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Alterations of N-3 Polyunsaturated Fatty Acid-Activated K₂P Channels in Hypoxia-Induced Pulmonary Hypertension

Gorm Nielsen¹, Christine Wandall-Frostholm², Veeranjanyulu Sadda¹, Aida Oliván-Viguera^{1,4}, Eric E. Lloyd³, Robert M. Bryan Jr.³, Ulf Simonsen², and Ralf Köhler^{1,4}

¹Cardiovascular and Renal Research, Institute for Molecular Medicine, University of Southern Denmark, Odense, Denmark

²Department of Biomedicine, Pulmonary and Cardiovascular Pharmacology, Faculty of Health Sciences, Aarhus University, Aarhus C, Denmark

³Department of Anesthesiology, Baylor College of Medicine, One Baylor Plaza, Houston, TX, USA

⁴Aragon Institute of Health Sciences I+CS and ARAID, Unit for Translational Research, Hospital Miguel Servet, Zaragoza, Spain

Abstract

Polyunsaturated fatty acid (PUFA)-activated two-pore domain potassium channels (K₂P) have been proposed to be expressed in the pulmonary vasculature. However, their physiological or pathophysiological roles are poorly defined. Here we tested the hypothesis that PUFA-activated K₂P are involved in pulmonary vasorelaxation and that alterations of channel expression are pathophysiotically linked to pulmonary hypertension.

Expression of PUFA-activated K₂P in the murine lung was investigated by quantitative reverse-transcription polymerase chain reaction (qRT-PCR), immunohistochemistry (IHC), by patch clamp (PC), and myography. K₂P-gene expression was examined in chronic hypoxic mice.

QRT-PCR showed that the K₂P2.1 and K₂P6.1 were the predominantly expressed K₂P in the murine lung. IHC revealed protein expression of K₂P2.1 and K₂P6.1 in the endothelium of pulmonary arteries and of K₂P6.1 in bronchial epithelium. PC showed pimoide-sensitive K₂P-like K⁺-current activated by docosahexaenoic acid (DHA) in freshly isolated endothelial cells as well as DHA-induced membrane hyperpolarization. Myography on pulmonary arteries showed that DHA-induced concentration-dependent and instantaneous relaxations that were resistant to endothelial removal and inhibition of NO and prostacyclin synthesis and to a cocktail of blockers of calcium-activated K⁺ channels but were abolished by high extracellular (30 mM) K⁺-concentration. Gene expression and protein of K₂P2.1 were not altered in chronic hypoxic mice while K₂P6.1 was up-regulated by fourfold.

In conclusion, the PUFA-activated K₂P2.1 and K₂P6.1 are expressed in murine lung and functional K₂P-like channels contribute to endothelium-hyperpolarization and pulmonary artery relaxation. The increased K₂P6.1-gene expression may represent a novel counter-regulatory mechanism in pulmonary hypertension, and suggest that arterial K₂P2.1 and K₂P6.1 could be novel therapeutic targets.

Keywords

endothelium; K₂P2.1; K₂P6.1; lung; polyunsaturated fatty acid; pulmonary hypertension; vasorelaxation

Introduction and background

Polyunsaturated fatty acid (PUFA)-activated two-pore domain potassium channels (K₂P) have been reported to be expressed in the vasculature almost one decade ago [1]. Although their functional roles have not been established yet, it is likely that the activation of these channels is capable of producing cell membrane hyperpolarization that leads to the closure of voltage-gated Ca²⁺-channels and thereby to vasorelaxation. Thus, PUFA-activated K₂P channels may oppose contractility and vasospasm. Among the different types of PUFA-activated K₂P found in the vasculature of particularly the murine lung, the PUFA-activated and mechanosensitive K₂P2.1 (a.k.a. TREK-1) has been shown to be highly expressed and K₂P2.1 immune reactivity was found in the bronchial and arterial smooth muscle and in the alveolar epithelium [2]. The related PUFA-activated K₂P4.1 (a.k.a. TRAAK) was detected on myelinated vagal afferents but not in the lung vasculature [2]. In arteries, K₂P2.1 has previously been implicated in endothelium-dependent relaxation of mesenteric artery, skin microvessels, and in the cerebral vasculature as concluded from corresponding defects in K₂P2.1-deficient mice [3,4]. However, this view has been challenged by a study on another K₂P2.1-deficient strain that revealed no significant alterations [5]. Thus, the physiological roles of K₂P2.1 in these vascular beds remain unknown. Moreover, a major importance of the channels compared to the established roles of other classical vascular K⁺ channels such as Ca²⁺-activated K⁺ channels (KCa2.3, KCa3.1, KCa1.1) [for review see 6,7,8], ATP-sensitive K⁺ channels [for review see 9], and K₂P3.1 (a.k.a. TASK-1) channels [10,11] for vasoregulation remains unclear in the physiological setting.

Pulmonary hypertension involves vasoconstriction of pulmonary arteries leading to increased workload of the heart followed by hypertrophy of the right ventricle and right heart failure [12,13]. Vasodilators such as prostacyclin analogues, endothelin receptor antagonists e.g. bosentan, and phosphodiesterase type 5 inhibitors e.g. sildenafil have proven useful for treatment of pulmonary arterial hypertension, but 5-year survival still remain below 70 percent despite these treatments or combinations thereof [14], and may be even less in other forms of pulmonary hypertension. Therefore, there is an unmet need for other drug targets. It remains elusive whether K₂P2.1 and/or the closely related PUFA-activated K₂P channels, K₂P4.1, and K₂P10.1 (a.k.a. TREK-2), become important in a pathophysiological setting such as hypertensive disease in which other vasodilator systems may fail or could be compromised [for extensive review see 6]. That this might be possible in principle is suggested by a study on spontaneous hypertensive rats (SHR), in which functional expression of an endogenous mechanogated K⁺-channel with a single-channel conductance of 90 pS and thus properties similar to K₂P2.1 was identified in aortic endothelial cells while this channel was barely found in normotensive Wistar Kyoto rats [15]. Besides K₂P2.1, a mechanistic role in vasoregulation has been assigned to the PUFA-activated K₂P6.1 (a.k.a. TWIK-2) in smooth muscle as concluded from a higher contractility to the vasospastic agent, the thromboxane mimetic, U46619, and to the L-type calcium channel activator, BAY K 8644, a more depolarized membrane potential in aortic smooth muscle cells, and a higher systemic blood pressure in mice deficient of K₂P6.1 [16]. Whether expression of K₂P channels are altered in pulmonary hypertension has not been addressed so far.

In the light of the previous findings and controversy regarding vascular K_{2p} channels as outlined above and the need to identify new pharmacological targets, we here investigated the expression of the PUFA-activated K_{2p}1.1 (a.k.a. TWIK-1), K_{2p}2.1, K_{2p}4.1, K_{2p}6.1, and K_{2p}10.1, under healthy conditions in the murine lung and tested the hypothesis that pulmonary hypertension in chronic hypoxic mice alters gene and protein expression of PUFA-activated K_{2p} channels.

Materials and Methods

Animal studies

Animal protocols were approved by the Animal Ethical Committee according to Danish legislation (permit no. 2011/561-2011). Wildtype mice (C57Bl/6J) were derived from our own breeding colonies at the animal core facilities at Aarhus University and University of Southern Denmark.

Hypobaric hypoxia induced pulmonary hypertension

Male mice (7–8 weeks) were either placed in a hypobaric chamber that was depressurized to 560 mbar, thus halving the oxygen partial pressure, or kept in similar but normoxic and normobaric chambers. The temperature in the chambers was maintained at 21–22°C and the chamber was ventilated with air at approximately 45 l min⁻¹. All mice had free access to water and rodent chow. After 4 weeks the mice were anaesthetized and right ventricular pressure and weight were measured as previously described for rats [17]. Thereafter, lungs were carefully dissected and either snap-frozen in liquid nitrogen for mRNA expression studies or fixed in 4% formalin for histological/ immunohistological analysis.

mRNA-expression studies

RNA extraction from snap-frozen tissue was performed using Trizol reagent (Invitrogen) according to the manufacturer's instructions. Total RNA was then treated with DNase I (Qiagen) for elimination of any residual genomic DNA. The concentration of the RNA was measured by optical density at 260 nm. Total RNA was then reverse transcribed using iScript cDNA Synthesis Kit (Bio-Rad). PCR-products were analyzed by gel electrophoresis using 1.5% agarose in Tris-borate-EDTA (TBE) buffer and staining with GelRedTM (Biotium). Semi-quantitative RT-PCR was done using iQTM CYBR[®] Green Supermix (Bio-Rad) and a Stratagene MX3000p cyclor. QPCR was done using an initial denaturing at 95°C for 10 minutes followed by 40 cycles of denaturing at 95°C for 20 seconds, annealing at 60°C for 20 seconds, and an extension step at 72°C for 20 seconds. Thereafter, a three-step series of 95°C for 60 seconds, 55°C for 30 seconds, and gradual increase to 95°C was run to determine melting points of qPCR products. Expression levels were normalized to expression of a reference gene, -actin. -actin mRNA was used as reference by calculating the Ct values (Ct = Ct(target) – Ct(reference gene)) and the ratios to reference gene (ratio = 2^{-Ct}). Results were expressed as percentage of reference gene (% reference gene). Specific RT-PCR primers were designed to span intronic sequences. Primer sequences and product lengths are listed in table 1.

Immunohistochemistry

Immunohistochemistry (IHC) was done on paraffin-embedded tissue sections cut in 5 µm slides, deparaffinized in Tissue-Tek[®] Tissue-Clear[®], rehydrated in a graded series of ethanol (99 – 70%) and finally in distilled water.

For K_{2p}2.1 staining, blocking of non-specific antibody binding was done with 3 % bovine serum albumin (BSA) in tris-buffered saline (20 mM Tris, 137 mM NaCl, pH 7.6) supplemented with 0.05% Tween 20 (TBST). A primary antibody against K_{2p}2.1 (sc-50412,

Santa Cruz Biotechnology, 1:100) was diluted in 1% BSA in TBST. The antigen-antibody complex was visualized by using a horseradish peroxidase-conjugated secondary antibody (P0448, 1:1000, Dako, Denmark) followed by 3,3'-diaminobenzidine (DAB+, K3468, Dako, Denmark).

For K₂p6.1 staining, Heat-Induced Epitope Retrieval was performed with Tris-EGTA (TEG) buffer heated in a microwave oven. Primary antibody (sc-11486, Santa Cruz Biotechnology) was diluted 1:200 in antibody diluent (Dako REAL™ Antibody Diluent) and blocking was done with 5% skimmed non-fat dry milk in TBST. All slides were counter-stained with Mayer's Hematoxylin (Bie & Berntsen, Denmark).

Patch clamp electrophysiology

Endothelial cells were isolated from pulmonary artery or carotid artery as follows: Freshly dissected carotid arteries or 1st order pulmonary arteries were mounted on glass capillaries and the lumen was filled with 0.5% trypsin in phosphate-buffered saline (Biochrom AG) and sutured. After incubation for 30 min at 37°C, the arteries were cut open longitudinally and the luminal surface was gently scraped with a 10- μ l pipette tip. Detached cells and cells clusters were aspirated and transferred into culture dishes containing DMEM cell culture medium supplemented with 10 % newborn calf serum and streptomycin/penicillin (all from Biochrom AG). Cells were allowed to settle down on cover slips for 1–3 hr at 37°C in 5% CO₂. Whole-cell membrane currents were recorded using an Axopatch patch-clamp amplifier (Axon Instruments, Foster City, CA) and the Clampex 9.2 data acquisition software, and were analysed by the Clampfit 9.2 software. Currents were recorded using an extracellular NaCl bath solution containing (mM): 137 NaCl, 4.5 Na₂HPO₄, 3 KCl, 1.5 KH₂PO₄, 0.4 MgCl₂, 0.7 CaCl₂, and 10 glucose (pH 7.4). The KCl-pipette solution contained (in mM): 140 KCl, 1 Na₂ATP, 1 MgCl₂, 2 EGTA, 0.72 CaCl₂ (0.1 μ M [Ca²⁺]_{free}), and 5 HEPES, pH 7.2. For K₂p-channel activation, docosahexaenoic acid (DHA) was added to the bath solution to a final concentration of 10 μ M. In another series of experiments, we added an isoflurane/bath solution (1:10 (v/v) and vigorously mixed) to the bath solution to a final concentration of 1:100 (v/v; i.e. 1%). For blocking experiments, pimozone (1-[1-[4,4-Bis(4-fluorophenyl)butyl]-4-piperidinyl]-1,3-dihydro-2H-benzimidazol-2-one) was added to the bath to a concentration of 2 μ M. 10 mM stock solution of DHA, arachidonic acid, γ -linolenic acid and pimozone were prepared with dimethylsulfoxide (DMSO). The final DMSO concentration did not exceed 0.3 %.

Wire Myography

For isometric tension recordings, small pulmonary arteries with a length of approximately 2 mm were mounted on two 40 μ m wires in microvascular myographs (DMT, Aarhus, Denmark). The baths, containing PSS (NaCl 119mM, KCl 4,7 mM, MgSO₄ 1,17 mM, NaHCO₃ 25 mM, KH₂PO₄ 1,18 mM, EDTA 0,026 mM, Glucose 5,5 mM), were heated to 37°C and equilibrated with 5% CO₂ to maintain the desired pH of 7.4. Segments were allowed to equilibrate for 10 minutes thereafter. Segments were stretched to 2.4 kPa for optimal measurements, corresponding to a transmural pressure of 18 mmHg. Viability of arterial segments was tested with a 124 mM KPSS (KCl 123,7 mM, MgSO₄ 1,17 mM, NaHCO₃ 25 mM, KH₂PO₄ 1,18 mM, EDTA 0,026 mM, Glucose 5,5 mM) twice at the start of the experiment.

After the initial test of viability, arterial segments were contracted with phenylephrine (100 nM). After reaching a level of stable contraction, to ensure preservation of endothelial function, we tested the ability of acetylcholine (10 μ M) to produce relaxation. Only segments exhibiting more than 50% relaxation to acetylcholine were included in the study. For the arterial segments in which the endothelium was to be disrupted, a human hair was

used to rub the inside of the vessel and endothelial function was tested again. These vessels were only included in the study when exhibiting less than 10% relaxation to acetylcholine. To investigate the role of DHA-activated K_{2p} -channels in tension, arterial segments were incubated with the nitric oxide synthase inhibitor L-NAME (N5-[imino(nitroamino)methyl]-L-ornithine, methyl ester, monohydrochloride, 100 μ M), the cyclooxygenase inhibitor indomethacin (10 μ M), and, when noted, the $KCa_{2.3}$ blocker UCL1684 (6,12,19,20,25,26-Hexahydro-5,27:13,18:21,24-trietheno-11,7-metheno-7H-dibenzo [*b,n*] [1,5,12,16]tetraazacyclotricosine-5,13-diium dibromide, 1 μ M), the $KCa_{3.1}$ blocker TRAM-34 (1-[(2-Chlorophenyl)diphenylmethyl]-1H-pyrazole, 1 μ M), and the $KCa_{1.1}$ blocker iberiotoxin (100 nM). Segments were contracted with the thromboxane analogue U46619 ((5Z)-7-[(1R,4S,5S,6R)-6-[(1E,3S)-3-Hydroxy-1-octenyl]-2-oxabicyclo[2.2.1]hept-5-yl]-5-heptenoic acid, 20 nM) and once the contraction was stable, cumulative concentration-response curves for DHA (2–50 μ M) were constructed.

Drugs

Iberiotoxin, isoflurane, indomethacin, U46619, arachidic acid, arachidonic acid and γ -linolenic acid were purchased at Sigma-Aldrich CO. LLC. (St. Louis, Missouri, USA.). Docosahexaenoic acid and L-NAME were purchased at Cayman Chemical Company (Ann Arbor, Michigan, USA) Pimozide and UCL1684 were purchased at Tocris Bioscience (Bristol, UK). TRAM-34 was a kind gift from Dr. Heike Wulff, UC Davis.

Statistics

All values are presented as means $\pm$ standard error of means (SEM). For comparisons of data set from two or more groups, we used one-way ANOVA followed by Bonferroni post hoc test. P-values of <0.05 were considered significant.

Results

Expression of K_{2p} genes

The RT-PCR showed the presence of mRNA transcripts of all five PUFA-activated K_{2p} s, $K_{2p1.1}$, $K_{2p2.1}$, $K_{2p4.1}$, $K_{2p6.1}$, $K_{2p10.1}$, together with transcript of classical endothelial KCa channels, $KCa_{3.1}$ and $KCa_{2.3}$ (Figure 1A). The results of qRT-PCR experiments that are presented in Figure 4 together with alterations of channel expression caused by chronic hypoxia further showed that $K_{2p2.1}$ and $K_{2p6.1}$ were the predominantly expressed channels while expression of $K_{2p1.1}$, $K_{2p4.1}$, and $K_{2p10.1}$ were rather weak.

To further characterize the tissue expression profile of the predominantly expressed channels, $K_{2p2.1}$ and $K_{2p6.1}$, we performed immunohistochemistry on paraffin-fixed lung sections (Figure 1B and C). Regarding $K_{2p2.1}$ (Figure 1B), we detected weak staining of the pulmonary endothelium and staining of perivascular connective tissue and alveoli. Signals for $K_{2p6.1}$ protein (Figure 1C) were likewise found in endothelial cells and to a higher extent in alveoli and in the epithelium of bronchioles and bronchi. The arterial smooth muscle layer of small pulmonary arteries did not give appreciable signals. In another vessel, the carotid artery (Figure 1D), $K_{2p2.1}$ and $K_{2p6.1}$ protein expression was detectable more clearly in the endothelium and we also observed non-homogeneous foci-like staining of arterial smooth muscle.

Electrophysiology

To verify the presence of functionally active PUFA-sensitive K_{2p} channels in murine endothelium of the main pulmonary artery and of carotid artery, we isolated endothelial cells from both vessels and performed whole-cell patch-clamp experiments the same day. In one third of these freshly isolated carotid artery endothelial cells (CAEC, Figure 2A–B) and

pulmonary artery endothelial cells (PAEC, Figure 2C–D), DHA at 10 μ M activated outward currents with variable amplitudes but clearly distinguishable from background currents. Current amplitudes at 0 mV were 13 ± 2 pA in CAEC (n=28) and 9 ± 4 pA in PAEC (n=4). These currents reversed at negative potentials near the K⁺ equilibrium potential, suggesting activation of K⁺ channels (Figure 2A–C). The outward current was increasingly noisy at more positive membrane potentials and at a holding potential of +80 mV we could distinguish single channel openings with amplitudes of 8–10 pA (Figure 2D). These small current densities suggested functional expression of a small number of channels per cell. We further characterized PUFA-activated currents in the CAEC because of a larger yield of cells from the larger carotid arteries. The DHA-activated current was blocked by pimozone (Figure 2B) or by flupentixol, both known blockers of PUFA-activated K_{2p} channels [18,19]. Pimozone at 2 μ M almost completely blocked the current (0.7 ± 0.5 pA, n=4, for a representative trace see Figure 2B). Flupentixol at 2 μ M blocked this current similarly (2 ± 1 pA (n=3). The PUFAs, arachidonic acid and α -linolenic acid, both at 10 μ M, also induced a similar outward current, although α -linolenic acid-induced currents were of smaller amplitude than arachidonic acid-induced currents (see suppl. Figure 1 for traces).

In another series of experiments on PAEC, we used 1% isoflurane as pharmacological activator of K_{2p2.1} channels but blocker of K_{2p6.1} to more specifically open K_{2p2.1} channels. As shown in figure 2E, 1% isoflurane activated a more outward rectifying current that increased also over time at a clamp potential of +80 mV (Figure 2F).

In current-clamp experiments on CAEC clusters of 3–10 cells, we determined membrane potential changes in response to DHA (Figure 2G and 2H). DHA at 10 μ M produced a slow (within 40 seconds) shift of the membrane potential from rather depolarized values to negative values (mV: -28 ± 4 mV, n=11; range: –10 to –50 mV), demonstrating the ability of the small DHA-activated K⁺ currents to cause substantial hyperpolarization. This hyperpolarization was reversed by pimozone (2 μ M) shifting the membrane potential back to depolarized values (1 ± 1 mV, n=5, Figure 2H). Taken together, these data suggested that DHA caused hyperpolarization through PUFA-activated K_{2p} channels, such as K_{2p2.1} and K_{2p6.1}. A further discrimination of the respective contribution of the different channels was not possible because of the present lack of specific inhibitors.

Wire-myography

To investigate whether activation of the PUFA-activated K_{2p} channels in these arteries produces vasodilation, we performed isometric wire myography (pulmonary artery, Figure 3A–B; for original traces see suppl. Figure 4) and also pressure myography (carotid artery, Suppl. Figure 2A–C) as complementary isobaric approach. To measure the vasorelaxation or vasodilation, respectively, that is mediated by activation of K⁺ channels and ensuing hyperpolarization we performed the experiments in the presence of blockers of prostacyclin and NO synthesis (L-NAME and indomethacin). In U46619 pre-contracted pulmonary arteries, DHA induced a dose-dependent vasorelaxation (Figure 3A, circles) that reached 55% at the highest dose tested (50 μ M). This effect did not depend on a functioning endothelium (Figure 3A, triangles), because disruption of the endothelium did not reduce the response. This endothelium-independent relaxation suggested that DHA may have activated smooth muscle K_{2p} channels or other K⁺ channels like the large-conductance Ca²⁺-activated channel KCa1.1 as also described previously [20,21]. To rule out a contribution of other KCa channels that are known to cause smooth muscle hyperpolarization, we performed a third series of experiments using a cocktail of KCa blockers (Iberiotoxin (KCa1.1), UCL 1684 (KCa2.3), and TRAM-34 (KCa3.1)) and found that under these conditions the DHA-induced relaxation was intact (Figure 3A, squares), suggesting an involvement of K_{2p} channels. From pressurized and phenylephrine-precontracted carotid arteries we obtained similar results (Suppl. Figure 2). DHA produced dose-dependent dilation under these

isobaric conditions (Suppl. Figure 2A). The DHA-induced dilation was likewise independent of the activity of endothelial (KCa3.1 and KCa2.3) and smooth muscle KCa channels (KCa1.1) (Suppl. Figure 2B) and was not inhibited by the ATP-sensitive blocker, glibenclamide (10 μ M, n=2, results not shown). Moreover, the saturated fatty acid arachidic acid failed to induce relaxation (Suppl. Figure 2B) known to be inactive on K_{2p}2.1 channels. Again similar to pulmonary arteries the DHA-induced dilations were resistant to inactivation of the endothelium, which was not the case for acetylcholine relaxation (Suppl. Figure 2C). To further demonstrate a role of K_{2p} channels by pharmacological means, we tested whether pimozone (10 μ M) was able to block the DHA response. However, pimozone produced *per se* substantial vasorelaxation of pulmonary arteries (not shown) that is related to its blocking actions on 5-HT receptor or other pathways and was therefore without use to study the contributions of PUFA-activated K_{2p} channels. In the light of these circumstances and the lack of selective K_{2p} blockers, we proved at least the K⁺ channels are involved in the DHA response by showing that 30 mM extracellular potassium (preventing any hyperpolarization) virtually abolished DHA relaxation (Figure 3B).

Expression of PUFA sensitive K_{2p} channels in the lungs of chronic hypoxic mice

The mice had pulmonary hypertension, since right ventricular systolic pressure were 26 ± 1 mmHg and 37 ± 2 mmHg ($P < 0.05$) in respectively, normoxic (n=7) and hypoxic mice (n=7), while the ratios of right ventricle to left ventricle plus septum in normoxic and hypoxic mice were, respectively, 0.28 ± 0.02 and 0.37 ± 0.01 ($P < 0.05$, n=8 in each group).

To assess the relative expression of the PUFA sensitive K_{2p} channels in the lung and to see whether they were differentially regulated in our murine model of pulmonary hypertension, we performed qRT-PCR. Our qRT-PCR showed K_{2p}2.1, K_{2p}6.1 and K_{2p}1.1 to be the predominately expressed PUFA-sensitive K_{2p} channels in the lung (Figure 4A and 4B). K_{2p}10.1 and K_{2p}4.1 transcripts were apparently much less as specific signals came up within the last cycles of our qRT-PCR. Gene expression of K_{2p}2.1 was not statistically different between the groups. In contrast, gene expression levels of K_{2p}6.1 were fourfold higher in the hypoxia group (Figure 4B). The low expression levels of K_{2p}1.1, K_{2p}10.1 and K_{2p}4.1 were not significantly altered by hypoxia.

Immunohistochemistry for the predominantly expressed channel, K_{2p}2.1, did not show any gross differences between the control mice and the mice subjected to hypoxia (Figure 4C). In contrast, signal intensity for K_{2p}6.1 was visibly stronger in the hypoxic lungs. The more intense staining was particularly apparent in the bronchiolar epithelium and the alveoli of the chronic hypoxic animals (Figure 4D).

Discussion

Our investigation of the expression profile of the PUFA-activated K_{2p} channels indicated relatively high mRNA expression of K_{2p}2.1, an intermediate level of K_{2p}6.1 and K_{2p}1.1, and relatively low mRNA levels of K_{2p}4.1 and K_{2p}10.1. The detection in lung tissue of significant amounts of K_{2p}2.1 and K_{2p}6.1 is in line with previous findings [1,2,22,23]. As to the tissue localization of the K_{2p}2.1 and K_{2p}6.1 channels, K_{2p}2.1 has been shown in the smooth muscle layers of intrapulmonary arteries and airways from mouse [2] and K_{2p}6.1 has been shown in the smooth muscle layer of larger pulmonary artery from rat [1] (the same study shows an absence of K_{2p}2.1 from pulmonary artery). In our own IHC stainings, the K_{2p}6.1 protein was widely expressed in the murine lung and particularly in the epithelium of bronchioles and alveoli but also in pulmonary endothelium and perivascular cells however not in smooth muscle. In carotid arteries, K_{2p}6.1 protein was found in both endothelium and smooth muscle. The IHC staining of K_{2p}2.1 was not completely clear-cut, since we also observed some staining in the K_{2p}2.1 KO animals (supplemental figure 3)

which was located mainly in the adventitial tissue. However, the scattered staining of the endothelial layer as observed in the wildtype mice was not seen in the K_{2p}2.1 KO mice. It was important therefore to further confirm K_{2p}2.1 protein expression functionally in the endothelial cells by electrophysiology. Although the focus of this study is on the role of K_{2p} channels in arterial tone, the localization of K_{2p}2.1 and K_{2p}6.1 in pulmonary tissues beyond arterial smooth muscle and endothelial cells leaves room for speculations on their involvement in other pathophysiologically important pulmonary processes such as bronchodilatory effects and mucocilliary clearance. As a case in point, KCa2.3 has been shown to have a dual vasodilatory and bronchodilatory effect [24] while Zhao *et al* have pointed to a role for K_{2p} channels in mucocilliary clearance [25].

Both K_{2p}2.1 and K_{2p}6.1 have been cloned and examined by patch-clamping in various cell cultures [22,23,26–29]. The present study, however, is the first to show functional expression of PUFA-activated K_{2p}-like channels in freshly isolated pulmonary and carotid artery endothelial cells. The patch-clamp electrophysiology identified a small but significant PUFA-activated K_{2p}-like current in PAEC and CAEC that was blocked by the unspecific PUFA-activated K_{2p} blocker pimozone. Isoflurane, an activator of K_{2p}2.1, produced more-outwardly rectifying and voltage-activated currents. The readily PUFA-activated currents were capable of significantly shifting the membrane potential of endothelial cell clusters to negative values, which is in accordance with PUFA-activated K_{2p} channels causing vasorelaxation through cellular hyperpolarisation. The DHA-activated outward K⁺ current showed similarity to the currents produced by cloned rat K_{2p}6.1 channels as reported previously [23,29] and because there was no mild outward rectification as typical for K_{2p}2.1 [22] we suggest that these currents are mainly carried by K_{2p}6.1 channels. However, the data showing isoflurane-induced currents suggest also functional expression of specifically weakly outward rectifying K_{2p}2.1 channels. It is likely, therefore, that both channels contribute to the currents seen. However, at this stage and considering the lack of truly specific blockers and activators we do not exclude the possibility that also the closely related PUFA-activated channels K_{2p}1.1, K_{2p}4.1, and K_{2p}10.1 contribute to some extent to these currents, although the low mRNA expression of these PUFA-activated K_{2p} channels does not argue in favor of a major contribution. In sum, the electrophysiology provided the first evidence of functional expression of PUFA-activated and volatile anesthetics activated K_{2p}2.1 channels and PUFA-activated K_{2p}6.1 channels in murine endothelium.

Infusion of n-3 polyunsaturated fatty acids in the fetal lamb pulmonary circulation has shown that they increase pulmonary blood flow and lower pulmonary vascular resistance [30]. In the present study DHA also relaxed isolated arterial segments. In previous studies, vasorelaxations induced by PUFA in the pulmonary circulation were in part suggested to be mediated by tetraethylammonium-sensitive K⁺ channels and by formation of a P450-derived product [30]. Moreover, a blocker of KCa1.1, iberiotoxin and glibenclamide, a blocker of ATP-sensitive K⁺ channels was found to inhibit relaxations induced by another PUFA, eicosapentaenoic acid (EPA) in human pulmonary arteries [31]. However, incubation with a cocktail of blockers of classical endothelial KCa3.1 and KCa2.3 channels and smooth muscle KCa1.1 failed to attenuate DHA-induced vasorelaxation, suggesting that other mechanisms play a role in DHA relaxation. In an effort to directly show the involvement of PUFA-activated K_{2p} channels we tested a blocker of PUFA-activated K_{2p} channels, pimozone, but the inherent vasodilatory effect of this compound which is also blocking a series of G-protein-coupled receptors impeded our attempts in that direction. Interestingly, endothelial denudation did not significantly affect the vasorelaxation caused by DHA. This indicates that DHA may interact with KCa1.1 [5,20] and/or K_{2p} channels [1] directly in the smooth muscle layers and is not strictly dependent on endothelial function to cause vasorelaxation. However, by increasing extracellular K⁺ concentration DHA relaxation was blunted. In sum, the observation that vasorelaxation is abolished by high K⁺ concentrations

but not by a cocktail of blockers of classical endothelial KCa3.1 and KCa2.3 channels and smooth muscle KCa1.1 still strongly suggests the contribution of a PUFA-activated K_{2P} channel in the endothelium (and possibly in the smooth muscle) to PUFA-induced vasorelaxation, although the lack of specific pharmacological tools for the K_{2P} channels makes it impossible to define the contribution of each specific K_{2P} channel.

Regarding endothelial physiology, we speculate that endogenously produced arachidonic acid (e.g. in response to shear stress stimulation [32]) could act as such a channel activator. To which extent such channel activation and the subsequent endothelial hyperpolarization may produce, in particular, endothelium-dependent vasodilation in the physiological setting can however not be answered by the present findings.

A major aim of the present study was to test whether K_{2P} channels are altered in a disease-related fashion in pulmonary hypertension. In our hypoxia model of murine pulmonary hypertension, we found no significant alterations of the mRNA expression of the weakly expressed PUFA-activated K_{2P} channels (K_{2P}1.1, K_{2P}4.1, and K_{2P}10.1) and of the K_{2P}2.1 channel. In contrast, K_{2P}6.1 was significantly up-regulated by fourfold in this model, an up-regulation that was predominant in the bronchiolar epithelium and the alveoli while there was no clear indication of a higher K_{2P}6.1 protein expression in the pulmonary vascular bed. The functional meaning of this up-regulation to the pulmonary hypertension in this murine model remains obscure. However, if considering the channels as potential target structures in pulmonary arteries to lower pulmonary pressure, our findings suggests that these PUFA-activated K_{2P} channel are at least conserved as potential drug targets in this condition. Future studies using specific but so far unavailable or unidentified activators are needed to support this possibility.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Expression of K_{2p} genes

A) RT-PCR showing mRNA-expression of K_{2p} genes as well as KCa3.1 and KCa2.3 genes in murine lung tissue. The expected sizes of the RT-PCR products are noted in the materials and methods section. B) Immunohistology of the predominantly expressed K_{2p} channel, K_{2p}2.1 in murine lungs. Upper panel: K_{2p}2.1 staining in larger airways and arteries of the lung. Lower panel: Immunohistology without the primary anti-K_{2p}2.1 antibody. C) Immunohistology of the K_{2p} channel, K_{2p}6.1 in murine lungs. Upper panel: K_{2p}6.1 staining in larger airways and arteries of the lung. Lower panel: Immunohistology where the primary anti-K_{2p}6.1 antibody has been pre-incubated with a peptide blocking the antigen specific site of the antibody. D) Immunohistology in carotid artery of K_{2p}2.1 (left) and K_{2p}6.1 (right). The scale bar in each picture corresponds to 50 µm.

Figure 2. Electrophysiological characterization of K_{2p}2.1-like channels in native endothelial cells

A) Whole-cell patch clamping in voltage-clamp mode showing the basal and DHA-induced current in murine carotid artery endothelial cells (mCAEC) B) Whole-cell patch clamping in voltage-clamp mode on mCAEC showing DHA-induced current blocked by pimozone (2 μ M) C) Whole-cell patch clamping in voltage-clamp mode showing the basal and DHA-induced current in murine pulmonary artery endothelial cells (mPAEC). D) Whole-cell patch clamping of mPAEC in voltage-clamp mode. The graph shows the current over the cell membrane when clamped to a holding potential of -80 mV for 200 ms followed by 80 mV for 200 ms. E) Whole-cell patch clamping in voltage-clamp mode showing the basal and isoflurane-induced current in mPAEC. F) Whole-cell patch clamping of mPAEC in voltage-clamp mode, similar to Figure 2D but showing both basal and isoflurane induced current. G) Whole-cell patch clamping in current-clamp mode showing membrane potential changes to DHA (10 μ M). H) Whole-cell patch clamping in current-clamp mode showing membrane potential changes in response to DHA (10 μ M) and to DHA in combination with pimozone (2 μ M).

Figure 3. Vasorelaxing effect of DHA

All measurements were done in the presence of L-NAME (100 μM) and indomethacin (10 μM). A) Isometric tension recordings in murine pulmonary artery, showing the relaxing effect of increasing concentrations of DHA both without KCa blockers (circles) as well as in the presence of 100 nM Iberiotoxin, 1 μM TRAM-34 and 1 μM UCL1684 (squares) and, finally, after removal of the endothelium (triangles). B) Isometric tension recordings in murine pulmonary artery, showing the relaxing effect of 50 μM of DHA in the presence of control (5.9 mM) and high (30 mM) potassium. ***, $p < 0.001$.

Figure 4. Expression profile of K_{2p} -channels in murine lung under hypoxia induced pulmonary hypertension

A) QRT-PCR showing K_{2p}2.1 mRNA to be predominantly expressed ahead of K_{2p}10.1 and K_{2p}4.1 mRNA during both hypoxia induced pulmonary hypertension as well as under normal conditions (normoxia). B) QRT-PCR showing the levels of K_{2p}1.1 and K_{2p}6.1 mRNA under the same conditions as in figure 4A. The K_{2p}6.1 mRNA is significantly higher during hypoxia induced pulmonary hypertension compared to normoxic conditions. C) Immunohistology showing similar pattern of expression of K_{2p}2.1 under both hypoxia and normoxia. D) Immunohistology of K_{2p}6.1 under both hypoxia and normoxia. A more intense staining of the bronchiolar epithelium is seen in lungs from mice with hypoxia induced pulmonary hypertension compared to the normoxic control group. The scale bar in each picture corresponds to 50 μ m. ***, $p < 0.001$.

Table 1**Primers**

List of primers used for RT-PCR and qRT-PCR.

Primer name	Forward primer	Reverse primer	Bp
-actin	TCCTAGCACCATGAAGAT	AAACGCAGCTCAGTAACA	190
K _{2p} 1.1	ATTTGCACGAAACCAGGAAA	TCCTCATAAGGCAGCTCCAC	210
K _{2p} 2.1	CAGCAATAAACGCAGGGATT	CTTGGCAATTCCTTTTCCA	250
K _{2p} 4.1	GCAGAGATGGGTGGCCTAAC	CAGGAGTGGTTGCTCCTTCT	117
K _{2p} 6.1	CTGGATGCCTTCGTGGAG	TCTGTCAGTGGGGTCGTGTA	176
K _{2p} 10.1	AGAACAACAATCGCCTTGAG	GATTTTGCCTCCTTCAGTGC	242
KCa3.1	CTGAGAGGCAGGCTGTCAATG	ACGTGTTTCTCCGCCTTGTT	217
KCa2.3	CCATGCCAAAGTCAGGAAAC	CATCTTGACACCCGAAGTT	70