**Impact of the mitochondria-targeted antioxidant MitoQ on hypoxia-induced pulmonary hypertension**

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**At a Glance Commentary**

**Scientific Knowledge on the Subject:** Reactive oxygen species (ROS), particularly superoxide, originating from mitochondria have been suggested to regulate hypoxic pulmonary vasoconstriction (HPV), chronic hypoxia-induced PH and possibly right ventricular (RV) remodeling. However, no treatment intervention targeting ROS is established, yet.

**What This Study Adds to the Field**: An increase of mitochondria-derived superoxide regulates acute HPV. The mitochondria-targeted antioxidant MitoQ can attenuate acute HPV, and RV remodeling, but not chronic hypoxia-induced PH. Thus, MitoQ is suggested to be beneficial as a treatment option for overactivated HPV or RV decompensation.

**This article has an online data supplement, which is accessible from this issue's table of content online at** [**www.atsjournals.org**](http://www.atsjournals.org/)**.**

**Abstract**

*Rationale*: Increased mitochondrial reactive oxygen species (ROS), particularly superoxide have been suggested to mediate hypoxic pulmonary vasoconstriction (HPV), chronic hypoxia-induced pulmonary hypertension (PH) and right ventricular (RV) remodeling.

*Objective:* We determined ROS generation in acute and chronic hypoxia and investigated the effect of the mitochondria-targeted antioxidant MitoQ under these conditions.

*Measurements and Main Methods*: The effect of MitoQ or its inactive carrier substance, triphenylphosphonium (TPP+), on pulmonary vasoconstriction during acute hypoxic ventilation (1% O2, 10 minutes) was investigated in isolated ventilated and blood free perfused mouse lungs. Mice exposed for 4 weeks to chronic hypoxia (10% O2) or after banding of the main pulmonary artery (PAB) were treated with MitoQ or TPP+ (50 mg/kg/day) by gavage. The concentration of different ROS was measured by electron spin resonance spectroscopy, as well by a fluorescent dye and a protein-based sensor.

*Results*: Total cellular ROS, and particularly mitochondrial superoxide levels were increased in pulmonary artery smooth muscle cells (PASMC), but decreased in pulmonary fibroblasts after acute hypoxic exposure. MitoQ, but not TPP+, significantly inhibited HPV and the acute hypoxia-induced rise in superoxide concentration. After chronic hypoxia superoxide levels were decreased in PASMC, while they increased in the RV. Correspondingly, MitoQ did not affect the development of chronic hypoxia-induced PH, but instead attenuated RV remodeling after chronic hypoxic exposure as well as after PAB.

*Conclusion*: Increased mitochondrial superoxide concentration in PASMC mediates acute HPV, but not chronic hypoxia-induced PH. MitoQ may be beneficial under conditions of exaggerated acute HPV and RV decompensation in PH.

**Abstract word count:** 250

**Keywords:** Hypoxic pulmonary vasoconstriction, Pulmonary hypertension, Mitochondria, MitoQ, Reactive oxygen species, Right ventricular remodeling.

**Introduction**

Hypoxic pulmonary vasoconstriction (HPV) is a unique physiological reaction of the precapillary pulmonary vessels to alveolar hypoxia and serves to maintain ventilation-perfusion matching, thereby optimizing the oxygenation of blood[1](#_ENREF_1), [2](#_ENREF_2). In contrast, generalized chronic hypoxia (*e.g.* in high altitude or in chronic obstructive pulmonary disease) leads to the development of pulmonary hypertension (PH), which is a progressive disorder characterized by an increase of pulmonary vascular resistance due to pulmonary vascular remodeling, ultimately resulting in right heart failure[3](#_ENREF_3). In addition to vascular remodeling, generalized HPV contributes to the increased vascular resistance in chronic hypoxia-induced PH.

Pulmonary arterial smooth muscle cells (PASMC) are key players in these responses, as they react to acute hypoxia with contraction and to chronic hypoxia with proliferation, even when isolated[4](#_ENREF_4), [5](#_ENREF_5). Mitochondrial release of ROS has been suggested to play a crucial role in both processes by interaction with protein kinases, phospholipases and ion channels inducing intracellular calcium release, or stabilization of transcription factors[6](#_ENREF_6), [7](#_ENREF_7). Additionally, ROS may be involved in the development of right ventricular (RV) remodeling[8](#_ENREF_8). However, it remains unclear if ROS are increased or decreased during acute and chronic hypoxia in the pulmonary vasculature and RV, and which species (superoxide or H2O2) triggers these responses[1-3](#_ENREF_1), [5-7](#_ENREF_5). Application of the unspecific ROS inhibitors N-acetylcysteine could inhibit hypoxia-induced PH[9](#_ENREF_9), while the superoxide dismutase (SOD)-mimetic Tempo (which decreases the concentration of superoxide, but might increase the concentration of H2O2) decreased RV remodeling, but not pulmonary vascular remodeling in chronic hypoxic rats[10](#_ENREF_10). A recent study showed that overexpression of the mitochondrial SOD2 increased and overexpression of mitochondria-targeted catalase decreased pulmonary vascular remodeling, respectively, suggesting that increased mitochondrial H2O2 contributes to hypoxia-induced PH[11](#_ENREF_11).

MitoQ is an orally available mitochondria-targeted antioxidant, which consists of an ubiquinone moiety linked to a triphenylphosphonium (TPP+) molecule[12](#_ENREF_12). The lipophilic TPP+ cation allows MitoQ to pass through the phospholipid bilayers and to accumulate within the mitochondrial inner membrane driven by the mitochondrial membrane potential[12](#_ENREF_12). In the mitochondrial inner membrane, the positively charged residue (TPP+) of MitoQ is adsorbed to the matrix surface, while the hydrophobic end (ubiquinone) is inserted into the hydrophobic core of the mitochondrial inner membrane[13](#_ENREF_13). The active antioxidative form of MitoQ, ubiquinol, is oxidized by ROS to the inactive form ubiquinone which is continually recycled by complex II of the respiratory chain to its active ubiquinol form[12](#_ENREF_12). MitoQ is an effective antioxidant against lipid peroxidation, peroxynitrite, the hydroperoxyl radical and superoxide, although its reactivity with hydrogen peroxide (H2O2) is negligible[13-15](#_ENREF_13). However, under specific conditions, particularly in the non-membrane bound form, MitoQ also may increase superoxide generation[16](#_ENREF_16),[12](#_ENREF_12). MitoQ has been demonstrated to be protective against several ROS mediated pathologies, including cardiovascular diseases[17](#_ENREF_17), [18](#_ENREF_18) and sepsis[19](#_ENREF_19) in experimental animal models, as well as in humans[20](#_ENREF_20). Although several studies addressed the role of superoxide and H2O2 in development of chronic hypoxia-induced PH, MitoQ offers the possibility to specifically target mitochondrial ROS release as a therapeutic approach.

Against this background we aimed to elucidate the role of mitochondria-derived ROS in HPV, in chronic hypoxia-induced PH and in development of RV remodeling. We hypothesized that the mitochondria-targeted antioxidant MitoQ is useful 1) to investigate the role of mitochondrial ROS in these process and 2) as a possible therapeutic tool.

**Methods**

All animal experiments were approved by the local authorities (Regierungspraesidium Giessen). C57BL/6J mice of either sex were studied. All reagents were purchased from Sigma Aldrich, unless stated otherwise. MitoQ was provided by Antipodean Pharmaceuticals Inc.

**Isolated blood-free perfused and ventilated mouse lung**

Lungs of mice were isolated ventilated and blood free perfused as described previously[21](#_ENREF_21). MitoQ or TPP+ was added to the perfusate 5 minutes before the respective hypoxic ventilation (1% O2, 5.3% CO2, rest N2) period, or the bolus injection of U46619, which was used as hypoxia-independent vasoconstrictive stimulus. For ROS measurements, CMH was added 5 minutes before the hypoxic ventilation and lung homogenate was taken 5 minutes after start of hypoxic ventilation. All gas concentrations are given for normobaric conditions.

**Chronic hypoxic exposure, pulmonary arterial banding and treatment with MitoQ**

For induction of chronic hypoxia-induced PH, mice were kept under normobaric hypoxia (10% O2) in a ventilated chamber for 28 days. Banding of the main pulmonary artery (PAB) was performed as described previously and mice were analyzed after three weeks[22](#_ENREF_22). During chronic hypoxic incubation and after PAB, mice were treated with MitoQ or TPP+ dissolved in tapped water at a concentration of 50mg/kg/day by gavage[12](#_ENREF_12). Control mice were kept in a normoxic chamber and also treated with MitoQ or TPP+.

Development of PH was determined by measurement of right ventricular systolic pressure (RVSP), pulmonary vasculature remodeling, and right ventricular (RV) hypertrophy, as described previously[23](#_ENREF_23). Global and right heart function were measured by transthoracic echocardiography as described previously[24](#_ENREF_24).

**Isolated pulmonary arterial smooth muscle cells (PASMC) and lung fibroblasts (LF)**

PASMC and LF were isolated from precapillary pulmonary arteries and cultured as described previously[24](#_ENREF_24), [25](#_ENREF_25).

**Measurement of ROS release by electron spin resonance spectroscopy and fluorescence approaches**

Intracellular and extracellular concentrations of ROS and reactive nitrogen species (RNS) were determined in PASMC and tissues by means of an ESR spectrometer (EMXmicro, Bruker, Rheinstetten, Germany), using the spin probe 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine (CMH, 0.5 mM)[26](#_ENREF_26), [27](#_ENREF_27). The superoxide portion of ROS was determined by subtracting the ESR signal of the samples incubated with 50 Units/ml polyethylen-glycol conjugated pSOD and CMH from the signal of the CMH-only samples. Hypoxia was applied by exposing the PASMCfor 5 minutes (acute hypoxia) or 5 days (chronic hypoxia) to 1% O2 within a hypoxic glove chamber (Coy Laboratory Products, Coy Drive, Michigan). In order to avoid reoxygenation, the hypoxic samples were frozen directly in liquid nitrogen within the hypoxic chamber.

To investigate the ROS/RNS levels within lung and heart tissues in chronic hypoxia, mice exposed for 4 weeks to hypoxia (10% O2) were sacrificed under hypoxia and superoxide concentration was measured by ESR spectroscopy as described above.

For isolated lung experiments only CMH and not the CMH minus CMH/pSOD signal was used, as perfusion of the lungs with pSOD for 2h prior to measurement was not possible. However, the CMH signal represents the total ROS/RNS signal without specification for superoxide[26](#_ENREF_26), [27](#_ENREF_27).

Mitochondrial superoxide concentration was measured by MitoSOX (Thermo Fisher Scientific, Waltham, USA) using fluorescence microscopy with a hypoxic perfusion system and the microplate reader Infinite M200 (Tecan Group Ltd., Männedorf, Switseland) during acute and chronic hypoxic experiments, respectively. The protein-based fluorescent sensor HyPercyto (Evrogen, Moscow, Russia) was used to detect cytosolic H2O2 concentration.

**MitoQ concentration in the lung and in the heart**

MitoQ concentration was measured by liquid chromatography and tandem mass spectrometry[28](#_ENREF_28).

**Proliferation assay**

**Proliferation of PASMC was evaluated by determination of the ratio of proliferating cells labeled by 5-ethynyl-uridine (EdU) to the total cell number labeled by Hoechst staining as described previously**[**23**](#_ENREF_23)**.**

**Statistical Methods**

Values are given as means±SEM. Statistical significanceof the data was calculated by Student's *t*test with Welsh's correction in experiments with two experimental groups and by one or two-way analysis of variance (ANOVA) in experiments with more than two experimental groups. Two-way ANOVA was used to verify interactions between the factors of treatment (TPP+ vs. MitoQ) and exposure (normoxia/hypoxia or PAB/sham). A *p* value less than 0.05 was considered significant.

Additional materials and methods are available in the online data supplement.

**Results**

**MitoQ inhibits acute HPV, as well as the hypoxia-induced increase of superoxide in PASMC**

The strength of HPV was determined as the increase in PAP (∆PAP) during hypoxic ventilation in isolated perfused and ventilated mouse lungs. The first hypoxic maneuver in the absence of any substance served as baseline and was set to 100%. MitoQ or TPP+ was applied in increasing concentrations during the subsequent hypoxic maneuvers. In the control group neither MitoQ nor TPP+ was applied. Application of 0.5µM MitoQ significantly attenuated the hypoxia-induced elevation of PAP (4th hypoxic maneuver) compared to ∆PAP in the absence of MitoQ (1st hypoxic maneuver) or ∆PAP in the presence of 0.5µM of TPP+ during the 4th hypoxic maneuver (Figure 1A). In contrast, higher doses of MitoQ or TPP+ (1µM) reduced acute HPV to a similar extent (5th hypoxic maneuver) compared to the control group (Figure 1A). Both MitoQ and TPP+ did not alter U46619-induced vasoconstriction (Figure 1B) displaying the specificity of the MitoQ effect for HPV. The strength of HPV and U46619-induced pulmonary vasoconstriction was similar in all experimental groups prior to the application of MitoQ or TPP+ (Figure E1A-B in the Online Data Supplement).[29](#_ENREF_29), [30](#_ENREF_30)

Exposure of PASMC to 1% O2 but not to 5, 10 and 15% O2 for 5 minutes induced an increase of the total intra- and extracellular superoxide concentration measured by ESR spectroscopy as difference of the signal of the spin probe CMH and the signal of CMH+pSOD (∆CMH), (Figure 1C). In contrast, the level of ROS/RNS measured by the CPH spin probe which is less lipophilic than CMH and thus regarded to measure preferentially cytosolic and/or extracellular, but not mitochondrial ROS/RNS[31](#_ENREF_31), and the level of extracellular superoxide measured as difference of the signal of CMH and the signal of CMH+SOD, which cannot diffuse into cells, was not altered after 5 minutes of hypoxic exposure (Figure E2A-B in the Online Data Supplement). Application of 0.1µM MitoQ specifically inhibited the acute hypoxia-induced increase of superoxide, while no effect of TPP+ in this concentration on the hypoxia-induced increase of superoxide was detected (Figure 1D). In higher concentrations (0.5µM) both, MitoQ and TPP+ caused a significant decrease of superoxide concentration in normoxia and hypoxia.

**Exposure to acute hypoxia induces an increase of cytosolic and mitochondrial superoxide specifically in PASMC**

We further focused on superoxide release in the intact organ and on the subcellular distribution of different ROS. In contrast to isolated PASMC, the ROS/RNS levels in lung homogenate from intact lungs exposed to acute hypoxic ventilation were decreased as determined by the spin probe CMH (Figure 2A). Accordingly, we detected decreased superoxide release from isolated lung fibroblasts (LF) during hypoxic exposure (Figure 2B). Staining of lung slices from isolated lungs revealed a specific increase of mitochondrial ROS in pulmonary vessels and bronchi, but not alveolar fibroblasts (E2C, D in the Online Data Supplement). With regard to the intracellular distribution, mitochondrial superoxide concentration measured with MitoSOX also showed increased mitochondrial superoxide release during acute hypoxic superfusion in isolated PASMC (Figure 2C).

Additionally, we increased the PASMC protein expression level of SOD1, which is located in the cytosol and mitochondrial intermembrane space, and SOD2, which is located in the mitochondrial matrix, and determined the superoxide concentration in these PASMC after 5min of exposure to 1% O2 by ESR spectroscopy using the CMH spin probe. Overexpression of either SOD1 or SOD2 prevented the acute hypoxia-induced increase of the CMH signal compared to the respective non-transfected controls (Figure E2E, F in the Online Data Supplement) suggesting that superoxide released into the mitochondrial matrix and into the mitochondrial intermembrane space/cytosol contributed to the acute hypoxia-induced increase of superoxide in PASMC.

Given that acute hypoxia increased the mitochondrial superoxide concentration and H2O2 has been suggested to act as downstream messenger during acute hypoxia[32](#_ENREF_32), we measured the level of cytosolic H2O2 by the HyPercyto fluorescent probe[33](#_ENREF_33). Acute hypoxia increased the concentration of H2O2 in PASMC during superfusion with hypoxic buffer (Figure 2D).

**Effect of MitoQ on chronic hypoxia-induced PH, PASMC proliferation and superoxide release in chronic hypoxia**

MitoQ concentration in mouse lungs measured after 4 weeks of treatment with MitoQ in a dose of 50mg/kg/day was in the range which was previously demonstrated to have protective effects in the heart[28](#_ENREF_28) (Figure 3A). Exposure of mice to chronic hypoxia (10% O2, 4 weeks) induced an increase of the RVSP to a similar degree in both the MitoQ and TPP+ treated groups (Figure 3B). In accordance, the degree of pulmonary vascular remodeling, expressed as percent of fully-, partially- and non-muscularized vessels, was increased after hypoxic exposure, but was not altered by MitoQ or TPP+ treatment (Figure 3C). TPP+ treatment did not have any effect on RVSP or CO during chronic hypoxia compared to untreated controls (Figure E3 A-G in the Online Data Supplement). Moreover, low doses of MitoQ did not prevent hypoxia-induced proliferation of PASMC, while higher doses showed an unspecific effect on proliferation, as TPP+ also decreased proliferation in high concentrations (Figure 3D).

Accordingly, treatment with the mitochondria-targeted superoxide dismutase mimetic MitoTEMPO decreased acute HPV, but did not affect chronic hypoxia-induced PH (see Supplemental Results and Fig. E4).

Given, that MitoQ and MitoTEMPO did not prevent the development of chronic hypoxia-induced PH, we measured the ROS levels in PASMC and in lung homogenate after chronic hypoxic exposure. Total superoxide concentration, as well as mitochondrial superoxide concentration was decreased after 5 days of chronic exposure of PASMC to 1% O2 measured by ESR spectroscopy (Figure 3E), and MitoSOX fluorescence, respectively (Figure 3F). The level of H2O2 measured by HyPercyto was also decreased in PASMC after 5 days of chronic hypoxic exposure (Figure 3G). Lung homogenate of mice after chronic hypoxic exposure showed a tendency for decreased superoxide levels which, however, did not reach significance (Figure 3H). All measurements were performed in a continuous hypoxic environment, without re-oxygenation (for details please refer to the methods section).

In low concentrations, in which MitoQ did not inhibit proliferation of PASMC, neither in normoxia nor in hypoxia (Fig. 3D), 100nM MitoQ decreased the superoxide concentration in normoxia compared to the normoxic untreated control (Fig. 3I). However, there was no difference in hypoxic superoxide concentration in untreated, TPP+-treated or MitoQ-treated PASMC (Fig. 3I).

**Effect of MitoQ on RV remodeling in chronic hypoxia and after PAB**

In contrast to the development of PH and vascular remodeling, RV remodeling differed after chronic hypoxic exposure in the MitoQ treated group compared to the TPP+ treated group (Figure 4). Morphologically, the ratio of the weight of the RV compared to the weight of the LV+septum (RV/LV+septum) was significantly increased after hypoxic exposure in the TPP+, but not in the MitoQ treated group (Figure 4A). Echocardiography revealed an increase of the right ventricular wall thickness (RVWT) after chronic hypoxic exposure in both groups, albeit to a significantly lower level in the MitoQ treated group (Figure 4B). Moreover, right ventricular internal diameter (RVID) and right ventricular outflow tract diameter (RVOTD) were not increased after chronic hypoxic exposure in the MitoQ group, in contrast to the TPP+ group, indicating less RV dilatation during chronic hypoxia in the presence of MitoQ (Figure 4C, D, H). Cardiac output (CO) and TAPSE were decreased after chronic hypoxic exposure, albeit to a similar degree in both the MitoQ and TPP+ treated groups (Figure 4 E, F). TPP+ treatment also attenuated RV remodeling, characterized by a decreased of the fulton index (RV/LV+septum) and RVWT (Figure E3D, E in Online Data Supplement), while only very slightly affecting RV dilatation compared to untreated control animals (Figure E3F, G in Online Data Supplement). The level of superoxide concentration was significantly higher in the RV homogenate from untreated mice exposed for 4 weeks to chronic hypoxia (Figure 4G).

Furthermore, MitoQ application after PAB, which served as hypoxia-independent stimulus for RV hypertrophy also improved RV remodeling (Figure 5). RVID was significantly reduced (Figure 5C), while TAPSE was significantly increased (Figure 5E) after PAB in MitoQ treated mice compared to TPP+ treated mice.

**Discussion**

This study showed that increased superoxide, most likely originating from mitochondria, regulates acute, but not chronic hypoxia-induced PH. This conclusion is based on the facts that 1) mitochondrial superoxide production was increased after 5 minutes of hypoxic exposure of PASMC and decreased after 5 days of hypoxic exposure, and 2) the mitochondria-targeted antioxidant MitoQ could inhibit the acute hypoxia-induced increase in superoxide, as well as acute HPV, but not the chronic hypoxia-induced PH. Moreover, our study indicates that mitochondrial ROS may play an important role in RV remodeling treatable independently from the pulmonary circulation.

To the best of our knowledge, this is the first report that investigates a specifically mitochondria-targeted antioxidant with regard to its effects on the response of the pulmonary vasculature to acute and chronic hypoxia.

Although there is recent evidence that mitochondria-derived superoxide plays an important role in acute and chronic hypoxic signaling in the pulmonary vasculature[1](#_ENREF_1), [5](#_ENREF_5), [6](#_ENREF_6), [34-37](#_ENREF_34), two opposing concepts were proposed, one promoting an increase in ROS during hypoxia and the other a decrease[1](#_ENREF_1), [5](#_ENREF_5), [6](#_ENREF_6), [34-37](#_ENREF_34). This controversy is mainly based on the facts that 1) measurement of ROS during hypoxia is prone to artifacts, 2) different cell types and ROS as well as intra- and extracellular ROS release and their intracellular distribution has to be taken into account and 3) specific pharmacological antioxidants for mitochondria were not available. Therefore, in our study we used two approaches to measure ROS including ESR technology, as well as a fluorescent dye and protein-based sensor. Moreover, the experiments in cells and tissue homogenates in this study were performed under continuous hypoxia, so that artifacts due to reoxygenation of the measurement sample are excluded. By application of these methods, our data support the hypothesis that an increase of intracellular ROS is the underlying mechanism of HPV (“ROS hypothesis”) and are inconsistent with the “redox hypothesis” suggesting that a decrease of ROS triggers HPV[1](#_ENREF_1). We identified that the concentration of mitochondrial superoxide, as well as cytosolic H2O2, which acts as a possible downstream signaling mediator of superoxide[32](#_ENREF_32), was increased in PASMC during acute hypoxia. Superoxide can either be converted to H2O2 by mitochondrial SODs, which can diffuse into the cytosol, or be released from the mitochondria into the cytosol via voltage-dependent anion channels[38](#_ENREF_38) where it can be converted to H2O2 by the SOD1. In contrast to PASMC, acute hypoxia decreased superoxide concentration in pulmonary fibroblasts. This finding may explain that the ROS concentration in the lung homogenates was decreased during acute hypoxia. Our results thus can explain the discrepancy between measurements of ROS concentration in the whole lung and PASMC[1](#_ENREF_1) by the fact of cell type specific regulation of ROS observed in our study. Our findings are consistent with others studies showing increased mitochondrial superoxide concentration in acute hypoxia[39-42](#_ENREF_39), and in agreement with our previous findings in rabbit PASMC, where acute hypoxia triggered the increase of mitochondrial matrix superoxide concentration measured by MitoSOX[30](#_ENREF_30" \o "Sommer, 2010 #5). Although the mechanism for the superoxide release during acute hypoxia is still not fully elucidated, it was recently suggested that increased hypoxic ROS originate from complex III of the respiratory chain[41](#_ENREF_41). Downstream targets of increased mitochondrial ROS triggering HPV have been explored in many studies and were recently summarized [2](#_ENREF_2), [5](#_ENREF_5), [43](#_ENREF_43). In accordance, acute HPV could be inhibited by application of MitoQ in a low dose, in which the carrier substance did not show any effects. MitoQ is a well-established mitochondria-targeted antioxidant[44](#_ENREF_44), inhibiting superoxide release, lipid peroxidation and peroxinitrate formation[12](#_ENREF_12). However, under some conditions, especially in an aqueous environment *in vitro*, MitoQ can act as pro-oxidant and produce superoxide[16](#_ENREF_16). However, it has been shown that this does not occur *in vivo*[12](#_ENREF_12). This might be caused by the fact that the ubiquinol moiety of MitoQ is located within the phospholipid bilayer in polarized mitochondria which prevents auto-oxidation of ubiquinol[14](#_ENREF_14" \o "James, 2005 #2769). Accordingly, MitoQ inhibited superoxide concentration in PASMC during acute hypoxia in our study. Moreover, the mitochondria-targeted SOD mimetic MitoTEMPO also inhibited acute HPV suggested to be due to its property to decrease mitochondrial superoxide release, but also cytosolic H2O2 release, which is quite unexpected for a SOD mimetic, but could be shown by us in this study and others, previously[45](#_ENREF_45).

The effect that inhibition of HPV reached a significant level at a slightly higher dose of MitoQ than the inhibition of hypoxia-induced superoxide release in PASMC might be explained by the fact that isolated PASMC may be more sensitive to MitoQ than PASMC in isolated organs, where the substance has a higher distribution volume and diffusion distance.

At high doses MitoQ and TPP+ have been shown to inhibit oxidative phosphorylation[46](#_ENREF_46) and interact with the mitochondrial sodium/calcium exchanger[47](#_ENREF_47), thereby likely decreasing mitochondrial membrane potential. However, low doses of TPP+, which were used in this current study *in vitro,* did not alter mitochondrial respiration[48](#_ENREF_48). Thus, the effect of TPP+ on acute HPV and the hypoxia-induced increase in superoxide at concentrations beyond 0.5µM could be related to its property to decrease mitochondrial membrane potential, as mitochondrial hyperpolarization has been observed during acute HPV[30](#_ENREF_30)

In addition to HPV, chronic hypoxic exposure of the pulmonary vessels causes PH due to pathological pulmonary vascular remodeling which is characterized, in part, by proliferation of PASMC in the media of pulmonary vessels. Increased proliferation of PASMC can also be observed *in vitro* when PASMC are exposed to hypoxia[23](#_ENREF_23). The pathogenesis of these changes is complex and includes multifactorial cellular pathways including the alteration of ROS production[49](#_ENREF_49). As in case of HPV, evidence for both a decrease[50](#_ENREF_50) and an increase of cellular ROS concentration triggering PH development [11](#_ENREF_11) has been provided. Recently, an elegant study showed that an increase of specifically mitochondrial H2O2 may promote development of chronic-hypoxia induced PH[11](#_ENREF_11). In our study, the level of superoxide and H2O2 measured by ERS spectroscopy, MitoSOX and HyPercyto fluorescence was significantly decreased in the cytosol and mitochondria of PASMC after 5 days of incubation in 1% O2 supporting the hypothesis that chronic hypoxia induces a decrease of total cellular ROS. Moreover, in contrast to systemic hypertension where MitoQ treatment prevented an increase of systemic blood pressure[17](#_ENREF_17), oral treatment with MitoQ reaching lung tissue levels which demonstrated protective effects in a previous study[28](#_ENREF_28). In addition, continuous subcutaneous MitoTEMPO treatment did not prevent development of chronic hypoxia-induced PH. Furthermore, MitoQ did not inhibit the chronic hypoxia-induced increase of PASMC proliferation. Thus, our data suggest that ROS in PASMC do not interact with proliferative pathways in PASMC. This conclusion is supported by the finding that decreased superoxide levels in normoxia during MitoQ treatment did not alter pulmonary vascular remodeling.

It cannot be excluded, however, that a cell-type specific and localized subcellular release of specific ROS may activate proliferation pathways. Upregulation of other ROS sources might contribute to this finding, as hypoxia-induced ROS release might also originate from specific isoforms of the NADPH oxidase[2](#_ENREF_2).

Interestingly, in chronic hypoxia MitoQ application decreased the development of RV hypertrophy and RV dilatation without affecting RV performance determined by TAPSE. TPP+ also decreased RV hypertrophy compared to untreated control animals, albeit to a lesser extent than MitoQ and had almost no effect on RV dilatation, suggesting that mitochondrial ROS specifically can influence RV remodeling. Accordingly, we found that chronic hypoxia increased superoxide concentration in the RV. Application of another mitochondrial antioxidant, MitoTEMPO resulted in the tendency to prevent the dilatation of RV in chronic hypoxia which might be due to a lower potency to inhibit different kind of ROS compared to MitoQ. Furthermore, MitoQ treatment attenuated the hypertrophy and dilatation of the RV as well as prevented development of RV dysfunction after PAB.

It has been shown previously that RV remodeling may rely on ROS dependent signaling pathways[8](#_ENREF_8), [51](#_ENREF_51), [52](#_ENREF_52), and that the transition from beneficial adaptive concentric RV hypertrophy to RV dilatation and failure may depend on the amount of ROS release[53](#_ENREF_53). Our study does not answer the question of by which mechanisms mitochondrial ROS release is activated in the RV, but suggests 1) that RV remodeling is differently regulated than pulmonary vascular remodeling and 2) a beneficial potential of MitoQ in RV remodeling by prevention of RV dilatation and thus RV failure in PH. Interestingly, the cytosolic SOD mimetic Tempol also reduced chronic hypoxia-induced RV hypertrophy in rats without affecting pulmonary vascular remodeling, but inhibiting HPV[10](#_ENREF_10), [54](#_ENREF_54). However, further studies will be necessary to explore the effect of MitoQ on RV hypertrophy and failure.

In conclusion, our study revealed that acute hypoxic exposure initiated an increase of mitochondrial superoxide concentration in PASMC which regulates HPV, while chronic hypoxic exposure was associated with a decrease of superoxide concentration in PASMC. Application of the mitochondria-targeted antioxidant, MitoQ, specifically attenuated HPV, and did not inhibit the development of pulmonary vasculature remodeling and chronic hypoxia-induced PH, but attenuated RV remodeling.

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**Figure 1.**

**Effect of MitoQ on hypoxic pulmonary vasoconstriction (HPV) in isolated ventilated and perfused mouse lungs and on superoxide concentration in pulmonary artery smooth muscle cells (PASMC) during acute hypoxia.**

A) Increase of pulmonary arterial pressure (ΔPAP) in isolated ventilated and perfused mouse lungs during acute hypoxic ventilation (1% O2, 5.3% CO2, balanced with N2) or B) after the bolus injection of the thromboxane mimetic, U46619, in the absence (Control group) or presence of the mitochondria-targeted antioxidant (MitoQ group) or the inactive carrier substance (TPP+ group). n=5-6 isolated lungs each group. \*\* p<0.01 compared to TPP+ treated group, †† p<0.01, ††† p<0.001 compared to respective control groups by two-way ANOVA with Tukey post hoc test.

C) Superoxide concentration in PASMC after 5 minutes of acute exposure to different oxygen concentrations. The superoxide concentration was measured by ESR spectroscopy as difference in the CMH signal with or without pegylated superoxide dismutase (∆pSOD)**.** n=3-4 individual cell isolations per group. \*\*\* p<0.001 by one-way ANOVA with Tukey post hoc test.

D) S**uperoxide concentration in** PASMC **after either 5 minutes of acute hypoxic** (1% O2, rest N2) or normoxic (21% O2) **incubation in presence or absence of the** mitochondria-targeted antioxidant (MitoQ) or the inactive carrier substance (TPP+). n=5-6 individual cell isolations per group. \* p<0.05, \*\* p<0.01 by two-way ANOVA with Tukey post hoc test. A.U.: arbitrary units.

**Figure 2.**

**Effect of acute hypoxia on subcellular, cell-type specific ROS concentration**

A) Total intra- and extracellular ROS/RNS determined by CMH intensity in lung homogenates from isolated ventilated and perfused lungs after 5 minutes of acute hypoxic (1% O2, 5.3% CO2, balanced with N2 [5min HOX]) or normoxic ventilation (21% O2, 5.3% CO2, balanced with N2 [NOX]). n=4 isolated lungs each group. \*\*\*\* p<0.0001 by Student's *t*test with Welsh's correction.

B) Superoxide concentration in primary mouse lung fibroblasts (LF) after 5 minutes of acute exposure to different oxygen concentrations. The superoxide concentration was measured by ESR spectroscopy as the difference in the CMH signal with or without pegylated superoxide dismutase (∆pSOD)**.** n=3-4 individual cell isolations per group. \*\*\*\* p<0.0001 by one-way ANOVA with Tukey post hoc test. A.U.: arbitrary units.

C) Superoxide concentration **in** PASMC **during perfusion with** hypoxic buffer. The level of cellular superoxide concentration is presented as the change in the MitoSox fluorescent signal compared to baseline and normoxic values. n=4 individual cell isolations. \* p<0.05 by Student's *t*test with Welsh's correction.

D) H2O2 concentration **in** PASMC **during perfusion with** hypoxic buffer. The level of cellular H2O2 is presented as the change in the HyPercyto fluorescent signal compared to baseline and normoxic values. n=4 individual cell isolations. \*\* p<0.01, \*\*\*\* p<0.0001 by Student's *t*test with Welsh's correction..

**Figure 3.**

**Effect of MitoQ on** **development of chronic hypoxia induced pulmonary hypertension (4 weeks, 10% O2).**

A) MitoQ concentration in mouse lungs and livers after 4 weeks of treatment with MitoQ (60mg/kg/day) via gavage measured by liquid chromatography and tandem mass spectrometry.

B) Right ventricular systolic pressure (RVSP), and B) pulmonary vasculature remodeling in mice exposed for 4 weeks to chronic hypoxia (10% O2  [4w HOX]) or normoxia (21% O2  [NOX]) and treated with the mitochondria-targeted antioxidant (MitoQ) or the inactive carrier substance (TPP+). n=8 animals per group. \*\*\* p<0.01, \*\*\*\*p<0.0001 by two-way ANOVA with Tukey post hoc test.

**D) Proliferation of PASMC after** 5 days of **hypoxic** (1% O2) or **normoxic** (21% O2) **incubation in the presence of** MitoQ or TPP+. **Proliferation is presented as percent of 5-ethynyl-uridine (EdU) positive proliferating cells compared to the total cell number labeled by Hoechst staining.** n=4 individual cell isolations. \*\*\* p<0.001, \*\*\*\* p<0.0001 by two-way ANOVA with Tukey post hoc test.

**E) Superoxide concentration in PASMC after 5 days of chronic hypoxic** (1% O2) or **normoxic** (21% O2) **incubation**. Superoxide concentration was measured by ESR spectroscopy as the difference in the CMH signal with or without pegylated superoxide dismutase (∆pSOD)**.** n=4 individual cell isolations. p<0.001 by Student's *t*test with Welsh's correction.

**F) Mitochondrial superoxide concentration determined by MitoSOX fluorescence in PASMC after 5 days of chronic hypoxic** (1% O2) or **normoxic** (21% O2) **incubation**. n=4 individual cell isolations. p<0.001 by Student's *t*test with Welsh's correction.

G) Cytosolic H2O2 concentration determined by HyPercyto fluorescence **in** PASMC **after 5 days of hypoxic incubation (1% O2).** n=4 individual cell isolations. \*p<0.05, \*\*\*p<0.001, \*\*\*\*p<0.0001 by Student's *t*test with Welsh's correction.

**H) Superoxide concentration per weight (g) in** lung homogenate from mice exposed **to chronic hypoxia** (10% O2) **or normoxia** (21% O2) **for 4 weeks**. The superoxide concentration was measured by ESR spectroscopy as difference in the CMH signal with or without pegylated superoxide dismutase (∆pSOD)**.** n=3.

I) **Superoxide concentration in PASMC exposed to chronic hypoxia** (1% O2) **or normoxia** (21% O2) for 5 days in presence of 100nM of TPP+ or MitoQ. n=4 individual cell isolations. \*\* p<0.01 by two-way ANOVA with Tukey post hoc test.

**A.U.: arbitrary units.**

**Figure 4.**

**Effect of MitoQ on remodeling of the right ventricle (RV) in mice exposed to chronic hypoxia (10% O2) for 4 weeks**

A) Ratio of RV mass to mass of left ventricle plus septum (RV/[LV+Septum]), B) right ventricular wall thickness (RVWT), C) right ventricular internal diameter (RVID), D) right ventricular outflow tract diameter (RVOTD), E) tricuspid annular plane systolic excursion (TAPSE), and F) cardiac output (CO) in mice exposed to chronic hypoxia (10% O2  [4w HOX]) or normoxia (21% O2  [NOX]) for 4 weeks and treated with the mitochondria-targeted antioxidant (MitoQ) or the inactive carrier substance (TPP+). n=8 animals per group, \*p<0.05, \*\*p<0.01, \*\*\*\* p<0.0001 and †p<0.05; †† p<0.01 interaction by two-way ANOVA with Tukey post hoc test.

G) Superoxide concentration in RV homogenate in mice exposed to chronic hypoxia (10% O2) or normoxia (21% O2) for 4 weeks. The superoxide concentration was measured by ESR spectroscopy as difference in the CMH signal with or without pegylated superoxide dismutase (∆pSOD)**.** Data presented in ratio to the organ mass (g). n=3 \* p<0.05 by Student's *t*test with Welsh's correction.

H) Representative echocardiographic images of RVWT and RVOTD measurements in the right parasternal long axis.

A.U.: arbitrary units.

**Figure 5. Effect of MitoQ on remodeling of the right ventricle (RV) in mice after pulmonary artery banding**

A) Ratio of RV mass to the mass of left ventricle plus septum (RV/[LV+Septum]), B) right ventricular wall thickness (RVWT), C) right ventricular internal diameter (RVID), D) right ventricular outflow tract diameter (RVOTD), E) tricuspid annular plane systolic excursion (TAPSE), F) Right ventricular systolic pressure (RVSP) and G) Cardiac output (CO) in mice after pulmonary artery banding (PAB) or sham operation (Sham). H) Representative echocardiographic images of RVWT and RVOTD measurements in the right parasternal long axis. n=8 animals per group, \*\*p<0.01, \*\*\*\* p<0.0001 and †p<0.05 interaction by two-way ANOVA with Tukey post hoc test.