**An uncovered contribution of Kv7 channels to pulmonary vascular tone in pulmonary arterial hypertension**

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**Abstract**

K+ channels play a fundamental role regulating membrane potential of pulmonary artery (PA) smooth muscle cells (PASMCs) and their impairment is a common feature in pulmonary arterial hypertension (PAH). Kv7 channels (*KCNQ1-5*) and their KCNE regulatory subunits are known to regulate vascular tone, but whether Kv7 channel function is impaired in PAH and how this can affect the rationale for targeting Kv7 channels in PAH remains unknown. Herein we have studied the role of Kv7/KCNE subunits in rat PA and their possible alteration in PAH. Using the patch-clamp technique we found that the total K+ current is reduced in PASMC from pulmonary hypertension (PH) animals (Su/Hpx) and Kv7 currents made a higher contribution to the net K+ current. Likewise, enhanced vascular responses to Kv7 channel modulators were found in PH rats. Accordingly, KCNE4 subunit was highly upregulated in lungs from PH animals and patients. Additionally, Kv7 channel activity was enhanced in the presence of Kv1.5 and TASK-1 channel inhibitors and this was associated with an increased KCNE4 membrane abundance. Compared with systemic arteries, PA showed a poor response to Kv7 channel modulators which was associated with reduced expression and membrane abundance of Kv7.4 and KCNE4. Our data indicate that Kv7 channel function is preserved and KCNE4 is upregulated in PAH. Therefore, compared to other downregulated channels, the contribution of Kv7 channels is increased in PAH resulting in an enhanced sensitivity to Kv7 channel modulators. This study provides insight into the potential usefulness of targeting Kv7 channels in PAH.

**Keywords:** Kv7 channels, KCNE subunits, pulmonary vasculature, vascular tone, ion channel remodelling, pulmonary arterial hypertension

## Introduction

In the cardiovascular system a wide variety of K+ channels are functionally expressed and their conductance plays a fundamental role in the control of membrane potential (Em) of vascular smooth muscle cells (VSMC) and vascular tone. Redundant channel function ensures that blockade or downregulation of a given channel can be partly compensated for by another(s) channel(s), safeguarding the tissues from reduced perfusion.

Pulmonary arterial hypertension (PAH) is a severe disease characterized by vascular remodelling in pulmonary arteries (PA) attributable to persistent vasoconstriction, proliferation, inflammation and *in situ* thrombosis 1. Downregulation of K+ channels in PA smooth muscle cells (PASMC) is considered an early contributor to the pathophysiology of the disease, that results in a more depolarized Em, increased intracellular calcium concentration ([Ca2+]i), vasoconstriction, hypertrophy and proliferation 2–6. In particular, downregulation of Kv1.5 channels and TASK-1 channels is a common denominator of human and experimental pulmonary hypertension (PH) and mutations in genes encoding for these channels have been identified in PAH patients 8,7,11,9,15,39,14,13,12,10.

Kv7 channels (encoded by *KCNQ* genes) are well-recognized regulators of vascular tone 16–20. Kv7 channel activation in VSMC causes K+ outward efflux and the subsequent hyperpolarization dampens cell excitability. Moreover, Kv7 channels can be regulated by different vasodilator and vasoconstrictor agents 21–24. Notably, the expression of Kv7.1, Kv7.4, and Kv7.5 channels predominates in VSMC 25. Kv7.4 and Kv7.5 α-subunits formed as homo or heteromers as well as the KCNE4 regulatory subunit have been shown to play an important role as regulators of vascular contractility 26–28. Kv7 channel dysfunction is associated with several cardiovascular disorders including diabetes, hypertension, and atrial fibrillation 23,29–31,27. Additionally, pharmacological modulation of Kv7 channels has been shown to influence blood vessel contractility and diameter 12,23,25,30,32, and Kv7 channel activators have been proposed as potentially useful drugs to treat cardiovascular diseases 33,16,18.

The functional expression of Kv7 channels in the pulmonary circulation of rats 34,35 and mice 36,24 has also been reported and, their activation has recently been implicated in the pulmonary vasodilator effect induced by drugs increasing cGMP levels 24. Interestingly, Sedivy *et al.,* reported that short term exposure to hypoxia reduced mRNA expression of Kv7.4 and suggested a loss of Kv7 channel function in the early onset of pulmonary hypertension 35. Reduced expression of Kv7.5 channels was later reported in PA from rats exposed to 9-day hypoxia 37. Curiously, against the potential decrease in Kv7 channel expression and activity, Kv7 channel activators have been shown to attenuate the development of chronic hypoxia-induced pulmonary hypertension in mice and to reverse established PH in SERT+ mice 36. Therefore, it remains unclear whether Kv7 channel function is impaired in PH and how this can affect the rationale for targeting Kv7 channels in PH. Thus, in the present study we aimed to study the activity and expression of Kv7 channels and their KCNE ancillary subunits in experimental PH and human PAH.

## Methods

The data that support the findings of this study are available from the first author (gemondej@ucm.es) upon reasonable request. An extended description of the materials and methods is available in the *online-only Data Supplement.*

**Animal model of PH.** The animals were handled according to animal welfare regulations and protocols approved by the authors’ Institutional Review Boards (PROEX-301/16). PH was induced in some male wistar rats by the combined exposition to the VEGFR2 inhibitor, SU5416 plus 10% O2 (hypoxia) for 2 weeks (Su/Hpx).

**Human samples.** Lung samples from idiopathic PAH patients and healthy controls deposited at the Biobank of Ciberes (Barcelona, Spain) were used.

**Electrophysiology.** Kv7 currents were recorded in freshly isolated PASMC using patch clamp technique with an Axopatch 200B amplifier (Axon instruments) and a Digidata 1322A as described 38,24,30.

**Vascular reactivity study.** PA and mesenteric arteries (MA) were mounted in a wire myograph with physiological salt solution at 37°C and bubbled with 95% O2 and 5% CO2. Vessels were stretched to give an equivalent transmural pressure of 30 mmHg or 100 mmHg for PA and MA, respectively.

**PASMC proliferation.** Cell proliferation was measured in rat PASMC from control or monocrotaline-induced PH by incorporation of the thymidine analog BrdU into newly synthesized DNA during cell proliferation and its subsequent detection according to the manufacturer's protocol (Roche Applied Science) as described (Morales-Cano et al 1014 cita 12).

**qRT-PCR assay**

Gene expression was determined by quantitative real-time PCR with a Taqman system (Applied Biosystems) in the Genomic Unit of the Complutense University.

**Western Blotting analysis**. Proteins from homogenized lungs were transferred to PVDF membranes, incubated with primary antibodies overnight and then with the secondary peroxidase conjugated antibodies. Blots were imaged using an Odissey Fc System and were quantified by densitometry using Quantity One software.

**Inmunocytochemistry.** Fixed MASMC or PASMC were incubated overnight with the primary antibody and afterwards incubated for 1 h with secondary conjugated antibody. Coverslips were analysed using a laser scanning confocal microscope. The details are provided in the *online-only Data Supplement.*

**Proximity ligation assay (PLA).** PLA technology was used to determine the co‐localization of Kv7.4 and Kv7.5 channels in freshly dissociated rat MASMC and PASMC using the Duolink *in situ* (PLA) detection kit 563 in accordance with the manufacturer's instructions. The details are provided Methods in the *online-only Data Supplement.*

**Statistical analysis.** Data are expressed as mean ± SEM. Statistical comparisons were performed using unpaired or paired *t* tests or two-way ANOVA followed by Bonferroni test.

## Results

**Total K+****current is decreased while Kv7 current is preserved in Su/Hpx-PASMC**

Whole cell patch clamp recordings from normal PASMC produced large rapidly activating voltage-dependent K+ currents (Figure 1). The total K+ current was dramatically reduced in PASMC from Su/Hpx rats (Figure 1A and 1B). This was associated with a more depolarized membrane potential in Su/Hpx-PASMC compared to control-PASMC (Figure 1C). To characterize the contribution of the Kv7 channels to the totalK+current, we used a specific Kv7 channel inhibitor, XE991 (10-5M) (Figure 1D). XE991 inhibited the K+current at test potentials more positive than -30mV in both control and Su/Hpx PASMC. The XE991-sensitive current amplitude, calculated by subtracting the current in the absence and in the presence of XE991 in PASMC from control or Su/Hpx treated animals is shown in Figure 1E. As shown in Figure 1F, the percentage of the current sensitive to XE991 was significantly higher in Su/Hpx-PASMC compared to control-PASMC indicating a greater contribution of Kv7 channels to the total current in experimental PH.

**Enhanced response to Kv7 channel modulators in PA from PH animals**

The cumulative addition of XE991 caused a minor effect in the basal tone of control-PA, whereas a clear vasoconstrictor effect was observed in PA from Su/Hpx rats (Figures 2A and 2B). In line with these results, we observed that the Kv7 channel opener retigabine had a negligible vasodilator effect in control-PA, but caused a marked vasodilator response in PA from Su/Hpx rats (Figure 2C and 2D). We also found that the in vitro incubation with SUGEN did not affect retigabine-induced relaxation and had not additive detrimental effects to hypoxia on Kv currents (Supplementary Figure S1).

Additionally, we tested whether retigabine had antiproliferative effects in PASMC from control or PH (induced by monocrotaline) rats. Notably, retigabine displayed marked antiproliferative effects in PASMC from PH but not from control animals (Supplementary Figure S2). Altogether these results, suggested a greater functional role of Kv7 channels in PA from PH as compared to control animals.

**Altered lung expression of Kv7 and KCNE ancillary subunits in PH**

We analysed the protein expression of Kv7.1, Kv7.4 and Kv7.5 channels by Western blot in lungs from Su/Hpx rats and human PAH patients compared to controls. We found no changes in the expression of Kv7.1 or Kv7.5 subunits but there was a clear downregulation of Kv7.4 channel expression in Su/Hpx-lungs compared to controls (Figure 3A). Nevertheless, no differences in any of the subunits were found in human samples (Figure 3B). Since these results could not help to explain the increased contribution of Kv7 channels in Su/Hpx-PA, we also analysed the levels of expression of the regulatory KCNE subunits. Interestingly, the expression of KCNE4, but not that of KCNE3, was upregulated in both Su/Hyp (Figure 3C) and PAH patients (Figure 3D) lung samples compared to controls.

**Enhanced response to Kv7 channel modulators under conditions mimicking the ion channel remodelling that occurs in PAH**

Downregulation of TASK-1 and Kv1.5 channels is a hallmark of PAH 8,7,11,9,15,39,14,13,12,10. We performed additional experiments with Kv7 modulators in the presence of TASK-1 and Kv1.5 channel inhibitors, ML-365 and DPO-1, respectively to mimic the characteristic ion channel remodelling of PAH. The effects of retigabine (10-5M) on K+currents were tested in the absence (vehicle) or in presence of ML365 (10-6M) plus DPO-1 (10-6M) in PASMC from control rats (Figure 4A). ~~Unexpectedly,~~ We observed a slight decrease in the K+current following the addition of the Kv7 activator, which turned into an increase in the current in the presence of ML365+DPO-1 (Figure 4A, right panel). Thus, the retigabine-sensitive current was negative under control conditions and positive in the presence of the TASK-1 and Kv1.5 channel inhibitors (Figure 4B). Moreover, retigabine had no apparent effect on Em under baseline conditions (Figure 4C and D). However, in the presence of ML365+DPO-1 (Figure 4C and E), which led to a marked membrane depolarization, a clear repolarising effect was observed following the addition of retigabine.

Thereafter, we analysed the vascular responses induced by XE991 and retigabine in the absence and in the presence of ML365+DPO-1. Remarkably, higher contraction to XE991 (Figure 5A and 5B) and relaxation to retigabine (Figure 5C and 5D) was observed in PA from control rats incubated with ML365+DPO-1 compared to vehicle. Therefore, the results in this *in vitro* model of ion channel remodelling resemble those observed in the PH experimental model and strongly suggest an increased contribution of Kv7 channels when TASK-1 and Kv1.5 channels are inhibited.

To further analyse the potential mechanisms responsible for the increased Kv7 activity we studied the subcellular location of Kv7.4 channel and the KCNE4 subunit in isolated PASMC in the absence and the presence of ML365+DPO-1 by immunofluorescence (Figure 5E). Kv7.4 channels staining was detected in PASMCs both in the absence or in the presence of ML365+DPO-1, showing some co-localization with the membrane marker WGA (Figure 5E), and rendering similar membrane abundance under both conditions (Figure 5G). Line scan fluorescence intensity analysis in control PASMCs revealed that the expression of Kv7.4 (red) was mainly observed between the peaks of highest intensity of the membrane marker WGA (green), suggesting that its expression was mostly cytosolic (Figure 5F-1). Such subcellular distribution was unaffected in cells treated with ML365+DPO-1 (Figure 5F-3). Immunofluorescence experiments also revealed KCNE4 expression in control PASMCs (Figure 5E), showing a mainly cytosolic distribution (Figure 5G). However, a higher location of KCNE4 in the plasma membrane was observed when cells were treated with ML365+DPO-1 (Figure 5E and 5F), leading to a net increase in the KCNE4 membrane abundance (Figure 5H).

**Kv7 channel activators produce a negligible relaxation in PA compared to MA**

A surprising finding in our study was the negligible effect of XE991 and retigabine in control-PA (Figure 2). In order to confirm this unexpected result, we tested the effects of different selective Kv7-enhancers in PA and, for comparative purposes, in MA. A similar pattern of attenuated pulmonary vasodilation was observed with all tested Kv7 enhancers (S1, retigabine, ML213 and ML277). However, all these Kv7 channels enhancers elicited a robust relaxation in MA (Supplementary Figure S3A and S3B). In addition, the negligible pulmonary relaxant response of retigabine in PA was also observed under different O2 concentrations and after applying different transmural pressures, even reaching values similar to those applied to the MA (Supplementary Figure S4).

**PA display lower expression of Kv7.4 and KCNE4 subunits compared to systemic arteries**

To elucidate the differences observed in the effects of Kv7 channel modulators between pulmonary and systemic vessels, we analysed the mRNA expression of the different Kv7 channels (*KCNQ1, KCNQ4* and *KCNQ5*) and KCNE ancillary subunits by RT-PCR in PA compared to MA (Supplementary Figure S3C). Our data revealed that PA have much lower expression of *KCNQ4* and *KCNE4* than systemic arteries. These data could explain the difference in Kv7 channel activity between these two types of arteries. We also compared the expression of other K+ channels in the pulmonary vasculature versus MA and found a similar expression level of *KCNA5* but a much higher expression of *KCNK3* in PA than in MA (Supplementary Figure S5).

**Differential cellular location of Kv7 channels in PASMC compared to MASMC**

We next assessed the possible differences in subcellular-location of Kv7 channels in isolated PASMC and MASMC by immunofluorescence. As stated above, we found that the expression of the Kv7.4 subunit was essentially cytosolic in PASMC, while in MASMC these channels were expressed at high level in the plasma membrane where they are functional (Supplementary Figure S3D). Line scan analysis showed that MASMC, in contrast to PASMC, had the highest levels of Kv7.4 channel expression matching with the plasma membrane marker WGA (Supplemental Figure S3E). We also observed that Kv7.5 channels and KCNE3 (Supplemental Figure S6) showed poor membrane localization in PASMC, in contrast to what has been reported in systemic vascular myocytes or heterologous systems 30,40,41. This may also contribute to the differential functional role of Kv7 channels in PA and systemic arteries. Finally, since functional Kv7.4/Kv7.5 heterotetramers have been suggested to play an essential role in the relaxation of systemic arteries 26,40,42,43 we assessed their existence in PASMC using a proximity ligation assay (PLA). Supplementary Figure S3F shows that Kv7.4/Kv7.5 heterotetramers are present in PASMC and their abundance is comparable to that found in MASMC.

**Discussion**

Our primary finding of the present study is that pulmonary vascular Kv7 channels have a higher functional role during the development of PH. This is associated with an upregulation of subunit KCNE4 and is observed under *in vitro* conditions that mimic the characteristic ion channel remodelling of PAH. Unexpectedly we found that, compared to systemic arteries, control PA displayed modest Kv7 current, low Kv7 channel expression and negligible response to Kv7 channel modulators. Therefore, the Kv7 channels present in the pulmonary circulation have a relatively poor role compared to those present in systemic arteries, but can become functionally highly relevant during the development of PAH.

A number of K+ channels are downregulated in experimental PH or human PAH, including Kv1.5, Kv2.1 and TASK-1 among others 44,7,9,11,2,13. In the Su/Hpx rats, the rodent model which best recapitulates PAH, we have previously reported a reduced activity of Kv1.5 10 and TASK-1 channels 45 in PASMC. Accordingly, herein we found a profound reduction of total K+ channel activity and a marked depolarization in PASMC from Su/Hpx rats. ~~In contrast, Kv7 currents were essentially preserved in PH rats~~. Moreover XE991 inhibited the K+ current to a greater extent in PH, indicating that Kv7 channels make a much higher contribution to the net K+ current in PH than in healthy PASMC. Likewise, retigabine-induced vasodilator and antiproliferative effects and XE991-induced vasoconstriction were greatly augmented in PA from PH rats. Thus, the vascular effects of the Kv7 channel modulators turned from negligible in control-PA to very robust in Su/Hpx-PA, again indicating a greater functional contribution of Kv7 channels in the setting of PH.

Previous studies suggest that drugs activating Kv7 channels may have beneficial effects in PH experimental models 35,36. Thus, Morecroft *et al.,* reported that flupirtine, a Kv7 channel activator, significantly attenuated the development of PH in chronic hypoxic mice and reversed established PH in mice over-expressing the 5-HT transporter (SERT) 36. In addition, Sedivy V et *al*., found that flupirtine caused pulmonary relaxant effects in rats exposed to short-term (3-5 days) hypoxia but not in those exposed to normoxia 35. Of note, the enhanced response to flupirtine in this hypoxic model was unrelated to an increased expression of Kv7 channels, but paradoxically, a robust reduction of *KCNQ4* was observed. Our data in the present study are consistent with these observations and suggest that the enhanced response to Kv7 modulators observed in PH models is not due to an augmented expression of the Kv7 α-subunits.

Kv7 channel activity is regulated by auxiliary subunits (KCNE1-5) and many arteries express KCNE3 and KCNE4 predominantly. Co-expression of KCNE4 increases Kv7.4 currents in heterologous expression systems 46,47. Of note, Jepps *et al.,* found that KCNE4 co-localizes with Kv7.4 channels and Kv7.4/Kv7.5 heteromeric channels in freshly isolated MASMC and that knocking down this regulatory subunit reduced Kv7 currents, depolarized MASMC and markedly attenuated the vascular responses to Kv7 channel modulators 46. Interestingly, we found an upregulation of KCNE4 in lungs from PH rats, which could contribute to the preservation of the Kv7 current in a condition where Kv7.4 expression is reduced. Remarkably, our study shows for the first time that Kv7 subunits are also expressed in human lungs and, consistent with our data in the animal model, we found an upregulation of KCNE4 in samples from PAH patients.

As stated above, the development of PAH is associated with downregulation of Kv1.5 and TASK-1 channels 7–12,2,13–15. To mimic these conditions we performed *in vitro* experiments in which the Kv7 channel function was assessed in the presence of Kv1.5 and TASK-1 channel inhibitors. Following acute exposure to these inhibitors the ability of retigabine to increase K+ current amplitude and hyperpolarise the membrane potential of PASMC became evident. In addition, this was associated with a profound enhancement of retigabine-induced PA vasodilation and XE991-induced pulmonary vasoconstriction. Altogether these data suggest that Kv7 channel activity is unmasked under conditions mimicking the ion channel remodelling characteristic of PH. The simplest explanation for this would be that: 1) this ion channel remodelling would bring the membrane potential up to the voltage threshold for activation of Kv7 channels and/or 2) the redundant nature of K+ channels would result in the Kv7 channels assuming a major role when other members (i.e. Kv1.5 and TASK-1) are not active. Alternatively, we studied if the acute inhibition of Kv1.5 and TASK-1 channels could regulate the trafficking of Kv7 channel subunits. No apparent changes in location or membrane abundance of Kv7.4 were observed, while KCNE4 trafficked from the cytosol to the plasma membrane, resulting in higher membrane abundance. Thus, the higher confinement of this enhancer subunit in the membrane would lead to a greater Kv7 channel activity compensating for the loss of Kv1.5 and TASK-1 channel function in PASMC. Our findings indicate that the activation of Kv7 channels under conditions mimicking the ion channel remodelling occurring in PAH would be crucial to control membrane potential and limit an exaggerated depolarization in PASMC. Moreover, the upregulation of KCNE4 found in our *in vivo* and *in vitro* models of PH, as well as in patient samples, is very likely to contribute to the enhanced Kv7 channel activity during the onset of the disease.

Currently, Kv7 channels are considered key regulators of membrane potential and vascular tone in many vessels Accordingly, MA were efficiently relaxed by a range of Kv7 channel openers. Surprisingly, we found that under control conditions PA were essentially insensitive to Kv7 channel modulators. This finding, confirmed in two independent labs, was consistent with differential expression profile of Kv7 channels in MA and PA. Thus, in comparison with MA, PA had similar expression of *KCNQ1*, *KCNQ5* and *KCNE3* but markedly smaller expression of *KCNQ4* and *KCNE4*, which are considered major contributors to the Kv7 current in systemic vessels 46,20. Additionally, Kv7.4 and KCNE4 were abundantly expressed at the membrane level (where the channel is functional) in MASMC 46, while in PASMC Kv7.4 channels expression was essentially cytosolic and KCNE4 appeared to be located in cytoplasmic accumulations around the nucleus and in plaques near the membrane. By contrast, Kv7.4/7.5 heterotetramers, which have been postulated as a predominant functional channel subtype in vascular smooth muscle cells 8,20,26,42 were similarly detected in MASMC and PASMC.

A few studies have previously assessed the role of Kv7 channels in the pulmonary circulation using rat 24,34,48 or mice models 36,38. Joshi et al., first reported that Kv7 channels blockers induced a marked vasoconstriction in rat PA but showed negligible effects in MA 34. Thereafter, these authors reported a substantial pulmonary vasodilator effect by Kv7 channel openers and suggested a greater functional role of Kv7 channels in the pulmonary as compared to the systemic circulation. This was associated with higher *KCNQ4* expression in PA than in MA. Herein we found that in control animals retigabine did not relax PA, and even inhibited a K+ current as previously observed in other vessels (Morales-Cano et al., 2015; Yeung et al 2008) which could be due to Kv7.1 channel inhibition as suggested (Yeung et al 2008). In an attempt to explain these discrepancies between ours and previous studies, we examined the pulmonary vasodilator responses to retigabine under different experimental conditions. The negligible response to retigabine was found to be independent of the vasoconstrictor stimulus, the transmural pressure (even when applying tension values similar to those used for MA) or oxygen levels. Alternatively, it is likely that differences in rat strains (Sprague-Dawley in the previous studies versus Wistar in this work) may account for the variable responses to the Kv7 channel modulators observed in the pulmonary arteries. In fact a lack of pulmonary vasodilation by the Kv7 channel opener flupirtine in control Wistar rats has been reported 35. In addition these authors reported that the Kv7 channel blocker linopirdine did not increase pulmonary arterial pressure in this rat strain unless lungs were previously primed with a stimulus to increase vasoreactivity (i.e pre-exposition to angiotensin II-hypoxia cycles). Likewise linopirdine or XE991 were unable to contract Wistar rat PA unless previously primed with U46619 48 or K+ channels inhibitors (herein). In summary, it is therefore likely that Wistar rats display reduced Kv7 channel function in the pulmonary circulation compared to Sprague-Dawley rats, which could contribute to their higher susceptibility to develop pulmonary hypertension 49–51.

**Perspectives**

Our study shows that Kv7 channels acquire a more relevant role controlling pulmonary vascular tone in the onset of pulmonary hypertension. As a result, targeting Kv7 channel may represent an attractive strategy in the treatment of PAH. In agreement with this idea preliminary studies in animal models suggest that Kv7 channel openers are able to prevent and even reverse pulmonary hypertension 35,36. While two of the commercially available pan-Kv7 channel openers (i.e. retigabine and flupirtine) have been withdrawn from the market in recent years a number of new molecules with higher selectivity for particular Kv7 channel subunits are under development 52,53. Remarkably, activation of Kv7 channels has been demonstrated to contribute to the vasodilation induced by drugs increasing cGMP 24 and cAMP levels 44 which represent current strategies for the treatment of PAH. It is therefore likely that the activation of Kv7 channels may contribute to the therapeutic effect of drugs increasing cGMP and cAMP in pulmonary hypertension patients. Our present study supports this notion and provides molecular insight into the mechanisms underlying the potential usefulness of therapies targeting Kv7 channels and in particular the Kv7.4 and Kv7.5 α-subunits co-assembled with KCNE4.

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## Conflict of interest

The authors declare no conflicts of interest.

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**Novelty and Significance**

1. **What Is New?**

* Compared to systemic vessels, pulmonary arteries display low Kv7 channel expression and activity.
* However, Kv7 channel function turns much more relevant in PAH.
* The ion channel remodelling in PAH involves a greater contribution of Kv7 channels

1. **What Is Relevant?**

* Since pulmonary hypertension is associated with a net loss of K+ channel activity, targeting the preserved Kv7 channels may be an interesting strategy in PAH.
* The development of available Kv7 channels enhancers in PAH would provide vasodilator effects with selectivity for diseased versus healthy pulmonary vessels.

1. **Summary**

* We have found Kv7 channels assume a remarkably higher functional role in the regulation of pulmonary arterial tone during the development of PAH.

**Figure legends**

**Figure 1. Kv7 current is preserved and provides a higher contribution to total K+ current in Su/Hpx-PASMC compared to control-PASMC.** A) Representative K+ current traces and B) K+ current-voltage relationships measured at the end of the depolarising pulses. C) Mean values of the resting membrane potential (*E*m). D) K+current-voltage relationships measured before and after the addition of XE991 (10-5M). Insets show representative current traces measured at +20 mV in the absence and presence of XE991 in control (left) and Su/Hpx-PASMC (right). E) XE991-sensitive current obtained by subtracting the K+ current in the presence of XE991 from the current in the absence of the drug. F) XE991-induced inhibition of K+ currents measured at 0 mV expressed as a percentage. \*P < 0.05, and \*\*\*P<0.001 vs control. Data was analysed by paired t-test in panel D and by unpaired t-test in panels B, C and E. n≥5 cells from n=3-5 different animals.

**Figure 2. Enhanced response to Kv7 channel modulators in Su/Hpx compared to control PA**. A) Representative recordings and (B) averaged data of the concentration-response curves to XE991 (10-8 -10-5M) in Su/Hpx and control PA. C) Representative recordings and D) averaged data of the concentration response curves to retigabine (10-8 -3x10-5M) in 5HT-precontracted PA from Su/Hpx and control rats. \*P < 0.05 and \*\*\*P < 0.001 vs control, respectively. n≥5 PA from n=3 different animals.

**Figure 3. Altered lung expression of Kv7 channels and KCNE ancillary subunits in experimental PH and human PAH.** Kv7 channel protein expression analysed by Western blot in lung samples from A) Su/Hpx rats and B) human PAH patients compared to controls. KCNE subunits protein expression analysed by Western blot in lung samples from C) Su/Hpx rats and D) human PAH patients compared to controls. \*P < 0.05 vs control.

**Figure 4. Retigabine increase K+ currents and induces membrane hyperpolarization in PASMC upon inhibition of Kv1.5 and TASK-1 channels.** A) K+current-voltage relationships measured before and after the addition of retigabine (10-5 M) in the absence and in the presence of ML365+DPO-1 (both at 10-6 M). B) Retigabine-sensitive current measured at 0 mV. C) Retigabine-induced hyperpolarization in absence and in presence of ML365+DPO-1. Representative recordings the effects of retigabine on Em in absence (D) or presence (E) of ML365+DPO-1.\* P<0.05 vs vehicle. Panels A, D and E were analysed by paired t-test and B and C by unpaired t-test. n=9 cells from n=4-5 different animals.

**Figure 5. Enhanced PA reactivity to Kv7 channel modulators in the presence of Kv1.5 plus TASK-1 inhibitors.** Representative recordings (A) and averaged data (B) of contractions induced by 10-5M XE991 in PA in the absence and in the presence of ML365+DPO-1 (both at 10-6M). Representative recordings (C) and averaged data (D) of the relaxation induced by increasing concentrations of retigabine (10-8 -3x10-5M) in precontracted-PA in the absence (vehicle) and in the presence of ML365+DPO-1. E) Kv7.4 channel and KCNE4 subunit protein expression (red) analysed using immunocytochemistry in isolated PASMCs in the absence and in the presence of ML365+DPO-1. Wheat Germ Agglutinin (WGA) was used as a membrane marker (green) and DAPI for nuclear staining (blue). F) Representative graph of Kv7.4 and KCNE4 channel (red) protein expression in the membrane determined by colocalization with WGA (green) in PASMC in the absence and in the presence of ML365+DPO-1. G) Kv7.4 channel and H) KCNE4 subunit membrane abundance determined by colocalization with WGA (yellow) in the absence and in the presence of ML365+DPO-1. \*P< 0.05, \*\*P < 0.01 and \*\*\*P < 0.001 vs control, respectively. n≥5 PA from 4-5 animals for functional experiments and n≥36 cell analysed from 3 different animals for immunocytochemistry.