METFORMIN REVERSES DEVELOPMENT OF PULMONARY HYPERTENSION
VIA AROMATASE INHIBITION

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Abstract Females are more susceptible to pulmonary arterial hypertension than males, although the reasons remain unclear. The hypoglycaemic drug, metformin, is reported to have multiple actions, including the inhibition of aromatase and stimulation of AMPK. Inhibition of aromatase using anastrazole is protective in experimental pulmonary hypertension but whether metformin attenuates pulmonary hypertension through this mechanism remains unknown. We investigated whether metformin affected aromatase activity and if it could reduce the development of pulmonary hypertension in the sugen 5416/hypoxic rat model. We also investigated its influence on proliferation in human pulmonary arterial smooth muscle cells. Metformin reversed right ventricular systolic pressure, right ventricular hypertrophy and decreased pulmonary vascular remodelling in the rat. Furthermore, metformin increased rat lung AMPK signalling, decreased lung and circulating estrogen levels, levels of aromatase, the estrogen metabolizing enzyme; cytochrome P450 1B1 and its transcription factor; the aryl hydrocarbon receptor. In human pulmonary arterial smooth muscle cells, metformin decreased proliferation and decreased estrogen synthesis by decreasing aromatase activity through the PII promoter site of Cyp19a1. Thus, we report for the first time that metformin can reverse pulmonary hypertension through inhibition of aromatase and estrogen synthesis in a manner likely to be mediated by AMPK.

Key words: Pulmonary hypertension, metformin, estrogen, aromatase, AMP-activated protein kinase
Introduction

Human pulmonary arterial hypertension (PAH) is a progressive disease of the pulmonary vasculature, which often leads to right heart failure. PAH is characterised by elevated pulmonary pressure (>25mmHg at rest), vascular remodeling and occlusive pulmonary vascular lesions. Although survival rates are improving slowly; there remains an unacceptably poor survival rate.¹

Females are more susceptible to PAH than males, as indicated in, for example, the REVEAL registry where around 80% of the patients registered were female.²,³ However male patients, have a poorer survival rate than females, implicating sex hormones in both PAH susceptibility and survival.⁴

Consequently the role of estrogens in PAH has been accumulating significant interest.⁵⁻⁸ There is an estrogen ‘paradox’ however as whilst exogenous estrogen may be protective against experimental pulmonary hypertension (PH) and right ventricular (RV) hypertrophy in rodent models,⁹⁻¹² endogenous estrogen is causative in female animal models.¹³⁻¹⁷ Estrogen is synthesised from testosterone through the enzyme aromatase and inhibition of aromatase attenuates experimental PH but only in females.¹⁷ Estrogen can be metabolised through the cytochrome p450 1b1 (CYP1B1) enzyme to form the mitogenic 16α-hydroxyestrone (16α-OHE1), a metabolite established to be pathogenic in PH.¹⁶ Furthermore, single nucleotide polymorphisms of CYP1B1 have been recently identified to be associated with PH and indicated to play a key role in sexual dimorphism in RV failure.¹⁸ One transcription factor for CYP1B1 is the aryl hydrocarbon receptor (AhR), suppression of which has been shown to be anti-proliferative (protective) in PH.¹⁹
Metformin is a well-established drug for use in type 2 diabetes mellitus (T2DM) and has been demonstrated to activate AMP-activated protein kinase AMPK in many tissues, although AMPK does not underlie the hypoglycaemic actions of metformin. AMPK is a ubiquitously expressed serine/threonine protein kinase, which plays a critical role in cellular and organ metabolism. In breast cancer and polycystic ovarian syndrome (PCOS) the therapeutic effects of metformin involves the enhancement of the AMPK pathway and inhibition of the nuclear translocation of CREB-regulated transcription coactivator 2 (CRTC2). CRTC2 (a downstream target of AMPK) can bind to the PII promoter site of the aromatase (Cyp19a1) gene and regulate aromatase activity and expression. In breast cancer and PCOS, it is hypothesised that an additional therapeutic mechanism of metformin may be aromatase inhibition. Metformin has been shown to reverse the development of experimental PH. Here we examine the hypothesis that metformin may reverse experimental PH through inhibition of aromatase in a manner mediated by AMPK activation.

Sugen (SU5416) is an inhibitor of vascular endothelial growth factor (VEGF) R2 receptors. A single dose of sugen combined with hypoxia followed by weeks of normoxia provides a refined model of PAH exhibiting high pulmonary pressures and occlusive pulmonary lesions (the sugen/hypoxic rat model ((hereafter termed SuHx))). As this is a gold-standard model for testing potential new therapies, we used this model to determine if any therapeutic effect of metformin was associated with aromatase inhibition in PH. We have previously shown that aromatase inhibition was only effective in female hypoxic and SuHx rat models of PH, having no effect in male rats with PH. As we were specifically looking to identify the inhibitory action of metformin on aromatase, we therefore selected females for this study. We
further investigated the mechanism of action of metformin in human pulmonary arterial smooth muscle cells (hPASMC) from non-PAH and PAH female patients.
Materials and Methods

Animals studies

Wistar Kyoto adult (nine weeks) female (Harlan, UK) rats were divided into three groups (n=12 per group), control (with no PH induced), vehicle dosed (0.9% (w/v) NaCl in distilled water for 21 days) and metformin dosed. The latter two groups received a single dose of Sugen-5416 (Novartis, UK) (20mg/kg), before being placed in a hypobaric chamber (atmospheric pressure 550 mbar) for two weeks and then placed in normal room pressure (1013 mbar) for three weeks whilst PH developed. Animals were then dosed, daily (oral gavage) with vehicle or with metformin (100mg/kg (dissolved in 0.9% (w/v) NaCl) p.o., od for a further 21 days). Full details of subsequent haemodynamic measurements (right ventricular systolic pressure (RVSP), right ventricular hypertrophy (RVH) can be found in the online supplement.

Immunohistochemistry

Immunohistochemistry was carried out as described previously and further details are described in the online supplement.16,17

Protein Analysis

Protein analysis was carried out by western blotting as described previously and in the online supplement.16,17

Gene expression analysis

mRNA was measured by RT-PCR as described previously, reagent/probe details are described in the online supplement.16,17
**Estrogen Immunoassay**

Levels of 17β estradiol were determined by competitive immunoassay in lung samples and plasma from female rats from each group, full details are in the online supplement.

**Human Pulmonary Arterial cells**

Human PASMCs were provided by Professor Nicholas W. Morrell (University of Cambridge) and cultured as described previously and in the online supplement.

**Aromatase activity Assay (tritiated water-release assay)**

Aromatase activity was assessed using a modification of methods described previously\(^3\) and in the online supplement.

**Statistics**

Data is represented as the group mean ± SEM. Data was analyzed by either a student’s t test (where there were only two groups), or a one-way ANOVA followed by a Bonferroni’s Post Hoc test.
Results

**Metformin reduces the pulmonary hypertension phenotype in the SuHx female rat model and reverses proliferation of hPASMCs**

SuHx vehicle-dosed rats developed increased RVSP and RVH and this was normalised by metformin treatment (Figures 1A, B). A majority of the pulmonary arteries within vehicle animals were remodeled and this was significantly reduced by metformin treatment (Figure 1C). Only in vehicle-treated animals were occluded lesions found (Figure 1D) (similar to human disease) suggesting severe PH. No occluded lesions were found in any of the metformin dosed animals (Figure 1D). Remodeling and occluded lesions were confirmed by immunohistochemistry (Figure 1E, 1F, 1G)

No differences were found in mean systolic arterial pressure (mSAP), glucose levels, cardiac output or heart rate (Figure S1A, 1B, 1C and 1D). Increased proliferation (indicated by PCNA) was found in vehicle exposed SuHx animals, particularly in occluded lesions and PCNA levels were decreased in metformin-treated rats (Figure 1H), illustrating the anti-proliferative effects of metformin within the SuHx model.

**In the SuHx PH rat model, metformin decreases estrogen levels and estrogen metabolism and enhances the lung AMPK pathway**

To determine if metformin was affecting CRTC2, aromatase and subsequent estrogen synthesis, we first measured lung gene expression of CRTC2. Metformin caused a significant decrease in CRTC2 mRNA levels compared with vehicle-dosed and control animals (Figure 2A). An increase in aromatase protein levels was found
in the lungs of SuHx animals, metformin reversed this increase in lung aromatase protein (Figure 2B). Plasma estrogen levels were increased in the SuHx vehicle group (Figure 2C). Metformin treatment decreased both circulating and local lung estrogen levels consistent with the decreased aromatase levels (Figure 2C, 2D). CYP1B1 protein levels in lung were significantly increased in the SuHx control group and this was decreased by metformin treatment (Figure 2E). Immunohistochemistry indicated lung aromatase, CYP1B1 and AhR levels (Figure 2F) were increased in vehicle exposed SuHx animals, particularly in occluded lesions; expression of each protein was decreased in metformin exposed rats. Metformin caused a significant increase in lung pAMPK (Figure 2G) levels as well as downstream phosphorylation of ACC (Figure 2H), indicative of an increase in AMPK activity. Protein levels of the AMPK kinase-LKB1 (upstream of AMPK) in the rat lung were unchanged by vehicle or metformin treatment in comparison to control animals (S2A), indicating metformin directly activates AMPK (without altering levels of the upstream kinase).

**Metformin is anti-proliferative in hPASMCs. Exposure of female hPASMCs to metformin decreases CRTC2 gene expression, decreases PII promoter activity of the CYP19A1 (aromatase) gene and decreases aromatase activity in female hPASMCs.**

Metformin decreased serum-induced proliferation in hPASMCs in both non-PAH (~58%) (Figure 3A) and PAH patient cells (~70%) (Figure 3B). Inhibiting aromatase with anastrozole also decreased PASMC proliferation in non-PAH (~56%) and PAH patient cells (~66%) (Figure 3A, 3B). All results were confirmed by studying BrdU incorporation (S3A, 3B). The AMPK activator AICAR also decreased proliferation in patient cells (~59%) (Figure 3B). In hPASMCs, metformin treatment decreased
CRTC2 gene expression (41%) in non-PAH cells and 61% in PAH cells (Figure 3C). Metformin, anastrazole and AICAR all significantly reduced activity of the PI1 promoter region of the aromatase gene in non-PAH hPASMC cells transfected with a luciferase reporter gene (Figure 3D). Aromatase activity in non-PAH hPASMCs was decreased by metformin, AICAR and anastrozole treatments (Figure 3E).

**Exposure of female hPASMCs to metformin decreases downstream estrogen metabolism and increases pAMPK levels**

AhR levels were increased in PAH hPASMCs in comparison to non-PAH hPASMCs (Figure 4A). Metformin significantly reduced AhR levels in PAH hPASMCs (Figure 4A). CYP1B1 levels were increased in PAH hPASMCs in comparison to non-PAH hPASMCs (Figure 4B) this was also decreased by metformin in PAH hPASMCs (Figure 4B). Metformin treatment of non-PAH hPASMCs increased pAMPK (Figure 4C) and downstream pACC (Figure 4D). LKB1 expression (upstream of AMPK) was unchanged (S2B). Incubation of hPASMCs with AICAR or the aromatase inhibitor anastrozole for 24h did not activate the AMPK pathway as assessed by pAMPK and pACC (Figure 4C, 4D respectively). However, we found AICAR to activate the AMPK pathway in hPASMCs, earlier at a 2 hour time point (S4).
Discussion

We report metformin can reduce PH in the rat SuHx model as well as proliferation in hPASMCs and describe a novel mechanism of action via aromatase inhibition.

Metformin reversed the elevation in both RVSP and RVH in the SuHx rat, reduced pulmonary vascular remodeling and completely deterred the formation of occluded pulmonary vascular lesions suggesting metformin inhibited the disease from reaching severity. Metformin had no effect on systemic blood pressure, plasma glucose levels and no effect on cardiac output and heart rate, demonstrating that the observed effects are pulmonary specific and independent of glycaemia. Metformin reduced proliferation in hPASMCs consistent with the anti-proliferative effects of metformin reported in other cell types, (MCF7s and glioma cells in cancers).\textsuperscript{32,33} We considered possible mechanisms by which metformin could be exerting its therapeutic and anti-proliferative effects.

For the first time we report metformin inhibits estrogen synthesis in the female SuHx rat and aromatase activity within hPASMCs. We have reported previously, aromatase expression is elevated in lungs from SuHx rats and inhibiting aromatase using anastrozole reversed PH in the female SuHx model.\textsuperscript{17} In the current study, metformin reduced elevated aromatase expression to normal in the lungs and importantly, it reduced both lung and plasma estrogen levels in the SuHx rat. This confirms aromatase inhibition is protective in PH, furthermore the aromatase decreasing effects of metformin did not have any adverse effect on the right ventricle in these studies. Indeed, metformin is the first line of treatment for thousands of people with diabetes and there are no reports of right ventricular dysfunction following chronic treatment.\textsuperscript{22} The inhibition of aromatase by metformin is of key
interest as women are more susceptible to PAH and estrogens and or estrogen metabolites may therefore be pathogenic in this disease. Additionally, genetic variation of aromatase and estrogen signaling is also involved in other related pathogenicities such as portopulmonary hypertension (PPHTN)\textsuperscript{34}. Recently it has been shown that iPAH (idiopathic pulmonary arterial hypertension) male patients have elevated circulating estrogen levels,\textsuperscript{18} associated with poorer 6 minute walk distance and RV function and consequently it is worth considering anastrazole or metformin (given its effects on aromatase) may well be effective in male patients and post-menopausal women at select doses. This data may indicate limitations with regards to differences seen in human vs animal studies as we have previously shown that male mice with PH have very low circulating estrogen levels and estrogen levels in male Su/Hx rats were undetectable unlike the human male patients.\textsuperscript{17}

We show metformin can both inhibit aromatase activity and decrease the activity of a reporter gene containing the PII promoter of the aromatase gene. It is well established that CRTC2 can bind to the PII promoter site of aromatase \textsuperscript{27} and we show metformin reduced CRTC2 gene expression in rat lung as well as hPASMCs. Phosphorylated CRTC2 stops nuclear translocation, although we were unable to measure phosphorylation, our studies demonstrate metformin can alter CRTC2 mRNA expression, which may offer a novel means by which metformin can regulate CRTC2. These results may reflect reduced CRTC2 activity, as suggested by our results of decreased PII driven expression and as supported by the work of others \textsuperscript{35} indicating metformin activated AMPK inactivates CRTC2 to decrease aromatase expression. Interestingly, CRTC2 mRNA was not found to be increased in our SuHx vehicle rat group however the same group had significantly increased aromatase
expression. It should be noted that protein levels might not reflect mRNA levels; we were unable, however, to measure CRTC2 protein due to lack of an efficient antibody. We have shown previously that animals with PH (both mouse hypoxic and rat sugen model), have increased aromatase expression in remodeled pulmonary arteries. In addition, female lungs from the animal PH models and female hPASMCs express increased levels of aromatase protein compared with male\textsuperscript{17} in comparison to respective controls. The increased aromatase we report did not correlate with increased local estrogen. There is increased metabolism of estrogen in the SuHx rat model \textsuperscript{16} and hence whilst there may be more synthesis of estrogen, it will be metabolised quickly to metabolites such as 16α-OHE\textsubscript{1} \textsuperscript{16} hence levels of actual estrogen may not necessarily be expected to mirror levels of aromatase.

Here we demonstrated an increase in whole lung aromatase in the SuHx rat [Sugen plus 3 weeks hypoxia plus 6 weeks normoxia] rat whereas in our previous study this did not reach significance in the SuHx rat lung [Sugen plus 2 weeks hypoxia plus 2 weeks normoxia].\textsuperscript{17} This most likely reflects the different protocols adopted between the two studies.

CYP1B1 is a major enzyme in estrogen metabolism, converting estradiol to pro-mitogenic estrogen metabolites.\textsuperscript{36} CYP1B1 plays a role in the development of PAH\textsuperscript{16} and metformin has been shown to inhibit CYP1B1 activity in breast cancer cells.\textsuperscript{37,38} We demonstrate CYP1B1 to be increased in PAH hPASMCs in comparison to control cells (as reported previously\textsuperscript{16}) and that metformin treatment was beneficial in reducing CYP1B1 expression in both the SuHx rat model as well as in hPASMCs.

AhR is a transcription factor known to regulate CYP1B1.\textsuperscript{39,40} To the best of our knowledge, only one study has reported a role for AhR in PH, whereby inhibition of
TGF-β1 using baicalin led to a decrease in hPASMC proliferation through a decrease in AhR.\textsuperscript{19} AhR antagonism is considered a promising mode of treatment and preventive target for diseases, such as gastric and breast cancer and nitric oxide dependent vasculature diseases.\textsuperscript{41,42} We found elevated expression of AhR in vehicle SuHx rats, particularly in occluded lesions and this was reversed by metformin. For the first time, we demonstrate increased AhR expression in hPASMCs from PAH patients in comparison to non-PAH patients and we show that metformin can reduce AhR expression in both rat lung and hPASMCs.

We found pAMPK levels were not changed in the lungs of female SuHx rats in comparison to control, but metformin treatment induced an increase in pAMPK and also the downstream substrate, pACC, suggesting the pathway is upregulated and may be protective in PH, as described previously.\textsuperscript{28} Metformin also increased phosphorylation of AMPK and ACC in hPASMCs. Interestingly hPASMC treatment with the AMPK activator, AICAR did not have the same effect at the 24 hour time point. This may reflect the mechanism by which AICAR activates AMPK, whereby AICAR is transported into the cell and phosphorylated to form ZMP.\textsuperscript{43} The accumulated ZMP binds directly to the γ subunit of AMPK, mimicking AMP and stimulating AMPK phosphorylation. Initial activation of AMPK would be sufficient to retard the cell cycle and thereby reduce proliferation, in the same way that stimulating cells with growth factors only transiently increases proximal signalling pathways whereas increased proliferation, which relies on the time taken to undergo cytokinesis remains increased long after those signalling pathways have returned to basal levels. The timescale of our experiments may, therefore, have exceeded the time course of ZMP accumulation and AMPK activation. Metformin inhibits mitochondrial ATP synthesis, reducing the cellular energy charge, which activates
AMPK. This process may well continue for 24 hours, such that the AMPK pathway may still be activated after 24 hours in response to metformin but not AICAR. Subsequently we demonstrated AICAR activates AMPK in hPASMCs at a much earlier time point of 2 hours (SF4).

Enhancement of the AMPK pathway is involved in regulation of several different pathways; therefore the therapeutic effects of metformin could involve additional mechanisms. For example, AMPK activation can affect the cyclooxygenase pathway as well as the mTOR pathway, both known to be dysregulated in PAH. Moreover, metformin targets the mitochondria of cells and this is where steroidogenesis takes places. Emerging data indicates metformin can dampen the entire steroidogenesis pathway, and activated AMPK is reported to inhibit HMG CoA reductase (which regulates cholesterol synthesis) thus leading to a decrease in steroidogenesis.

It is possible that all the therapeutic effects of metformin could be driven via its ability to increase AMPK, and this work is supported by other studies which indicate AMPK to be protective in PH, however, conflict exists, whereby it has also been shown that a decrease in AMPK may be protective against PH.

We previously showed that aromatase inhibition was only effective in female hypoxic and SuHx rat models of PH, having no effect in male rats with PH. As we were specifically looking to identify the additional inhibitory action of metformin on aromatase, we therefore selected female rats. In addition, metformin has already been shown to reverse PH in male hypoxic and monocrotaline PH rats via other mechanisms of action, therefore it would most certainly have had an effect in males in our model via other mechanisms. This would not, however, have determined any additional effect on aromatase activity. We were unable to measure different
estrogen metabolites in the lung tissue from our experimental rats as there are no available assays with sufficient sensitivity. We and others have however provided evidence that increased CYP1B1 may contribute to PAH pathology via increased accumulation of mitogenic metabolites such as 16α-OHE1.16,50,51

In summary, we have demonstrated metformin to be protective in PH in the rat SuHx model and anti-proliferative in hPASMCs. We show aromatase expression and activity as well as estrogen metabolism (via AhR/CYP1B1 axis inhibition) in both rat and human models were down regulated by metformin. We report that metformin increases pAMPK, which can affect CRTC2 gene expression, and aromatase PII promoter activity. This mechanism is summarized in S5. As metformin is already in widespread use in the clinic and well tolerated by patients, our study denotes this drug to be beneficial in experimental PH and merits further investigation as a therapy for PAH. The study also suggests a role for AhR in PH which is worthy of further investigation.
**Perspectives:**

The precise role of estrogen, its formation and its metabolites, in pulmonary arterial hypertension requires to be fully understood in order to understand why females are more susceptible to the disease. Our studies show metformin can inhibit aromatase expression and activity to decrease estrogen but also its metabolism and this is beneficial in reversing the disease phenotype in females. In pulmonary hypertension, this is a novel mechanism which is highly relevant given the role of estrogen metabolites and estrogen synthesis in the disease. Furthermore, metformin is already in widespread use as an anti-cancer drug and well tolerated by patients and thus suggests a practical therapy for females with this devastating disease.

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**Disclosures:** None
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Novelty and significance

What Is New?

- We have identified that metformin can reverse pulmonary hypertension in females through inhibition of aromatase and decrease of estrogen and its metabolites, this mechanism has not been reported in PH previously.
- We report, for the first time, that AhR is increased in female patient pulmonary arterial smooth muscle cells in comparison to non-patient pulmonary arterial smooth muscle cells and metformin can decrease AhR.

What is relevant?

- The ability of metformin to affect steroidogenesis to reverse PH in females is extremely relevant as a gender bias exists in this disease.
- Given the importance of AhR in mediating toxicity, it is very relevant to note that metformin can decrease levels of AhR and that AhR may be modulated by AMPK in this disease.

Summary

Our study shows that metformin may provide an alternative therapy for females with pulmonary arterial hypertension through its target of AMPK and estrogen synthesis and metabolism.
Figure Legends

**Figure 1.** Metformin treatment attenuates SuHx induced pulmonary hypertension in female rats and is anti-proliferative. (A) Right ventricular systolic pressure (RVSP, n=8-10 per group), (B) Right ventricular hypertrophy (RVH, n=10-13 per group) and (C) percentage of remodeled arteries in lungs without treatment (control) and SuHx treatment with vehicle or metformin, n=7-12. (D) Percentage of occluded vessels found in each group, n=7. (E) Representative images showing elastic laminae stained indicative of extent of remodelling of arteries. (F) Representative image of occluded vessel (only found in vehicle exposed animals) stained with anti-von Willebrand factor. (G) Representative image of complex vascular lesions (similar to human disease) (only found in vehicle exposed SuHx animals) stained with anti-von Willebrand. (H) Representative images and immunoratio quantification of proliferation (stained by PCNA) within the female rat lung. Solid scale bar represents 20µm, dashed scale bar represents 50µm. Data represents mean ±SEM. * P<0.05, **P<0.01 ***P<0.001 as indicated, determined by one way ANOVA followed by Bonferroni post test.
**Figure 2.** Metformin decreases estrogen levels and metabolism and enhances the lung AMPK pathway in female rat lung

Effect of Su/Hx treatment with or without metformin on (A) relative gene expression levels of CRTC2 (n= 6-9 per group), (B) aromatase protein levels (representative immunoblot and quantification of n= 4-8 per group shown), (C) circulating estrogen levels, (D) Local estrogen levels within lung, n=8-10, (E) CYP1B1 protein levels (representative immunoblot and quantification of n= 4-8 per group shown), (F) Localisation and expression of aromatase (brown staining), CYP1B1 (red staining) and AhR (pink staining) in lung vessels (representative images shown), (G) AMPK phosphorylation levels and (H) ACC phosphorylation levels. Representative immunoblots and quantification of n=7-8 per group are shown.

Scale bar represents 20µm. C=Control (no sugen/hypoxic treatment), V=Vehicle (sugen/hypoxic treatment), M=Metformin (sugen/hypoxic treatment with metformin). Data is displayed as a mean ±SEM. * P<0.05, **P<0.01 ***P<0.001 as indicated, determined by one way ANOVA followed by Bonferroni post test.
Figure 3. Metformin is anti-proliferative in hPASMCs, through an aromatase and AMPK axis and exposure of female hPASMCs to metformin decreases CRTC2 gene expression, aromatase activity through the PII promoter region of cyp19a1. (A) Normalised cell counts of non-PAH (A) and PAH (B) female hPASMC depicting proliferation due to 10% (v/v) serum, and anti-proliferative effects of 24 hour treatment with either metformin, AICAR or anastrozole (in the presence of 10% serum), n=4-5 different cell lines, each count repeated in triplicate. (C) Relative gene expression levels of CRTC2 in non-PAH and PAH hPASMCs exposed to metformin for 24 hours, n=4 per group. (D) Luciferase activity of non-PAH cells transfected with PII promoter site of cyp19a1 gene construct and exposed to treatment. (E) Aromatase activity in non-PAH hPASMCs exposed to metformin, AICAR or anastrozole for 24 hours, repeated in 2 different non-PAH cell lines. Data is displayed as mean ±SEM. * P<0.05, **P<0.01 ***P<0.001 as indicated, analysed by one way ANOVA followed by Bonferroni post test.
Figure 4. Exposure of hPASMC to metformin decreases AhR, CYP1B1 and enhances the AMPK pathway. (A) Representative immunoblot and quantification, showing basal levels of AhR protein expression in female non-PAH and PAH hPASMCs and effect of metformin on AHR expression n=3-5 per group. (B) Representative immunoblot and quantification, showing basal levels of CYP1B1 protein expression in female non-PAH and PAH hPASMCs and effect of metformin, n=3-6 per group. Phosphorylated AMPK (C) and phosphorylated ACC (D) after 24 hours of exposure to the indicated agents. N=2-3 different cell lines per group. C=Control, M=Metformin, AC=AICAR, A=Anastrozole. Data is displayed as mean ±SEM. * P<0.05, **P<0.01 ***P<0.001 as indicated, determined by unpaired t-test when comparing only two groups all others analysed by one way ANOVA followed by Bonferroni post test.
A) Cell counts (normalised to control)

B) Cell counts (normalised to control)

C) CRTC2 mRNA levels

D) Normalised luciferase activity

E) Aromatase activity fold change
Online Supplementary Data

METFORMIN REVERSES DEVELOPMENT OF PULMONARY HYPERTENSION VIA AROMATASE INHIBITION

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Materials and Methods

Ethics
All experimental procedures were carried out in accordance with the United Kingdom Animal Procedures Act (1986) and with the "Guide for the Care and Use of Laboratory Animals" published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996), and ethical approval was also granted by the University of Glasgow Ethics Committee. Rodents were housed in a 12-hour light dark cycle with access to food and water ad libitum. Experimental procedures using hPASMCs conform to the principles outlined in the Declaration of Helsinki.

Animal studies

In vivo Hemodynamic Measurements
Wistar Kyoto female rats, aged 9 weeks, were purchased from Harlan, UK. The animals were housed in a 12-hour light-dark cycle with access to food and water ad libitum. Animals were housed together to promote synchronisation of their estrous cycles two weeks prior to the study. In order to ensure animals were synchronising together, a blunt, shortened tip of a Pasteur pipette was placed at the vaginal orifice. One drop of PBS was gently expelled into the vagina and aspirated back before being transferred to a microscope slide. Smears were examined microscopically and classified as to the stage of the cycle.

In vivo Hemodynamic Measurements
Animals were anaesthetically induced in 3% (v/v) isoflurane and then maintained at 1.5-2% (v/v) isoflurane supplemented with a constant flow of 5% (v/v) oxygen. Under anaesthesia, prior to hemodynamic measurements being taken, a small volume (2-3 drops) of blood was removed from the tail vein for glucose measurements, using an Accu-Chek mobile glucose monitor and strips (https://www.accu-chek.co.uk/gb/). There was no requirement for analgesic or tranquilizing drugs in this study. Haemodynamic measurements were done in a blinded fashion. Right ventricular systolic pressure (RVSP) measurements were taken using a Polyimide Mikro-Tip pressure volume catheter (ADI instruments spr-869NR); 12.5cm effective length, with a pressure sensor (2F) and four electrodes, with a pressure sensor centered between E2 and E3. The catheter was used as per the manufacturer's instructions and attached to corresponding software (LabChart Pro). This catheter was inserted into the jugular vein and guided into the right ventricle of the heart to measure RVSP. After RVSP was determined, the carotid vein was isolated and the same catheter used to determine mean systemic arterial pressures. This pressure-volume (PV) loop system also generated the cardiac output (CO) data. Blood was collected immediately in a heparinised syringe for plasma analysis.

Right ventricular hypertrophy and tissue harvest
Immediately following hemodynamic assessment, the heart and lungs were flushed with ice-cold PBS at a low pressure to clear peripheral blood cells. The right lung was excised for molecular analysis. The left lung was inflated with 10% (v/v) neutral buffered formalin (NBF) and left in NBF solution for 48 hours before paraffin processing and embedding for immunohistological analysis. Right ventricular hypertrophy (RVH) was assessed by the Fulton Index (dry weight of the right ventricle/(dry weight of the left ventricle + septum).1

Immunohistochemistry
Sections (3μm) were cut and stained using Millers elastin/picro Sirius Red for
identification of vascular thickening, characterized by an increase in the vessel wall diameter in more than 50% of the arterial wall. The total number of remodelled vessels was expressed over the total number of vessels present in a lung section as assessed by a blinded investigator. In order to determine the presence of occluded vessels, sections were stained with rabbit anti-von-willebrand factor (Dako, A0082 1:1000). The total number of occluded vessels were counted in lung sections of each animal by a blinded investigator. Additional sections were stained using anti-aromatase (Abexxa abx13974 1:200), anti-CYP1B1 (Abcam, Ab32649 1:500), anti-AHR (Abcam Ab153744 1:200) and anti-PCNA (Abcam Ab2426 1:1000) antibodies. An anti-rabbit IgG secondary antibody was used for each primary antibody (Vector Laboratories IMMpress kit) and protein immuno-localisation was visualized with the DAB substrate kit (Vector labs UK (sk-4600). PCNA staining was quantified using ImunoRatio software (jvsmicroscope.uta.fi/immunoratio/).

Human Pulmonary Arterial cells
Human PASMCs were provided by Professor Nicholas W. Morrell (University of Cambridge). Female hPASMCs were explanted from the distal pulmonary microvasculature from subjects with either no reported presence of PAH, referred to as non-PAH cells or from patients with reported PAH. Patient characteristics are shown in Table 1. Assays were performed between passages 4 and 7. Cells were seeded in 24-well plates (for cell proliferation or luciferase assay) and 6 well plates (for protein, RNA or aromatase activity analysis) at a density of 10,000 cells per cm². Cells were grown to ~60% confluency and then synchronized by serum-deprivation (0.2% (v/v) charcoal-stripped FBS) in phenol-red free DMEM (Invitrogen, UK) for 24 hours for all experiments apart from the aromatase activity protocol, in which cells were treated without serum deprivation. Proliferation studies were carried out in charcoal-stripped media using cell counting and BrdU incorporation studies.

Charcoal-Stripped Fetal Bovine Serum
Fetal bovine serum (FBS; Sera Labs, UK) was charcoal-stripped twice to remove estrogens. Dextran-coated charcoal (1% (w/v), Sigma-Aldrich, UK) in FBS was agitated gently overnight at 4ºC. Samples were centrifuged at 1811 g at 4ºC for 30 minutes. The stripped serum was decanted and filtered through a 0.22µm filter.

Effect of Metformin on PASMC proliferation
Cell counts:
PASMCs plated in 24-well plates were used for these studies. Following serum deprivation, cells were replenished with fresh phenol-red free DMEM (Invitrogen, UK) supplemented with 2% (v/v) or 10% (v/v) FBS and metformin (2 mM), AICAR (3 mM), anastrazole (1 μM), 17β-estradiol, TMS (2,3',4,5'-tetramethoxystilbene) or AhR antagonist CH223191 (1-Methyl-N-[2-methyl-4-[2-(2-methylphenyl)diazenyl]phenyl-1H-pyrazole-5-carboxamide) (10μM). Cells were incubated for 24 hours before cell proliferation was assessed, in a blinded fashion, by cell counting using a haemocytometer.

BrdU incorporation assay:
Cells were seeded in 96 well plates at a density of 2000 cells per well. Cells were grown to ~60% confluency and then synchronized by serum-deprivation for 24 hours. Cells were then treated for a further 24 hours in medium supplemented with 2% (v/v) or 10% (v/v) FBS and metformin (2 mM), AICAR (3 mM) or anastrazole (1 μM).
BrdU incorporation was measured in each well as per the manufacturer’s instructions (Millipore UK). Absorbance in each well was measured using a spectrophotometric plate reader at dual wavelengths of 450-540 nm.

**Aromatase activity Assay (tritiated water-release assay)**
Female non-PAH hPASMCs were plated in six-well plates and grown to 60% confluency. Cells were cultured in medium supplemented with 2% serum and 2mM metformin, 3mM AICAR or 1µM Anastrazole. Aromatase activity in these cells was measured using the $^{3}$H$_{2}$O release assay in the presence or absence of 30 µM [3H]androst-4-ene-3, 17-dione (NET926001MC, PerkinElmer) for 18 hours. Media was removed and placed in 30% TCA and 100% chloroform and left overnight for steroid extraction. The upper phase was removed, added to 5% (w/v) charcoal dextran and centrifuged at 3000g for 30 minutes at 4°C. The upper phase was removed and radioactivity determined using a liquid scintillation counter. Activity was corrected for by protein concentration.

**Plasmid hPASMC transfection studies**
Non-PAH cells were transfected with the CYP19A1 p11-516 reporter construct (generated and provided by Dr Kristy Brown of Monash University) using lipofectamine (as per the manufacturer’s instructions). Eight hours post transfection; cells were stimulated with experimental agents for 24 hours. Luciferase reporter assay was carried out using a Promega Luciferase Assay system E1500 as instructed by the manufacturer. Further details are described in the online supplement.

Female non-PAH hPASMCs were plated in six-well plates and grown to 60% confluency. Cells were synchronized by serum-deprivation for 24 hours (0.1% (w/v) charcoal stripped serum). PII promoter plasmid was provided by Dr Kristy Brown of Monash University. Cells were transfected with 400ng of plasmid in lipofectamine (Invitrogen) as per manufacturer’s instruction. Briefly, cells were washed twice with 500µl optimem (Invitrogen) before addition of 500µl of 10% (v/v) fetal bovine serum DMEM containing no antibiotics to each well. Next, 100µl of transfection mix (containing 400 ng of plasmid) was added to each well and cells transferred back to the humidified cell culture chamber. After 8 hours the transfection media was removed and replaced with medium containing 10% (v/v) charcoal-stripped FBS in the presence or absence of metformin (2mM), AICAR (3 mM) or anastrazole (1µM). Cells were transferred back to a humidified cell culture chamber at 37°C and incubated for 24 hours. To measure the luciferase activity, a luciferase reporter assay was carried out using a Promega Luciferase Assay system E1500 according to the manufacturer’s instructions. Briefly, growth media was aspirated and each well washed with PBS. PBS was aspirated, and 100µl of 1x passive lysis buffer (PLB) was added into each well. The 6 well plates were transferred to -80°C for 5 minutes to flash freeze the lysates. Cells were placed on ice, on a shaker for 10 minutes and each well was scraped into a microcentrifuge tube and centrifuged at 12,000rpm for 5 minutes at 4°C. Thereafter 20µl of supernatant was transferred into a single well of a white 96-well plate (in duplicate). 100µl of Luciferase Assay Reagent (LAR) was added per well and light reaction measured using LUMItstar OPTIMA microplate reader (BMG Labtech).
Protein Analysis
Whole lung rat samples were homogenized and hPASMCs were lysed in ice-cold 0.1% (w/v) lauryl maltoside (Abcam, UK) solution in PBS (v/v). Protein concentrations were determined on a nanodrop (ND-1000 spectrophotometer (Thermo Scientific, UK). 20µg of protein was loaded for hPASMCs and whole lung homogenates, for protein identification by SDS-PAGE and immunoblotting. Protein expression was quantitated in immunoblots probed with rabbit anti-aromatase (Abbexa, abx13974 1:200), anti-CYP1B1 (Abcam ab32649 1:200) anti-phospho-AMPK Thr172 (Cell Signaling, #2535, 1:500), anti-phospho-ACC Ser79 (Cell signaling #3361 1:800), anti-LKB1 (Cell Signalling #3047 1:1000), anti-AhR (Abcam ab153744 1:100), total-AMPK (Cell signaling 2532 1:1000) and total-ACC (Cell signaling 3676 1:1000), antibodies by overnight incubation at 4°C, subsequently incubated with anti-rabbit secondary antibodies and immunoblots developed using Pierce™ ECL Western Blotting Substrate (Life Technologies)and normalized to GAPDH (abcam, ab8264, UK; 0.2µg/ml). Densitometrical analysis was performed using TotalLab TL100 software.

Gene expression analysis
For quantitative analysis of gene expression by qRT-PCR, total RNA was extracted from rat lung samples from the different treatment groups (control, vehicle and metformin) and hPASMCs treated in the presence or absence of either 2% or 10% charcoal stripped serum supplemented with 2 mM metformin, 3 mM AICAR or 1 µM anastrazole, using an RNeasy Micro Kit with on-column DNase digestion (Qiagen, UK). Random hexamer primed cDNA was prepared using the Applied Biosystems TaqmanTM RT kit (Applied Biosystems, CA). Quantitative real time PCR (qRT-PCR) was performed on the ABI Prism Sequence Detection System (Applied Biosystems). Expression of CRTC2 mRNA was determined using the Life Technologies Universal Probe Library (Roche Applied Sciences, Burgess Hill, UK) using primers listed in Table 2 below. The expression level of each gene was corrected using GAPDH expression as internal control. All samples were performed in duplicate. CT values were determined with Opticon2 software.

Estrogen Immunoassay
The levels of 17β estradiol were determined by competitive immunoassay in lung samples and plasma from female rats from each group, (control, vehicle and metformin). 400µg of protein was loaded and assayed in duplicate as per the manufacturer’s instructions (Demeditec, USA). The plate was read at a wavelength of 405nm for kinetic and end point measurements (SpectraMax M2 plate reader, Molecular Devices, California, USA). Protein samples from lung tissue were made in 1% LM buffer. We contacted Demeditec to discuss that we had detected estradiol levels in these samples which were within the standard curve range/detection limit. They stated that although the kit was designed for serum and plasma, there is no technical reason as to why estradiol would not be detected in the protein sample from tissue.
References


### Table S1. hPASMC patient information

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<th>Available patient information</th>
<th>Age</th>
<th>Condition</th>
<th>Treatment</th>
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<tr>
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<td>64</td>
<td>Emphysema (mild)</td>
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<td>Squamous cell carcinoma</td>
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<td>R899X, presented at 23 year old. Systolic PAP 90, diastolic PAP 30, mean PAP 46, mixed venous STATS 66.3%, NYHA II, 6MWD 400m</td>
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### Table S2: Assay Id numbers for qRT-PCR.

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<td>Rn01455374_m1</td>
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<tr>
<td><strong>Human</strong></td>
<td>Hs02758991_g1</td>
<td>Hs01064500_m1</td>
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Figure S1. mSAP, glucose levels, cardiac output and heart rate are not significantly altered in female Sugen/hypoxic rats exposed to metformin. (A) mSAP was unchanged between the group, this was also found for glucose levels (B), cardiac output (C) and heart rate (D). Glucose levels were measured at day 7, 14 and 21 (data shown for day 21) of treatment in each group and no significant difference in blood glucose levels, taken from tail vein, was found. n=6-13 per group. Data was analysed by one way ANOVA followed by Bonferroni post test.
Figure S2. Representative immunoblots and quantification relative to GAPDH of LKB1 (A) in whole lung from control (no sugen/hypoxic) and sugen/hypoxic animals without (vehicle) or with metformin treatment. n=4-8 per group. C= Control, V= Vehicle, M= Metformin. (B) Representative immunoblots and quantification, showing effects of 24h incubation with metformin, AICAR or anastrozole on LKB1 in female non-PAH hPAMSCs (n=2-3 different cell lines per group). C=Control, M= Metformin, AC= AICAR, A= Anastrozole. Data is displayed as a mean ±SEM
Figure S3. Non PAH female hPASMCs (A) and PAH female hPASMCs (B) were incubated in 2% (v/v) FBS or 10% (v/v) FBS in the presence or absence of the indicated agents for 24h and BrdU incorporation assessed. n=2 cell lines/group, repeated seven times per group. Data is displayed as mean ±SEM. * P<0.05, **P<0.01 ***P<0.001 as indicated, determined by one way ANOVA followed by Bonferroni post test.
Figure S4. Non-PAH female hPASMCs (A) were incubated in 2% (v/v) FBS in the presence (or absence–time point 0) of AICAR (3 mM) for either 2, 4, 6 or 10 hours. The activation of AMPK was assessed by measuring pAMPK protein expression over tAMPK protein expression. n=3 cell lines/group. Data is displayed as mean ±SEM. * P<0.05, **P<0.01 ***P<0.001 as indicated, determined by one way ANOVA followed by Bonferroni post test.
Figure S5. Schematic illustrating the proposed pathway of mechanism of metformin action in experimental PH. Metformin increases pAMPK and one effect of this is to cause a decrease in CRTC2. CRTC2 binding to the PII promoter of aromatase is decreased, which means less estrogen is synthesised from testosterone by aromatase. Metformin treatment decreased AhR expression. AhR is a transcription factor for the estrogen metabolising enzyme CYP1B1. CYP1B1 expression was decreased by metformin, possibly due to the decrease in its transcription factor. We have previously suggested decreased CYP1B1 activity can protect against PH by decreasing accumulation of estrogen metabolites.\textsuperscript{2,3}