was only exaggerated in the context of hypoxia in Cyp1B1$^{-/-}$ mice (Figure 6G).

We further found that Pparγ was reduced by about 40% in Cyp1B1−/− mice, both in normoxia and in addition to the ~50% reduction seen in hypoxia (Figure 6H). However, the fatty acid transporter CD36 is not altered by Cyp1B1 (although it is regulated by hypoxia), nor is AKT phosphorylation, which has been reported as both upstream and downstream [31] of Pparγ, regulated by CYP1B1. The lower Pparγ levels provides a simple explanation for lack of weight gain; high Pparγ is required to form new adipocytes [32], although we did not explicitly measure fat mass.

In all of these experiments, both male and female mice were used; results were not different by sex.

3.7 | Cyp1B1$^{-/-}$ Mouse Lung Has Alterations in Lipid Metabolism, Cytokine, Circadian Rhythm, and Vasoreactivity Genes

We performed RNA-Seq on normoxic lungs from the above experiment, with three mice per group. There were relatively few differences between Cyp1b1$^{-/-}$ and control mouse lungs. 174 genes at $p < 0.05$ and fold change > 1.3 by Kruskal-Wallace, and similar by *t*-test. However, the false discovery rate, determined by scrambling group identifiers, was 61% ± 19%, and so most of these are likely to be spurious. One of the best ways to sort the (likely) real results from the spurious is by using overrepresented gene ontology groups – if genes fall into coherent pathways, they're unlikely to be random. We found four primary categories of genes altered; lipid metabolism (**7A**), cytokines (**7B**), circadian rhythm (**7C**), and genes related to vasoreactivity (**7D**).

4 | Discussion

In this study, we demonstrated that both estrogen and inhibition of Cyp1b1 reduce Pparγ protein levels through ubiquitination rather than through regulation of transcription (Figures 1 and 3). This reduction in Pparγ is functionally important, significantly reducing capacity for fatty acid oxidation (Figure 2). In addition to increasing ubiquitination of Pparγ, though, we found that siCyp1b1 affects phosphorylation and activation of numerous Pparγ cofactors, including increasing AKT, p38, and p42/44 (but not AMPK) and decreasing JNK (Figure 3). This suggests that Cyp1b1 drives a broader alteration of Pparγ transcriptional targets, not just a broad reduction. We found that estrogen impact on Pparγ was dependent on cell confluence, receptor type, and Bmpr2 mutation (Figure 4). We also found that genes impacted by siCyp1b1 were substantially different than those impacted by estrogen treatment in pulmonary

FIGURE 5 | Impact of estrogen and Cyp1b1 siRNA are distinct in genes affected, but have substantial overlap in pathways affected. (A) Principal components analysis shows that the impact of estrogen or the estrogen metabolite 16αOHE is similar in cells with scrambled or siCyp1b1, but is along a nearly orthogonal vector to that separating scrambled siRNA from siCyp1b1. (B) Both estrogens and siCyp1b1 alter roughly a thousand transcript levels at $p < 0.05$ and fold change > 1.2, but with overlap in only about a quarter of the genes so changed in each group. (C) Significantly overrepresented gene ontology groups in cells with siCyp1b1 compared to control–gene lists are taken from (B). Radius corresponds to Log_{10}(False Discovery Rate), angular width corresponds to number of genes in the group compared to total number of annotated genes with altered regulation. Overlap is because many genes fall into multiple ontology groups; degree of overlap is approximate because this is a two dimensional figure. Color is for clarity, and roughly groups ontology groups by overlap. Gray wedge at the bottom is differentially regulated genes which do not fit in any overregulated gene ontology group. (D) Similar to (C), but for gene ontology groups altered by E2 or 16αOHE. (E) Selected angiogenesis genes, to demonstrate similarities and differences between estrogen and siCyp1b1 effect. Blue lines and markers are for scrambled siRNA cells; red for siCyp1b1. Each gene depicts from left to right, linked by lines, vehicle, 16αOHE, and E2 treated cells; each point is the average of three RNA-seq experiments. (F) Similar to (E), but for selected metabolism genes.

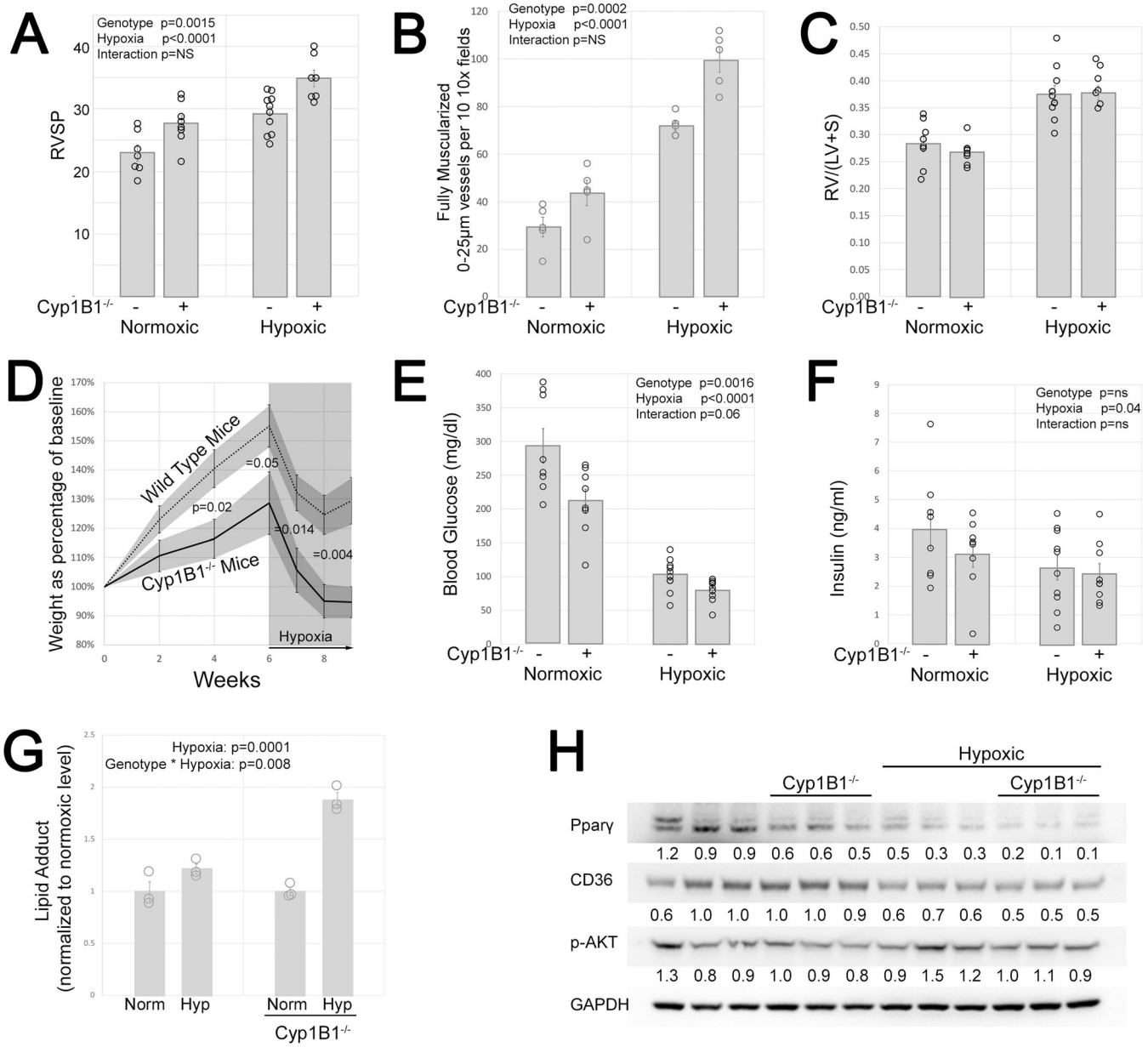

FIGURE 6 | Cyp1b1 knockout mice have elevated RVSP, reduced Pparγ, and increased muscularization of small vessels. (A) Right ventricular systolic pressure (RVSP). Each circle is a measurement from one animal. All animals were fed western diet for nine weeks; the hypoxic mice were placed in normobaric 10% O_2 for the last 3 weeks. Genotype effect $p = 0.0015$, hypoxia effect $p < 0.0001$, but interaction not significant by two way ANOVA. Pairwise comparisons using Tukey-Kramer HSD. (B) Small vessel muscularization. Each circle is a measurement from one animal, which consists of counting ten 10× fields. Genotype effect $p = 0.0002$, hypoxia effect $p < 0.0001$, but interaction not significant by two way ANOVA. Pairwise comparisons using Tukey-Kramer HSD. (C) Fulton index (RV/LV+S) is increased in hypoxic mice, but is not impact by Cyp1B1 knockout. (D) Weights of mice, normalized to their starting weight (starting weights were not different between groups). Cyp1B1$^{-/-}$ mice gained weight more slowly on high fat diet than wild-type mice did, weight difference significant at every time point past 2 weeks. (E) Blood glucose at sacrifice–mice were not fasted. Lower levels in hypoxic WT animals is normal/expected. Lower levels in Cyp1 significant at $p = 0.0016$, hypoxia effect $p < 0.0001$, but interaction not significant by two way ANOVA. (F) Insulin levels at sacrifice. No difference based on genotype, but hypoxia results in slightly lower levels ($p = 0.04$). (G) Levels of lipid peroxidation in mouse lung by malondialdehyde (MDA) assay. MDA levels go up in hypoxia ($p = 0.0001$), but go up more in Cyp1B1$^{-/-}$ mice ($p = 0.008$ for genotype-hypoxia interaction). (H) Cyp1B1 knockout reduces PPARγ protein levels ~40%, both in normoxic and hypoxic mouse lung. Hypoxia reduces PPARγ protein by ~50% on its own. Cyp1B1 knockout does not appear to meaningfully alter CD36 of phospho-AKT levels in mouse lung.

microvascular endothelial cells, although in similar gene ontology groups (Figure 5), implying that some of the effect of low Cyp1b1 in driving penetrance in patients may not be estrogen dependent. Moving on to Cyp1b1 knockout mice, we found that they spontaneously developed mild pulmonary hypertension with a high fat diet (Figure 6), associated with some other metabolic oddities. Gene expression changes in these mice were broadly along similar pathways to those seen in cell culture (Figure 7), although not as strongly different (likely because of dilution effects from using whole lung).

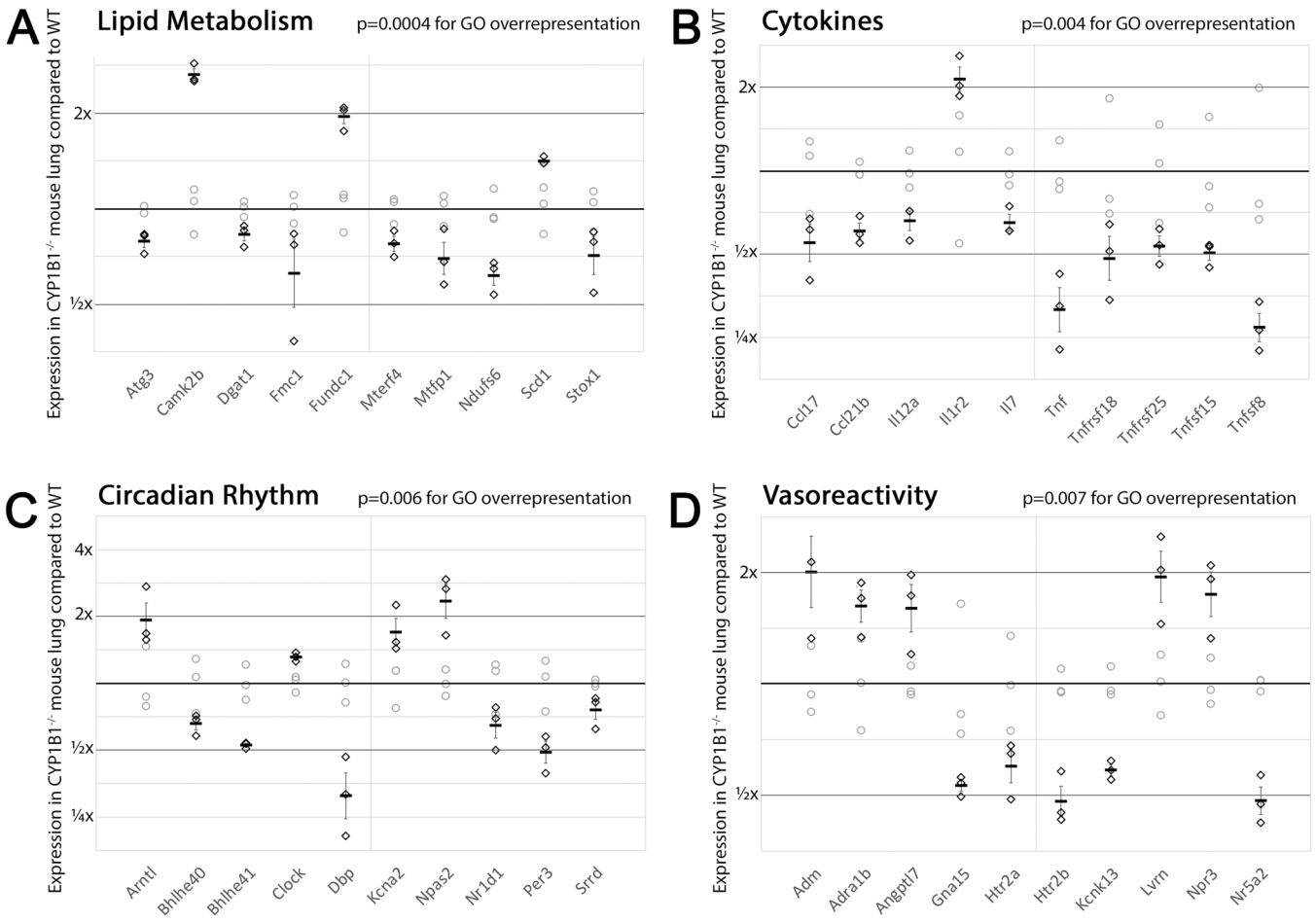

FIGURE 7 | CYP1B1 knockout mice have alterations in genes related to lipid metabolism, cytokines, circadian rhythm, and vasoreactivity. For all parts, expression has been normalized to average expression of wild-type mice. Wild type mice are represented by gray circles, and Cyp1B1$^{-/-}$ mice by black diamonds, with each symbol indicating expression in an individual animal. Average expression in Cyp1B1$^{-/-}$ mice is indicated by black horizontal bars with error bars, indicating SEM. All genes in each part fall within overrepresented gene ontology groups, with *p* values and gene-ontology names at the top of each part.

The study of Cyp1b1 in the context of pulmonary arterial hypertension began with our 2008 publication that female Bmpr2 mutation carriers that were affected had 10× lower Cyp1b1 expression in peripheral blood mononuclear cells than those who were unaffected [12], which we followed up in 2009 by showing that affected mutation carriers had much higher levels of 16αOHE than unaffected mutation carriers [9]. This was true to such a degree that there was no overlap in 2-OHE/16αOHE ratio between female affected and unaffected mutation carriers [9]. Results were similar in males, although not as extreme as in females [11]. This suggests that unless estrogen metabolism is affected by pulmonary hypertension, this alone may account for much of the penetrance question.

The Glasgow group has also shown that 16αOHE levels are higher in patients, including men, and shown Cyp1b1 polymorphisms associated with pulmonary hypertension [33–35]. Although they're thus in agreement with much of our work, a central point of disagreement is about whether Cyp1b1 activity is good or bad–they believe that increased Cyp1b1 activity, rather than decreased, is resulting in the increased 16αOHE levels (which we agree on). The reason this is a difficult controversy to resolve is that the background literature on how Cyp1b1 metabolized estrogen is unclear, and it is extremely difficult to measure estrogen metabolites out of tissue [36]. A third group, Pittsburgh, believes we're both wrong–that Cyp1b1 has very little to do with 16αOHE levels one way or another [37]. In this view, Cyp1b1 primarily regulates production of a different estrogen metabolite, 4-OH estradiol [38], and is primarily important in metabolizing amino acids into mid-chain hydroxyeicosatetraenoic acids (HETEs) [39], thus driving inflammation. The data in the current manuscript leans a little towards the Pittsburgh interpretation – estrogens are important, and Cyp1b1 is important, but they don't appear to be strongly interacting, based both on the fact that Cyp1b1 regulation of Pparγ protein appears to be independent of estrogen, and on the gene expression data (Figure 5). That said, both in our previous publications and in this, the correlation between estrogens and Cyp1b1 seems strong enough to defy coincidence, although the correlation may be reversed – with Cyp1b1 being regulated by the relevant estrogens rather than regulating them [40]. In our RNA-seq in endothelial cells, E2 treatment resulted in a 30% increase in Cyp1b1 expression.

Our transcription data has indirect evidence for HETE as being an important consequence of Cyp1b1 knockout. One of the

things that HETE does is regulate cell-cell adhesion, which dominates our cell culture RNA-seq and turns up in our whole lung RNA-seq as well. Loss of vascular barrier function is both associated with pulmonary hypertension [41], and has been reported as a consequence (in brain) of Cyp1b1 knockout [42], secondary to alterations in HETE, and so it's plausible (although we do not have direct evidence for it) that alteration in HETE is part of what's driving both phenotype and gene expression changes with loss of Cyp1b1.

Considering the gene expression changes seen in whole lung from Cyp1b1 mice, the lipid metabolism genes with altered expression levels aren't those that one primarily thinks of as key regulators of lipid metabolism. This isn't unexpected; our prior results suggested that Cyp1B1 was regulating fatty acid metabolism on a protein, not transcriptional, level. These gene alterations are thus likely secondary effects. The alterations in vasoreactivity genes are similarly probably second order effects from both the defects in lipid metabolism and from the elevation in pressures, although many of these have been implicated directly in PAH before. Note altered regulation of the two serotonin receptors, Htr2a and Htr2b, as well as Npr3, all of which have been previously implicated in PAH [43–46]. In fact, Glasgow has previously shown a direct interaction between serotonin and Cyp1b1, although running the other direction [35]. Alterations in cytokines are also to be expected–they are a required part of the remodeling process – although the specific cytokines altered here aren't generally associated with PAH, with the exception of IL1 [47, 48], whose receptor is increased 2×. The last group–circadian rhythm–is not clearly related to PAH, but there are several papers indicating that cyp1b1 directly metabolizes melatonin [49, 50], and potentially circadian rhythm [51], and so this could be a fairly direct effect of an alternate cyp1b1 target.

Taking a broader view, this manuscript suggests that one reason that women are more likely to develop PAH is because the increased estrogens and decreased CYP1B1 drive down PPARg and thus fatty acid oxidation, which then predisposes to disease. Is there any evidence that men and women are different in how they handle fatty acids? Quite a bit, actually, although the source of the disparity is controversial, and it's mostly in exercise literature. Men are reported to have higher resting fatty acid oxidation than women [52], although fat oxidation during aerobic exercise was higher in women than men [53]. For at least some researchers, adding estrogen to men changed their metabolic phenotype to be similar to women [54]. However, there is a lot of disagreement on exactly what's happening in sex differences in fatty acid oxidation in which tissue under which circumstances [55]. Overall, though, our data suggesting estrogen and CYP1B1 drive PPARG and thus metabolism is not inconsistent with human data.

This study has several limitations, the largest being all of the data we didn't collect. We did not collect urines, and were not able to measure estrogen metabolites from the tissues (mass-spec methodologies for these metabolites from whole tissues is apparently quite difficult). Since we were primarily thinking about impact of Cyp1b1 on metabolism, we did not explicitly collect data on alteration in HETEs. In addition, we were unable to prevent some level of EndoMT in our endothelial cell culture. Finally, for many of the westerns, we have data on levels of phosphorylated protein, but not total protein. This means that we can't distinguish between whether relative phosphorylation was unchanged and protein level decreased, or whether protein was unchanged and phosphorylation was decreased.

Despite these limitations, this project has added a great deal to our understanding of contribution of estrogens, and particularly Cyp1b1, to pulmonary arterial hypertension. In vitro, in both mouse and human cells, we find that knockdown of Cyp1b1 results in reduction in Pparγ protein through increased ubiquitination, functionally associated with reduced fatty acid oxidation. Cyp1b1 knockout mice develop moderately elevated pressures when stressed with a high fat diet. Gene expression changes in both cells and in mice suggest that the phenotype is driven by metabolism and inflammation. Given the apparent at least partial disconnect between estrogens and the metabolic effects of Cyp1b1, though, the Cyp1b1 substrates that are driving the ubiquitin binding of Pparγ are unclear, and an important topic for future studies.

Author Contributions

Conceptualization: Eric Austin, James West, Xinping Chen and Anna Hemnes. Methodology: Jingyuan Chen, Xinping Chen, Vineet Agrawal, Christy S. Moore, Tom Blackwell, Santhi Gladson, and Anandharajan Rathinasabapathy. Validation: Jingyuan Chen, Christy S. Moore and Tom Blackwell; Formal Analysis, James West and Jingyuan Chen. Investigation: Jingyuan Chen, Xinping Chen, Vineet Agrawal, Christy S. Moore, Tom Blackwell, Santhi Gladson, Nivedita Rathaur, and Anandharajan Rathinasabapathy. Data Curation: James West and Jingyuan Chen. Writing – original draft preparation: James West and Jingyuan Chen. Writing – review and editing: Anna Hemnes, Jingyuan Chen, Vineet Agrawal, and James West. Visualization: James West and Jingyuan Chen. Supervision: James West and Santhi Gladson. Funding acquisition: Anna Hemnes, Eric Austin, and James West.

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Ethics Statement

All the mice utilized in this study were housed at Vanderbilt University Medical Center animal care facility, which is certified by the Association for Assessment and Accreditation of Laboratory Animal Care International. All animal protocols were approved by the Institutional Animal Care and Use Committee at Vanderbilt University Medical Center in compliance with National Institute of Health guidelines.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are openly available in Gene Expression Omnibus at GSE166006 (cells) and GSE225402 (mice). Other data will be happily shared on request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.