Uncoupling Protein 2 Deficiency Mimics the Effects of Hypoxia and Endoplasmic Reticulum Stress on Mitochondria and Triggers Pseudo-Hypoxic Pulmonary Vascular Remodeling and Pulmonary Hypertension

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Running title: UCP2-Deficiency Mimics Hypoxia

Subject codes:
[130] Animal models of human disease
[131] Apoptosis
[162] smooth muscle proliferation and differentiation
[140] Energy metabolism
[156] Pulmonary biology and circulation

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In April 2013, the average time from submission to first decision for all original research papers submitted to Circulation Research was 13.5 days.
ABSTRACT

Rationale: Mitochondrial signaling regulates both the acute and chronic response of the pulmonary circulation to hypoxia, and suppressed mitochondrial glucose oxidation (GO) contributes to the apoptosis-resistance and proliferative diathesis in the vascular remodeling in pulmonary hypertension (PHT). Hypoxia directly inhibits GO, while endoplasmic reticulum (ER)-stress can indirectly inhibit GO by decreasing mitochondrial calcium (Ca\textsuperscript{2+}m levels). Both hypoxia and ER-stress promote proliferative pulmonary vascular remodeling. Uncoupling protein 2 (UCP2) has been shown to conduct calcium from the ER to mitochondria and suppress mitochondrial function.

Objective: We hypothesized that UCP2 deficiency reduces Ca\textsuperscript{2+}m in pulmonary artery smooth muscle cells (PASMCs), mimicking the effects of hypoxia and ER-stress on mitochondria in vitro and in vivo, promoting normoxic hypoxia inducible factor-1\alpha (HIF1\alpha) activation and PHT.

Methods and Results: Ucp2KO-PASMCs had lower Ca\textsuperscript{2+}m than Ucp2WT-PASMCs at baseline and during histamine-stimulated ER-Ca\textsuperscript{2+} release. Normoxic Ucp2KO-PASMCs had mitochondrial hyperpolarization, lower Ca\textsuperscript{2+}-sensitive mitochondrial enzyme activity, reduced levels of mitochondrial reactive oxygen species and Krebs’ cycle intermediates and increased resistance to apoptosis, mimicking the hypoxia-induced changes in Ucp2WT-PASMC. Ucp2KO mice spontaneously developed pulmonary vascular remodeling and PHT and exhibited a pseudo-hypoxic state with pulmonary vascular and systemic HIF1\alpha activation (increased hematocrit), not exacerbated further by chronic hypoxia.

Conclusion: This first description of the role of UCP2 in oxygen sensing and in PHT vascular remodeling may open a new window in biomarker and therapeutic strategies.

Keywords: Mitochondria, uncoupling protein, pseudo-hypoxia, metabolism, pulmonary vascular remodeling, pulmonary hypertension, hypoxia

Nonstandard Abbreviations and Acronyms:
\begin{itemize}
\item \alpha KG: alpha-ketoglutarate
\item CO: cardiac output
\item ER: endoplasmic reticulum
\item GO: glucose oxidation
\item HIF1\alpha: hypoxia inducible factor I-alpha
\item IDH: isocitrate dehydrogenase
\item Kv: voltage gated potassium channels
\item MCU: mitochondrial calcium uniporter
\item mPAP: mean pulmonary artery pressure
\item mROS: mitochondrial derived reactive oxygen species
\item NFAT: nuclear factor of activated T-cells
\item PAECs: pulmonary artery endothelial cells
\item PASMCs: pulmonary artery smooth muscle cells
\item PHT: pulmonary hypertension
\item PVR: pulmonary vascular resistance
\item TPR: total pulmonary resistance
\item UCP: uncoupling protein
\end{itemize}
INTRODUCTION

The role of mitochondria is increasingly being recognized in vascular health and disease \(^1\) and is particularly critical in the pulmonary circulation. As recognized oxygen sensors, mitochondria form the basis of hypoxic pulmonary vasoconstriction (HPV), a feature of all mammals and the most important difference between the pulmonary and systemic circulation (all systemic vessels dilate to hypoxia while pulmonary arteries constrict) \(^2\). In response to acute decreases in oxygen levels, mitochondria alter the production of mitochondria-derived reactive oxygen species (mROS), which reach the cell membrane and regulate redox-sensitive voltage-gated potassium channels (Kv). The resulting Kv inhibition depolarizes the cell, increases influx of \(\mathrm{Ca}^{2+}\) in the cytoplasm and causes contraction of pulmonary artery smooth muscle cells (PASMC) \(^2\). Mitochondria may also play an important role in the chronic hypoxic response and in pulmonary vascular disease via the regulation of the hypoxia inducible factor (HIF1\(\alpha\)) \(^3\). Several aspects of the HIF1\(\alpha\) axis are redox-sensitive and \(\alpha\)-ketoglutarate (\(\alpha\)KG), a Krebs’ cycle product, is an important cofactor in the hydroxylation reaction that destabilizes HIF1\(\alpha\). A primary suppression of mitochondrial glucose oxidation (GO) reduces mROS and \(\alpha\)KG, activating HIF1\(\alpha\) even in normoxia \(^3\). Aberrant HIF1\(\alpha\) activation has been shown in normoxic animal and human pulmonary arteries in pulmonary hypertension (PHT) \(^4, 5\). In cancer, where suppression of GO is critical for the apoptosis-resistance that characterizes most tumors, there is normoxic HIF1\(\alpha\) activation. This “pseudo-hypoxic” HIF1\(\alpha\) activation is reversed by activation of GO, decreasing cancer angiogenesis in vitro and in vivo \(^3\). Like in cancer, GO suppression also leads to apoptosis suppression and a proliferative diathesis that underlies the vascular remodeling in PHT \(^6-8\). Thus, it is possible that the HIF1\(\alpha\) activation in PHT is also a consequence of suppressed mitochondrial signaling.

The basis of GO suppression in PHT is unknown. Hypoxia, particularly if severe and sustained, directly inhibits GO by decreasing oxygen levels in mitochondria. Chronic hypoxia can inhibit GO by an additional, recently described mechanism: hypoxia, along with several conditions that trigger PHT, like viral infections or inflammation, causes endoplasmic reticulum (ER) stress \(^8, 9\). ER stress can lead to mitochondrial suppression by decreasing the influx of \(\mathrm{Ca}^{2+}\) from the ER to mitochondria due to an ER structural remodeling, decreasing mitochondrial \(\mathrm{Ca}^{2+}\) (\(\mathrm{Ca}^{2+}\)m). This inhibits many \(\mathrm{Ca}^{2+}\)-dependent mitochondrial enzymes including pyruvate dehydrogenase (PDH, the gate-keeper of GO) or isocitrate dehydrogenase (IDH) and \(\alpha\)KG dehydrogenase (both regulating \(\alpha\)KG levels) \(^8-10\).

We hypothesized that UCP2 deficiency could mimic the effects of hypoxia or ER stress on mitochondria in the pulmonary circulation. This is because UCP2 can function as a \(\mathrm{Ca}^{2+}\) channel in vascular mitochondria, facilitating influx of \(\mathrm{Ca}^{2+}\) from the ER to mitochondria \(^11-13\). UCP2, despite its name, does not appear to have uncoupling functions and has only modest homology to UCP1 \(^11-13\). Mitochondria deficient in UCP2 may be deficient in \(\mathrm{Ca}^{2+}\)m, and thus have suppressed GO in the absence of hypoxia or ER stress, inducing a “pseudo-hypoxia” and apoptosis-resistance state and predisposing to proliferative pulmonary vascular remodeling (Figure 1). The description of human UCP2 polymorphisms leading to decreased gene expression, makes this hypothesis more interesting and clinically relevant \(^14\).

METHODS

All experiments were performed with approval by the University of Alberta Committee on Animal Policy and Welfare. Mice were purchased from Jackson Laboratory and have previously been validated for effective UCP2 knockout \(^15\). Please see the online data supplement for detailed methods.

**In vitro experiments.**

Cell isolation, culture, and qrt-PCR were performed using standard techniques as previously described \(^7, 8\).
**FRET analysis.**

PASMCs were transfected with the 4mtD3CPV cameleon plasmid, and $Ca^{2+}_{m}$ was assessed as previously described under baseline or histamine stimulation.

**Electron microscopy.**

Cells were fixed with Karnovsky’s fixative, pelleted and post-fixed with 1% osmium tetroxide. Cells were then serially dehydrated in ethanol, rinsed in propylene oxide, and incubated in propylene oxide and resin before embedding resin. Samples were then sliced, mounted on metal gratings and imaged on a Phillips EM 400T/ST transmission electron microscope.

**Mass spectroscopy.**

PASMCs were pelleted, suspended in 800µL of ice-cold 80% methanol and 20% ddH$_2$O and subjected to two freeze-thaw cycles. Centrifugation pelleted out cell debris and the supernatant was evaporated to obtain metabolites. Metabolites were resuspended in ddH2O and normalized to protein quantification. Seven microliters of sample was injected using a 4000 QTRAP mass spectrometer (AB Sciex) equipped with a UHPLC 1290 system (Agilent) via SRM for Krebs’ cycle intermediates.

**PDH activity.**

PDH activity was measured with a commercially available MitoProfile Dipstick assay kit (Mitosciences) as previously described.

**HIF1α luciferase.**

HIF1α transcriptional activity was performed with the HIF1α dual luciferase plasmid (SABiosciences) using Xfect transfection reagent (Clontech) and was assessed with the dual-luciferase reporter assay system (Promega) as previously described.

**In vivo hemodynamic assessments.**

Mice were randomized to normobaric hypoxia (10% O$_2$) or room air as previously described. Cardiac output was assessed on isoflurane-anesthetized animals by echocardiography using the Vevo770 imaging system with a 707B (30MHz) probe. The cardiac output was calculated after determining the left ventricular outflow tract diameter (LVOT), aortic velocity time integral (AoVTI), and heart rate (HR) using the formula:

$$CO = 7.85 \times LVOT^2 \times AoVTI \times HR / 10000$$

Mean pulmonary artery pressures (mPAP) were assessed in closed-chest animals with a Millar catheter (microtip, 1.4F, Millar Instruments Inc., Houston, TX) as previously described. Animals were placed in a supine position on a heated table under inhaled isoflurane anesthesia and the right jugular vein was cannulated and the catheter advanced into the pulmonary arteries. Pressures from the right atrium, right ventricle and pulmonary arteries were also recorded continuously and mean PAP was calculated electronically (Power Lab, with Chart software 5.4, ADInstruments). Total pulmonary resistance was calculated by the mPAP/CO ratio. Systemic blood pressure was assessed with the CODA2 (Kent Scientific Corp.) mouse tail cuff system as previously described.

**Histological examination.**

Media wall thickness, muscularization, and medial proliferation were assessed using standard protocols as previously described.

**Immunoblots.**

Tissues were collected and immunoblotting was performed as previously described.
Whole lung respiration.
Freshly isolated lungs from Ucp2WT and Ucp2KO mice were minced in 1mL of Hanks buffer with 10mM glucose and placed in a sealed glass chamber (Warner Instruments) connected to a Strathkelvin 782 oxygen meter. Rate of oxygen consumption was calculated using the oxygen meter’s software (version 4.0, Strathkelvin Instruments LTD). Respiration rates were normalized to tissue weight.

Hematocrit.
Blood was collected from isoflurane-anesthetized mice via cardiac puncture and injected into a RapidLab 348 blood gas analyzer (Bayer Healthcare).

Statistics.
All values were expressed as mean±SEM. For in vitro analysis, differences were assessed by Student’s t-test. Normality of our in vivo data was confirmed by the Shapiro-Wilk test. Inter-group differences were assessed by ANOVA using Fisher’s least square differences post-hoc analysis. All analyses were performed using SPSS 19. Significance was considered at p<0.05.

RESULTS
We isolated mitochondria from whole lung, spleen and isolated PASMCs from resistance pulmonary arteries (>4th division) of mice lacking Ucp2 (Ucp2KO) and their wild-type controls (Ucp2WT). The absence of UCP2 in KO mice PASMC was confirmed by qRT-PCR (Online Figure IA) and immunoblots (Online Figure IB). UCP3, which may also influence ER-mitochondria calcium transfer, did not differ between genotypes (Online Figure IB). We then measured intra-mitochondrial calcium (Ca\textsuperscript{2+}_m) in Ucp2WT- and Ucp2KO-PASMCs in normoxia and sustained hypoxia (48hrs). FRET imaging showed that Ucp2KO-PASMCs had lower levels of Ca\textsuperscript{2+}_m than Ucp2WT-PASMCs under basal, normoxic conditions (Figure 2A). Unlike Ucp2WT-PASMCs, which had the expected Ca\textsuperscript{2+}_m reduction in hypoxia, hypoxia did not further reduce Ca\textsuperscript{2+}_m in Ucp2KO-PASMCs (Figure 2A). Similar results were obtained with the Ca\textsuperscript{2+}_m-sensitive dye Rhodamine-2AM (Online Figure II). Pharmacological UCP2 inhibition with genipin reduced Ca\textsuperscript{2+}_m in Ucp2WT-PASMCs but not Ucp2KO-PASMCs (Figure 2A). Thus, UCP2 deficiency reduces Ca\textsuperscript{2+}_m in normoxic PASMCs, mimicking the effects of hypoxia and ER-stress on PASMCs as previously published.

To determine whether UCP2 facilitates mitochondrial Ca\textsuperscript{2+} uptake specifically from the ER in PASMCs, we analyzed Ca\textsuperscript{2+}_m upon histamine-stimulation, which releases ER-calcium through IP\textsubscript{3} signaling. Experiments were performed in Ca\textsuperscript{2+}-free solution to eliminate extracellular Ca\textsuperscript{2+} sources. The histamine-induced increase in Ca\textsuperscript{2+}_m was blunted in Ucp2KO-PASMCs (Figure 2B). Inhibition of the mitochondrial Ca\textsuperscript{2+} uniporter (MCU) with ruthenium red (RR) inhibited the responses similarly between genotypes, suggesting that the differences were not due to a potential MCU down-regulation.

Chronically hypoxic PASMCs have an ER stress-induced disruption of the normal communication between the ER and mitochondria, including Ca\textsuperscript{2+} exchange, due to an ER structural remodeling and an increase in the minimal distance between the two organelles. Using transmission electron microscopy, we showed that the minimal ER-mitochondria distance was similar between Ucp2WT- and Ucp2KO-PASMCs under normoxia, suggesting that our observations cannot be attributed to ER-mitochondrial spatial remodeling (Figure 2C). In contrast, hypoxia increased the ER-mitochondria distance similarly in Ucp2WT- and Ucp2KO-PASMCs (Figure 2C). In addition, the expression of glucose regulated protein-78 (GRP78), which is upregulated in ER-stress states in PASMCs, was not elevated in Ucp2KO-PASMCs, further supporting a lack of ER stress at normoxic baseline in these cells (Online Figure III). Evidence that spatial ER-mitochondria disruption in hypoxia does not further reduce...
Ca\textsuperscript{2+} in Ucp2KO-PASMCs (Figure 2A) supports the fact that a lack of UCP2 in normoxia induces a “pseudo-hypoxia” state by maximally inhibiting ER-mitochondrial calcium transport. In other words, a primary loss of Ucp2 or an ER stress-induced disruption of the ER-mitochondria unit may both, and via the same mechanism, result in a similar reduction of Ca\textsuperscript{2+} and GO.

Several key metabolic enzymes are Ca\textsuperscript{2+}-dependent, including PDH and the Krebs’ cycle enzymes IDH and α-KG dehydrogenase\textsuperscript{10}. PDH drives mitochondrial GO and its inhibition is associated with PHT\textsuperscript{17}. In keeping with reduced Ca\textsuperscript{2+}, Ucp2KO-PASMCs had lower PDH activity than Ucp2WT-PASMCs (Figure 3A). PDH inhibition decreases the acetyl-CoA influx into the Krebs’ cycle, while Krebs’ cycle activity can be further decreased by IDH and αKG dehydrogenase inhibition. Indeed, a metabolomics assessment of Krebs’ cycle function showed reduced levels of many metabolites, including αKG, in Ucp2KO-PASMCs compared to the Ucp2WT-PASMCs under normoxia (Figure 3B). Once again, the reduction of Krebs’ cycle metabolites in normoxic Ucp2KO-PASMCs (compared to wild-type) was similar to the chronic hypoxia-induced reduction in Ucp2WT-PASMC metabolites. Thus, lack of Ucp2 mimics a hypoxic state and hypoxia does not cause a further suppression of mitochondrial function over and above what lack of Ucp2 does.

A similar pattern was present when we studied two other important indices of mitochondrial function: mitochondria-derived reactive oxygen species (mROS; which are produced by the electron transport chain in proportion to Krebs’ cycle-derived electron donors) and mitochondrial membrane potential (ΔΨm). Ucp2KO-PASMCs had lower levels of mROS and increased ΔΨm compared to Ucp2WT-PASMCs in normoxia (Figure 3C). The increased ΔΨm in Ucp2KO-PASMCs which, like the decreased mROS levels, is described in human and animal PHT-PASMC\textsuperscript{6} and is in keeping with reduced mitochondrial levels of the positively charged Ca\textsuperscript{2+}. Once again, normoxic Ucp2KO-PASMCs mimicked hypoxic Ucp2WT-PASMCs and hypoxia did not further suppress any tested parameter in Ucp2KO-PASMCs (Figure 3-A-C).

The hyperpolarized ΔΨm and the decreased mROS (both of which directly inhibit the opening of the voltage- and redox-gated mitochondrial transition pore, through which pro-apoptotic mediators like cytochrome c and apoptosis inducing factor efflux from the mitochondria\textsuperscript{19}) promote an apoptosis-resistant state in both cancer\textsuperscript{3, 18, 20} and pulmonary hypertension\textsuperscript{1, 7, 8, 17}. To determine whether Ucp2KO-PASMCs are resistant to apoptosis, PASMCs were stressed in serum-starved conditions (0.1% FBS). Apoptosis, measured by TUNEL, was increased in Ucp2WT-, but not Ucp2KO-, PASMCs (Figure 3D).

We then determined whether lack of Ucp2 could explain the activation of two transcription factors that have been shown to be critical in both hypoxic and non-hypoxic PHT: HIF1α\textsuperscript{4} and the nuclear factor of activated T-cells (NFATc2)\textsuperscript{21}. Upon dismutation, mitochondria-produced superoxide becomes H\textsubscript{2}O\textsubscript{2}, a more stable and diffusible mROS that reaches several extra-mitochondrial targets, including Kv-channels and transcription factors like HIF1α. Ucp2KO-PASMCs had reduced H\textsubscript{2}O\textsubscript{2} (Figure 4A) and αKG levels (Figure 3B), as well as normoxic HIF1α-activation, similar to hypoxic Ucp2WT-PASMCs. HIF1α activation was confirmed with measurement of nuclear HIF1α levels (Figure 4B), with direct measurement of its transcriptional activity using a dual-luciferase assay (Figure 4B), and by the mRNA expression of the HIF1α downstream target, Vegf (Online Figure IVA). Ucp2KO-PASMCs had a reduced hydroxylated to total HIF1α ratio compared to Ucp2WT-PASMCs (Online Figure IVB), suggesting that at least some of the inhibitory effects were through activation of prolyl hydroxylases, which hydroxylate and target HIF1α for degradation. in an αKG-dependent manner. The activation of HIF1α in normoxic PASMCs lacking UCP2 was similar to that in hypoxic wild-type PASMCs and was maximal, as it was not increased further by hypoxia (Figure 4B).
Like HIF1α, NFAT is influenced (albeit indirectly) by mitochondrial signals. While dephosphorylation by the Ca²⁺-sensitive cytoplasmic enzyme calcineurin activates NFAT, driving it to the nucleus, phosphorylation by the metabolic sensor glycogen synthase kinase-3β [GSK3β, which is inhibited when GO is suppressed in PASMCs] results in nuclear export and transcriptional inhibition. Consistent with reduced mROS and inhibited Kv-channels, Ucp2KO-PASMCs had elevated levels of cytoplasmic Ca²⁺ compared to Ucp2WT-PASMCs (Figure 5A). Moreover, Ucp2KO-PASMCs had elevated phosphorylated (inactive) GSK3β compared to Ucp2WT-PASMCs (Figure 5B). The increased cytoplasmic Ca²⁺ and inhibited GSK3β were associated, as expected, with NFATc2 activation (increased nuclear levels) in normoxic Ucp2KO-PASMC, similar to hypoxic wild-type PASMC (Figure 5C).

The activation of HIF1α, NFATc2 as well as the hyperpolarized ΔΨm and the decreased mROS and GO in Ucp2KO-PASMCs essentially create the cellular in vitro PASMC phenotype as has been shown in several human and animal PHT models. They promote proliferation and apoptosis-resistance and thus would be expected to cause PHT in normoxic mice in vivo. We thus measured mean pressure (mPAP) and cardiac output (CO) in close-chest anesthetized mice. Ucp2KO mice had elevated mPAP and total pulmonary resistance (TPR=mPAP/CO) compared to Ucp2WT controls (Figure 6A/B, Online Figure V), without differences in systemic blood pressure (Online Figure VI). The elevated pressures resulted in a trend towards right ventricular hypertrophy, which did not reach significance (Online Figure VII). Lack of Ucp2 did not worsen pulmonary hemodynamic parameters upon exposure to chronic hypoxia, a standard PHT model (Figure 6A-B, Online Figure VI, Online Figure VII), in keeping with our in vitro findings.

To confirm our in vitro studies to the whole organ level that is more relevant to our in vivo studies, we performed ex vivo lung respiration studies. Whole lung tissue from Ucp2KO mice had lower respiration rates (oxygen consumption) compared to Ucp2WT mice (Online Figure VIII), studied under identical conditions, in keeping with our in vitro studies that showed a suppression of several indices of mitochondrial activity.

To determine whether the hemodynamic changes were due to pulmonary vascular remodeling (as opposed to vasoconstriction), we examined the resistance pulmonary arteries of these mice in normoxia and hypoxia. Normoxic Ucp2KO mice had increased media thickness of resistance pulmonary arteries (Figure 6C). This was associated with increased nuclear levels of NFATc2 (Online Figure IX) and increased proliferation indices (Ki67) in smooth muscle actin-positive cells (Figure 6D). There was also increased arterial muscularization in Ucp2KO mice in normoxia, which was not exacerbated in hypoxia (Figure 6E). Taken together, these results indicated that the pulmonary vascularity in Ucp2-KO mice resembles that of wild-type mice under chronic hypoxia. In addition, normoxic Ucp2KO mice have modestly, but significantly elevated hematocrit compared to Ucp2WT mice (Figure 6F), suggesting that lack of Ucp2 mimics hypoxia in the kidney as well, where the HIF1α product erythropoietin is produced, inducing perhaps a global pseudo-hypoxia state.

**DISCUSSION**

We show for the first time that the mitochondrial protein UCP2 is important in the vascular biology of the pulmonary circulation. We specifically show that lack of UCP2 causes a decrease in the entry of Ca²⁺ from the ER to the mitochondria in PASMCs, resulting in decreased Ca²⁺, and a subsequent suppression of mitochondrial function. This suppression of mitochondrial function, and its associated metabolic switch toward glycolysis, has previously been shown to be critical for the pro-proliferative and
anti-apoptotic diathesis that characterizes the response of the pulmonary circulation to several triggers leading to PHT \(^1,4,6-9,17,21\). The effects of UCP2 deficiency in PASMC mitochondria resemble the effects of hypoxia and ER stress as recently described \(^8,9\). Lack of UCP2 induces a cellular and an in vivo phenotype that is identical to that caused by hypoxia as measured by several parameters, including the activation of HIF1\(\alpha\) and NFATc2. Our results are in keeping with the work that has shown that UCP2 conducts mitochondrial Ca\(^{2+}\) and support the emerging metabolic hypothesis of pulmonary vascular oxygen sensing and PHT \(^1,6\).

The activity of the Ca\(^{2+}\)-sensitive PDH is decreased in normoxic Ucp2KO-PASMCs (Figure 3A, B) and this has been shown in many PHT models, where the PDH activator small molecule dichloroacetate has been shown to reverse PHT \(^4,7,22\). PDH regulates the influx of pyruvate into the Krebs’ cycle, stimulating the production of electron donors, ETC activity, and thus the production of diffusible mediators like mROS or \(\alpha\)KG. Overall, ROS production by PASMCs, particularly in response to acute hypoxia, has been a controversial issue likely relating to experimental conditions and the specific nature of the ROS measured in several studies \(^2,23-25\). Nevertheless, our data herein are in keeping with several observations in human and animal PAH that describe both a suppression of mitochondria-derived ROS and a hyperpolarization of mitochondria \(^4,7-9,17,21,26\). Yet, it is important to clarify that a decrease in mROS does not necessarily mean a decrease in the overall levels of cellular ROS produced by extramitochondrial sites in the cytoplasm and the cell membrane. Since mitochondrial function is overall suppressed in Ucp2-deficient cells, the decrease in mROS likely occurs through additional mechanisms, where PDH inhibition may be a contributing factor. Our data suggest that a potential deficiency of Ucp2 in humans could explain, at least in part, the PDH inhibition and perhaps the resulting suppression of GO and activation of glycolysis that has been described in patients with idiopathic pulmonary arterial hypertension \(^7,27\).

In humans, Ucp2 expression is influenced by recently described polymorphisms in the Ucp2 promoter. These polymorphisms, which decrease Ucp2\(^2\) expression, are associated with increased incidence of atherosclerosis \(^28\), diabetic retinopathy \(^29\) and hypertension \(^30\). In animal models of UCP2 deficiency however, the expression of the disease phenotype requires the exposure to predisposing conditions or triggers, consistent with the multifactorial nature of vascular disease. For example, mice lacking Ucp2 develop systemic hypertension only in appropriately stressed conditions \(^31\), and thus do not conflict with the normal systemic hemodynamics observed in our study. It is also intriguing that Ucp2 polymorphisms in humans are also associated with overall metabolic disturbances like obesity and diabetes \(^14\), while an unexplained insulin-resistance state has recently been described in patients with pulmonary arterial hypertension \(^32\). As the pulmonary vasculature exists in a much more oxidized environment and the PASMC are different than the systemic vascular mitochondria (perhaps explaining why hypoxic pulmonary vasoconstriction is restricted to the pulmonary circulation) \(^33\), the threshold for expressing a disease phenotype may be lower in the pulmonary arteries than in systemic vessels, explaining why PHT but not systemic hypertension is shown in normal/baseline conditions in Ucp2KO mice.

The lack of UCP2 appears to cause HIF1\(\alpha\) activation in PASMC (Figure 4B, Online Figure IV) as well as in the kidney, explaining the increased hematocrit in the Ucp2KO mice (Figure 6F). This activation in the pulmonary vasculature is believed to contribute to the enhanced glycolytic rates and glucose uptake that is apparent with lung 18-fluorodeoxyglucose-positron emission tomography in animal \(^34\) and human pulmonary arterial hypertension \(^27\). The activation of HIF1\(\alpha\) in rat and human pulmonary arterial hypertension tissues is well-described but remains unexplained particularly because it often takes place in the absence of hypoxia \(^4,5\).
Our data suggest that the suppression of downstream mitochondrial signaling in cells lacking UCP2, including the decreased mROS and αKG levels, may explain this “pseudo-hypoxic” HIF1α activation. We have recently shown that in cancer cells (where HIF1α activation occurs even under normoxia) the suppression in mitochondrial function activates HIF1α by a mechanism that includes a prolyl-hydroxylase dependent mechanism (decrease in αKG production) and a prolyl-hydroxylase independent mechanism (that involves mitochondrial H2O2-mediated regulation of p53 as well as inhibition of GSK3β)3. Lack of UCP2 causes a direct decrease in H2O2 (Figures 4A) and αKG production (Figure 3B) from mitochondria as well as an indirect inhibition of GSK3β (Figure 5B) that follows the inhibition of GO, and thus the mechanism described in cancer cells can be applied to the PASMCs. Interestingly HIF1α activation induces the expression of pyruvate dehydrogenase kinase, a major inhibitor of PDH, thus causing inhibition of GO35. This has been used to suggest that the metabolic remodeling in cancer is a result, not a cause of HIF1α activation. Our current data, where the primary abnormality is in mitochondria (lack of UCP2), support a mitochondria-driven regulation of HIF1α. It is likely that both pathways are important and mitochondrial PDK and HIF1α activation are a part of positive feedback loop that promotes a sustained inhibition of GO and HIF1α activation even under normoxia.

Our data are also compatible with the discovery that epigenetic suppression of mitochondrial-specific superoxide dismutase (MnSOD) can trigger normoxic HIF1α activation in the fawn-hooded rat, a model of spontaneous PHT26. Our observation that lack of UCP2 causes a decrease in the mitochondrial production of hydrogen peroxide (Figure 4A) is similar to the effects of MnSOD inhibition that decreases hydrogen peroxide, a diffusible mitochondria-derived redox mediator. In other words, both an epigenetic suppression of MnSOD or deficiency of other proteins that lead to suppression of mROS production, like UCP2 proposed here, can explain the intriguing observation of normoxic HIF1α in the pulmonary circulation and its role in promoting or predisposing to PHT.

The degree of spontaneous PHT that we found in the normoxic UCP2KO mice is modest, but it is important because spontaneous PHT (i.e. without additional triggers like hypoxia, monocrotaline or Sugen5146 with hypoxia) is very rare in animal models. This modest increase in mean PA pressure may explain the absence of significant RV hypertrophy (Online Figure VII). Nevertheless, recent work suggests that UCP2 is upregulated in RV pathobiology36. The fact that our mouse model was a global UCP2 knockout, suggests that it is possible that UCP2 may influence the development RV hypertrophy, which was beyond the scope of this study. Further studies are required to identify the precise or potential causal role of UCP2 in RV hypertrophy.

A limitation of this study is that we focused on PASMC biology and did not address the role of Ucp2 in pulmonary artery endothelial cells (PAEC). Nevertheless, it is now recognized that PHT PAEC show similar metabolic remodeling to PASMCs, characterized by suppressed GO27. UCP2 has also been shown to regulate Ca2+ in handling in vascular endothelial cells as well11-13. In other words, we speculate that PAEC will have a similar to PASMC metabolism in the Ucp2KO mice.

We also did not explore the role of inflammation on PHT-development in these mice. Indeed, inflammation may contribute to PHT-pathogenesis and Ucp2-deficient macrophages have basal activation of the pro-inflammatory transcription factor NFκB and exaggerated immune responses37. In humans, Ucp2 polymorphisms are also associated with autoimmune diseases38, a well-recognized feature of PHT17. While we do not address a potential role of activated macrophages or other immune cells in the development of PHT in Ucp2KO mice, our in vitro studies and their agreement with the in vivo data suggest that a primary mechanism for spontaneous PHT is intrinsic to PASMC. Importantly, the master transcription factor NFAT, which is activated both in inflammatory/immune cells and in PASMCs in the remodeled pulmonary circulation in both animal models and human PHT (promoting PASMC
proliferation)\textsuperscript{21}, was also found to be activated in $\textit{Ucp2KO}$ PASMC in vivo and in vitro (Figure 5C, Online Figure IX). This activation was compatible with both the increase in intracellular Ca\textsuperscript{2+} and the decrease in the total/phosphorylated GSK3\textbeta ratio in $\textit{Ucp2KO}$ PASMC (Figure 5A-B).

Since the $\textit{Ucp2KO}$ mouse model spontaneously develops PHT and also shares many of the mitochondria abnormalities described in many animal models and human PHT, it may be an important tool for future studies in the field. Clinical studies could explore whether $\textit{Ucp2}$ polymorphisms contribute to PHT in humans, offering new biomarker and potentially therapeutic strategies. Our work does not prove a link between UCP2 and pulmonary arterial hypertension specifically, but may trigger future studies in this direction. On the other hand our work may offer important clues for the mechanism that hypoxia causes PHT. The fact that hypoxia does not appear to have any additional effects to the PASMC over and above those induced by the lack of UCP2, suggest that chronic hypoxia may utilize the mitochondria calcium signaling system for its effects on the pulmonary circulation. Importantly, the fact that $\textit{Ucp2KO}$ mice have what appears to be a generalized pseudo-hypoxic environment makes them an attractive model for oxygen sensing studies or for the effects of hypoxia on organs other than the lung as well.

**SOURCES OF FUNDING**
EM is funded by operational grants from the Canadian Institutes for Health Research (CIHR), the Alberta Innovates Health Solutions (AIHS) and by the Canada Research Chairs program (CRC). RP and PD have received support from the CIHR and AIHS, respectively. SB is funded by the CIHR and the CRC.

**DISCLOSURES**
The authors have no disclosures to declare.

**REFERENCES**


FIGURE LEGENDS

Figure 1: A model of how UCP2-deficiency can mimic hypoxia-induced ER stress. UCP2-deficiency reduces calcium transfer from the ER (shown in blue) into the mitochondria (shown in red), which mimics the physical disruption of the ER-mitochondria unit caused by PHT-associated ER-stress inducers like hypoxia, bone-morphogenic receptor II mutations, inflammation, or viruses. The mechanism and the consequences of such a hypoxia and ER stress induced-disruption of the ER-mitochondria unit was recently described in 8, 9. Reduced Ca$^{2+}_{\text{m}}$ inhibits mitochondrial metabolic enzymes like PDH and some Krebs’ cycle enzymes, lowers mROS, and hyperpolarizes $\Delta\Psi_{\text{m}}$, suppressing apoptosis. Altered mitochondrial derived signals activate transcription factors like HIF1$\alpha$ and NFAT, potentiating the pro-proliferative and anti-apoptotic signaling.

Figure 2: Ucp2-deficiency reduces ER-mitochondrial calcium transport. (A) FRET analysis of Ca$^{2+}_{\text{m}}$ in Ucp2WT- and Ucp2KO-PASMC at basal normoxic conditions, hypoxia, and with the UCP2 inhibitor genipin. Lack of UCP2 causes a decrease in Ca$^{2+}_{\text{m}}$, similar to that caused by either hypoxia or genipin in Ucp2WT-PASMC. Hypoxia or genipin, do not have any additional effects on Ucp2KO PASMC (n>50 cells/group; *p<0.05 compared to normoxic vehicle). Bar = 20$\mu$m. (B) Representative traces of YFP and CFP fluorescence after histamine addition on PASMCs treated with either vehicle or ruthenium red (left). Percent increase in Ca$^{2+}_{\text{m}}$ in response to histamine is calculated by the YFP/CFP ratio (right). The response to histamine is blunted in Ucp2KO-PASMC, whereas the ruthenium red inhibits the response to histamine by a similar degree in Ucp2WT- and Ucp2KO-PASMC (n>15 cells/group, *p<0.05 compared to WT normoxic vehicle, +p<0.05 compared to KO normoxic vehicle). (C) Transmission electron microscopy of Ucp2WT- and Ucp2KO-PASMCs (left) and minimum ER-mitochondrial distance (right) (n=50 ER-mitochondria contact points/group, * and + p<0.05 compared to normoxic controls). E=endoplasmic reticulum, M=mitochondria. Arrows denote sites of ER-mitochondrial contact.

Figure 3: Ucp2-deficiency mimics a hypoxia-induced suppression of mitochondrial function and apoptosis. (A) Lack of UCP2 in Ucp2KO-PASMC decreases PDH activity similar to hypoxia in the Ucp2WT-PASMCs. Hypoxia does not have additional effects on Ucp2KO-PASMCs (n=3; *p<0.05 compared to normoxic vehicle). (B) Percent decrease in Krebs’ cycle metabolites of hypoxic Ucp2WT-PASMCs and normoxic and hypoxic Ucp2KO-PASMCs compared to normoxic Ucp2WT-PASMCs. Lack of UCP2 decreases all Krebs’ metabolites, similar to hypoxia in Ucp2WT-PASMCs. Hypoxia does not have any additional effects on UCP2 KO-PASMCs (n>3 merged samples/group). (C) mROS (left) and $\Delta\Psi_{\text{m}}$ (right) in Ucp2WT-PASMC and Ucp2KO-PASMC in normoxia and hypoxia. Lack of UCP2 decreases mROS and increases $\Delta\Psi_{\text{m}}$ similar to hypoxia in Ucp2WT-PASMCs. Hypoxia does not have any additional effects on Ucp2KO-PASMCs (n>150 cells/group, *p<0.05 compared to normoxic vehicle). (D) Serum-starvation induces apoptosis in Ucp2WT-PASMC, but not Ucp2KO-PASMCs (n>25 fields/group, * and # p<0.05 compared to 10% FBS and 0.1% FBS, respectively).

Figure 4: Ucp2KO-PASMCs have normoxic HIF1$\alpha$ activation. (A) Representative confocal microscopy images show the H$_2$O$_2$-sensitive dye Amplite (green) and the nuclear stain Hoechst 33342 (blue). Normoxic Ucp2KO-PASMCs have lower H$_2$O$_2$ levels than normoxic Ucp2WT-PASMCs, but similar to hypoxic Ucp2WT-PASMCs. Hypoxia does not further reduce Amplite in Ucp2KO-PASMCs (n~150 cells/group). *p<0.05 vs. normoxic Ucp2WT-PASMCs. Bar = 20$\mu$m. (B) Representative confocal microscopy images show HIF1$\alpha$ (green) co-localizing with the nuclear stain DAPI (blue). Bar=20$\mu$m. Top: Normoxic Ucp2KO-PASMCs have similar percentage of HIF1$\alpha$-positive nuclei compared to hypoxic Ucp2WT-PASMCs and this is not altered by hypoxia (n~30 fields/group from 3 separate experiments). Bottom: Normoxic Ucp2KO-PASMCs have similar HIF1$\alpha$-driven luciferase signal compared to hypoxic Ucp2WT-PASMCs. Hypoxia does not further increase the HIF1$\alpha$-driven luciferase signal in hypoxic Ucp2KO-PASMCs (n=15-18 wells/group). *p<0.05 vs. normoxic Ucp2WT-PASMCs.
**Figure 5:** Ucp2KO-PASMCs have normoxic NFATc2 activation. **A)** Representative confocal microscopy images show the cytosolic Ca\(^{2+}\)-sensitive dye Fluo3 (green) and the nuclear stain Hoechst 33342 (blue). Normoxic Ucp2KO-PASMCs have cytoplasmic Ca\(^{2+}\) levels higher than normoxic Ucp2WT-PASMCs, but similar to hypoxic Ucp2WT-PASMCs. Hypoxia does not further increase cytoplasmic Ca\(^{2+}\) in Ucp2KO-PASMCs (n=30-80 cells/group). *p<0.05 vs. normoxic Ucp2WT-PASMCs. Bar = 20\(\mu\)m. **B)** Representative immunoblots showing total and inactive (i.e. phosphorylated) GSK3\(\beta\) (P-GSK3\(\beta\)). Data are presented as a ratio of active to inactive enzyme. Normoxic Ucp2KO-PASMCs have similar GSK3\(\beta\) inhibition compared to hypoxic Ucp2WT-PASMCs. Hypoxia does not further inhibit GSK3\(\beta\) in Ucp2KO-PASMCs. (n=3 experiments, *p<0.05 vs. normoxic Ucp2WT-PASMCs. **C)** Representative confocal microscopy images show the expression of NFATc2 and the nuclear stain DAPI (blue). Normoxic Ucp2KO-PASMCs have a similar percentage of NFATc2-positive nuclei compared to hypoxic Ucp2WT-PASMCs. Hypoxia does not further increase the percentage of NFATc2-positive nuclei in Ucp2KO-PASMCs (n~30 fields/group). *p<0.05 vs. normoxic Ucp2WT-PASMCs. Top panel: Bar = 50\(\mu\)m; Bottom panel: Bar = 20\(\mu\)m.

**Figure 6:** Ucp2-deficient mice develop spontaneous PHT. **(A-B)** Ucp2KO mice have elevated mean pulmonary artery pressure (mPAP) and total pulmonary resistance (TPR) compared to Ucp2WT controls in normoxia, similar to hypoxic Ucp2WT mice. Hypoxia does not cause further changes in hemodynamics in UCP2KO mice (Each point represents one animal, *p<0.05 compared to normoxic Ucp2WT mice). **(C)** Ucp2KO mice have greater medial wall thickness compared to Ucp2WT controls in normoxia, similar to hypoxic Ucp2WT mice (n~50 vessels/group, *p<0.05 vs. normoxic Ucp2WT-mice). **(D)** Ucp2KO mice have higher proliferation rates (%Ki67-positive) in lung smooth muscle actin (SMA)-positive cells compared to Ucp2WT controls in normoxia, similar to hypoxic Ucp2WT mice (n>35 vessels/group, *p<0.05 compared to normoxic Ucp2WT mice). **(E)** Ucp2KO mice have greater medial wall thickness compared to Ucp2WT controls in normoxia, similar to hypoxic Ucp2WT mice (n>70 vessels/group). **(F)** Ucp2KO mice have higher hematocrit compared to Ucp2WT controls in normoxia, similar to hypoxic Ucp2WT mice (Each point represents one animal, *p<0.05 vs. normoxic Ucp2WT mice).
Novelty and Significance

What Is Known?

- In pulmonary hypertension, vascular cells in the remodeled pulmonary arteries are characterized by a suppression of mitochondrial function and a switch towards a glycolytic phenotype.

- This metabolic switch causes a cancer-like resistance to apoptosis and promotes proliferation leading to a proliferative vascular remodeling.

- Even in the absence of hypoxia, remodeled pulmonary arteries in pulmonary hypertension show activation of the Hypoxia Inducible Factor 1α (HIF1α).

What New Information Does This Article Contribute?

- Absence of UCP2 in pulmonary artery smooth muscle cells (PASMCs) is associated with a decrease in mitochondrial calcium levels and a suppression of mitochondrial function.

- The resulting suppression of downstream mitochondrial signaling causes activation of HIF1α, even under normoxia.

- UCP2-deficient mice spontaneously develop proliferative pulmonary vascular remodeling and pulmonary hypertension.

We found that UCP2 mediates calcium transfer from the endoplasmic reticulum to the mitochondria of PASMCs and its absence may explain the metabolic remodeling in pulmonary hypertension and the normoxic activation of HIF1α. Future studies exploring the recently described UCP2 polymorphisms, associated with decreased levels of the gene, may lead to discovery of novel biomarkers and treatments in human pulmonary hypertension. A role of UCP2 should also be considered in diseases characterized by a pseudohypoxic HIF1α activation.
Figure 1
Uncoupling Protein 2 Deficiency Mimics the Effects of Hypoxia and Endoplasmic Reticulum Stress on Mitochondria and Triggers Pseudo-Hypoxic Pulmonary Vascular Remodeling and Pulmonary Hypertension

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Circ Res. published online May 7, 2013;

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/early/2013/05/07/CIRCRESAHA.112.300699

Data Supplement (unedited) at:
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Supplemental Material

**Methods:**

**Cell Culture:** PASMCs from five *Ucp2*WT- or *Ucp2*KO-mice were freshly isolated from fourth generation pulmonary arteries, with an enzymatic cocktail containing papain (1mg/ml) (Sigma Aldrich), dithiothreitol (DTT; 0.5mg/ml) (Sigma Aldrich), collagenase (0.6 mg/ml) (Worthington), and bovine serum albumin (0.6 mg/ml) (Sigma-Aldrich) and pooled as previously described. PASMCs were grown in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco, Invitrogen) supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich) and 1% antibiotic/antimycotic (Gibco, Invitrogen) and placed in a humidified incubator set at 37°C in either normoxic conditions (21% O2; pO2~120mmHg) or mild-moderate (physiologic) hypoxia conditions (4% O2; pO2~40mmHg), while the pH and PCO2 remain within normal limits.

**FRET analysis:** PASMCs were plated on glass dishes and transfected with the 4mtD3CPV cameleon plasmid using a Xfect transfection reagent (Clontech) under normoxic conditions as previously described. After 24 hours, media was replaced with treatment media containing vehicle (DMSO) (Sigma Aldrich) or genipin (50 µM, Wako) and placed in normoxia or hypoxia. After 48 hours, media was diluted with 4% paraformaldehyde in a 1:1 ratio and cells were fixed under treatment conditions for 60 minutes. Cells were washed with distilled water and mounted on a slide using Prolong Gold (Invitrogen) and imaged 48 hours later on a Zeiss LSM 510 confocal microscope. Excitation occurred at 458 nm and the emission filters were 480 to 520 nm for cyan (when Ca2+ is not bound) and 535 to 590 nm for FRET (yellow when Ca2+ is bound). The ratio of yellow/cyan intensities was used to standardize the rate of infection for each cell, as previously described.

**Histamine stimulation:** Media from cells cultured under normoxic or hypoxic conditions was washed and replaced with calcium-free buffer [NaCl (125mM), KCl (5mM), Na3PO4 (1mM), MgSO4 (1mM), Glucose (5.5mM), HEPES (20mM)] as previously described, containing either vehicle or ruthenium red (10µM, Calbiochem). A series of 60 images (8sec/image) was performed. Histamine (1mM) was added to give a final concentration of 100µM on the 6th
image, in order to establish a 5-image baseline fluorescence. The percent increase was determined by the maximum YFP/CFP ratio after histamine minus the average baseline YFP/CFP ratio divided by the average baseline YFP/CFP ratio.

**Electron Microscopy:** Cells were grown to 80% confluence on 100mm dishes. Media was aspirated and replaced with Karnovsky’s fixative [2% paraformaldehyde (Sigma Aldrich) and 2% gluteraldehyde in phosphate buffer] for 1 hr at room temperature. Cells were then washed with phosphate buffered saline, scraped and centrifuged. Pelleted material was transferred to a scintillation vial and post-fixed with 1% osmium tetroxide in phosphate buffer for 1 hour on ice. After washing in PBS, cells were serially dehydrated in ethanol and rinsed in propylene oxide, before being incubated for 18 hours in 1:1 ratio of propylene oxide and resin. After 24 hours, cells were placed in 100% embedding resin under a vacuum for 6-8 hours before being transferred to a cutting tube and polymerizing at 60 degrees Celsius for 48 hours. Samples were then sliced, mounted on metal gratings and imaged on a Phillips EM 400T/ST transmission electron microscope. Quantifications were performed at 40,000x magnification. Representative images are at 105,000x magnification.

**PDH Activity:** PDH activity was measured with a commercially available MitoProfile Dipstick assay kit (Mitosciences) as previously described. Briefly, 50µL of protein (1mg/ml) was incubated with a dipstick containing the PDH complex antibody in a 96 well plate, followed by activity buffer. A flat top scanner was used to measure the band intensity.

**Metabolite extraction:** PASMCs were pelleted and suspended in 800µL of ice-cold 80% methanol and 20% ddH2O. Samples were vigorously vortexed and placed in liquid N2 for 10 minutes to freeze. Samples were then thawed on ice for 10 minutes and the freeze-thaw cycle was repeated two times. Samples were centrifuged at 13,000g to pellet cell debris, lipids and proteins. Supernatant was evaporated and metabolites were re-suspended in HPLC-grade H2O. Metabolites were normalized to protein concentration.

**Mass Spectroscopy:** Seven microliters of sample was injected using a 4000 QTRAP mass spectrometer (AB Sciex) equipped with a UHPLC 1290 system (Agilent) via SRM for Krebs’
cycle intermediates. Samples were delivered to the MS with mobile phases A (20mM NH₄OH, 20mM NH₄Ac in 95%/5% H₂O/CH₃CN) and B (98% CH₃CN, 2% H₂O) via a 2.0mm i.d. x 10cm HILIC Luna NH₂ column (Phenomenex) at 250µl/min using negative ion LC/MS/MS analytical run. The dwell time was 5ms per SRM transition, and collision energy was optimized for each SRM transition. Total cycle time was 2.09 s.

**HIF1α luciferase:** PASMCs (20,000 cells/well) were seeded into a black, clear bottom 96-well plate and allowed to adhere. Cells were transfected with the HIF1α dual luciferase plasmid (SABiosciences) using Xfect transfection reagent (Clontech) under normoxic conditions. After 24 hours, media was replaced with treatment media and cells were placed in normoxia or hypoxia. HIF1α activity was assessed with the dual-luciferase reporter assay system (Promega). After 48 hours, cells were lysed by a freeze-thaw cycle in passive lysis buffer (provided in kit) and luminescence was measured with a luminometer. HIF1α activity was assessed by HIF1α-driven firefly luminescence normalized to a constitutively-driven Renilla luminescence to standardize the transfection as previously described²,⁵.

**qRT-PCR:** qRT-PCR was performed as previously described¹,². mRNA isolated from PASMCs were added to a microwell plate with TaqMan probes and reagents. qRT-PCR was performed with the ABI Prism 7700 Sequence Detector (Applied Biosystems), and β₂-microglobulin was used as a housekeeping gene (Applied Biosystems).

**Medial Wall Thickness:** The percent medial wall thickness was determined as previously described¹,². Briefly, 5µm-thick lung sections were stained using hematoxylin and eosin (H&E) stain. Vessels >50µm and <300µm were identified and measured at the two ends of the shortest external diameter of the distal PAs, and the average was taken [(2 x wall thickness/external diameter) x 100].

**Muscularization:** Lung sections (5µm) were stained for smooth muscle actin and von Willebrand Factor (endothelial cell marker). Vessels (<50µm) were classified as fully (100%),
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partially, or non-muscularized (0%), based on the percentage of vWF surrounded by SMA in each vessel as previously described \(^1\).

**Systemic blood pressure:** Systemic systolic and diastolic blood pressures were measured in non-anesthetized mice with the CODA2 (Kent Scientific Corp.) mouse tail cuff system as previously described \(^2\). Animals were restrained in a holding chamber and an occlusion cuff was placed at the base of the tail. A volume pressure cuff was placed distally. Systolic and diastolic blood pressures were obtained by averaging at least five volume-pressure recordings per animal.

**Immunoblots:** Tissues were collected and immunoblotting was performed as previously described \(^1,2,4,6\). The films were digitized and quantified with 1D Image Analysis Software (Kodak, Rochester, NY). Expression was normalized to \(\alpha\)-actin to correct for loading differences. Antibodies: UCP2 1:1000; COXIV 1:1000; GRP78 1:200 (both Santa Cruz Biotechnologies), HIF1\(\alpha\) 1:1000; hydroxylated HIF1\(\alpha\) 1:1000 (both Cell Signaling), UCP3 1:1000 (Proteintech), SMA 1:2000 (Abcam).
Online Figure I: UCP expression in *Ucp2WT* and *Ucp2KO* tissues. 
(A) *Ucp2* mRNA expression in UCP2WT- and KO- PASMCs. (B) Immunoblot on isolated mitochondria showing presence and absence of UCP2 in spleen (top left), lung (top right) and in isolated PASMCs (bottom left) from *Ucp2WT* and *Ucp2KO* mice, respectively. Very faint bands can be seen in the UCP2KO samples, likely because UCP2 and 3 have a >70% homology, creating a recognized problem of UCP2 antibodies. UCP3 expression was not upregulated in the UCP2KO PASMCs, as assessed by a commercial UCP3-antibody (bottom right). Even though the faint band can represent UCP3 in these otherwise well-validated and studied Ucp2KO mice \(^9\) (where we also show complete absence of Ucp2 mRNA), it is important to document that UCP2 levels are quite low and compatible with the cellular phenotype that we describe in this work.
Online Figure II: Rhodamine-2AM staining for Ca\(^{2+}\)\(_m\) in *Ucp2WT* and *Ucp2KO* PASMCs in normoxia and hypoxia. Representative confocal microscopy images show Rhodamine-2AM (red) and the nuclear stain Hoechst 33342 (blue). Normoxic *Ucp2KO*-PASMCs have Ca\(^{2+}\)\(_m\) levels similar to hypoxic *Ucp2WT*-PASMCs. Hypoxia does not further reduce Ca\(^{2+}\)\(_m\) in *Ucp2KO*-PASMCs (n=100 cells/group). *p<0.05 vs. normoxic *Ucp2WT*-PASMCs. Bar = 20µm.
Online Figure III: Expression of ER-stress marker glucose regulated protein-78 (GRP78) in Ucp2WT- and Ucp2KO- PASMCs at baseline. (A) Grp78 mRNA expression in Ucp2WT- and Ucp2KO-PASMCs do not significantly differ (n=3 experiments), P>0.05. (B) Immunoblot showing GRP78 protein expression does not change between isolated Ucp2WT- and Ucp2KO-PASMCs (n=3 experiments). P>0.05.
Online Figure IV: HIF1α expression and activity in Ucp2WT- and Ucp2KO- PASMCs. (A) mRNA expression of the HIF1α downstream target Vegf is higher in Ucp2KO-, compared to Ucp2WT-PASMCs (n=3 experiments).*p<0.05. (B) Immunoblot showing hydroxylated-HIF1α (inactivated) (top) and total HIF1α (bottom) in Ucp2WT- and Ucp2KO-PASMCs. Ucp2WT-PASMCs have a higher ratio of hydroxylated to total HIF1α compared to Ucp2KO-PASMCs.
Online Figure V: In vivo hemodynamic traces.
Representative traces of the right atrium (RA), right ventricle (RV) and pulmonary arteries (PAs) for *Ucp2*WT- and KO- mice in normoxia and hypoxia. Normoxic *Ucp2*KO mice have higher PA pressures compared to normoxic *Ucp2*WT mice. Hypoxia does not increase PA pressures in *Ucp2*KO-mice more than in *Ucp2*WT mice.
Online Figure VI: Systemic blood pressures in *Ucp2WT* and *Ucp2KO*-mice.
There were no statistically significant differences in systolic (left) and diastolic (right) blood pressures of *Ucp2WT-* and *Ucp2KO-* mice in normoxia and hypoxia (n=5 animals/group). 
*p*>0.05.
**Online Figure VII: Right ventricular hypertrophy.**
*Ucp2KO mice* have a non-significant trend in RV hypertrophy (RV/LV+Septum) compared to *Ucp2WT* controls in normoxia (p=0.089). Hypoxia similarly increases RV hypertrophy in *Ucp2WT* and *Ucp2KO* mice (Each point represents one animal, *p*<0.05 compared to normoxic *Ucp2WT* mice).
Online Figure VIII: Whole lung respiration.
Representative oxygen traces of Ucp2WT (black) and Ucp2KO (red) lung tissue (left). Lung tissue from Ucp2WT mice had a higher rate of oxygen consumption compared to lung tissue from Ucp2KO mice (n=lungs from 5 animals/group). *p<0.05.
Online Figure IX: Ucp2KO-mice have normoxic NFATc2 activation in SMA-positive cells in the distal pulmonary arteries.

Representative confocal microscopy images show NFATc2 (red), SMA (green) and the nuclear stain DAPI (blue). Arrows represent SMA-positive/NFAT-positive nuclei. SMA-positive cells in the distal PAs of normoxic Ucp2KO-mice have similar number of NFATc2-positive nuclei compared to SMA-positive cells in the distal PAs of hypoxic Ucp2WT-mice. Hypoxia does not further increase the NFATc2-positive nuclei in SMA-positive cells in the distal PAs of normoxic Ucp2KO-mice. (n~30 vessels/group) *p<0.05 vs. normoxic Ucp2WT-mice. Bar = 50µm.
Supplement References


