Src tyrosine kinase is crucial for potassium channel function in human pulmonary arteries

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Abstract

The potassium channel TASK-1, together with other potassium channels controls the low resting tone of pulmonary arteries. The Src family tyrosine kinase (SrcTK) may control potassium channel function in human pulmonary artery smooth muscle cells (hPASMCs) in response to changes in oxygen tension and the clinical use of an SrcTK inhibitor has resulted in partly reversible pulmonary hypertension. This study aimed to determine the role of SrcTK for hypoxia-induced inhibition of potassium channels in hPASMCs.

We show that SrcTK is colocalized with the TASK-1 channel. Inhibition of SrcTK decreases potassium current density and results in considerable depolarization, while activation of SrcTK increases potassium current in patch-clamp recordings. Moderate hypoxia and the SrcTK-inhibitor decrease the tyrosine-phosphorylation state of the TASK-1 channel. Hypoxia also decreases the level of phospho-SrcTK (tyr419) and reduces the co-localization of the TASK-1 channel and phospho-SrcTK. Corresponding to this, hypoxia reduces TASK-1 currents before but not after SrcTK inhibition and, in the isolated perfused mouse lung, SrcTK inhibitors increase pulmonary arterial pressure.

We propose that the Src family tyrosine kinase is a crucial factor controlling potassium channels, acting as a cofactor for setting a negative resting membrane potential in hPASMCs and a low resting pulmonary vascular tone.

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Introduction

In the pulmonary artery, vascular tone is regulated in part by the resting membrane potential of the pulmonary artery smooth muscle cells (PASMCs). As in all excitable cells, potassium (K⁺) channels are primary candidates for the regulation of the resting membrane potential by maintaining the resting membrane potential close to the K⁺ equilibrium potential. Activation or inhibition of the K⁺ channels results in hyperpolarization or depolarization of the cell membrane, leading to initiation of vasodilation and vasoconstriction, respectively. At least 4 classes of K⁺ channels have been identified in PASMC [1]. The background or leak K⁺-selective channels are exceptional amongst the K⁺ channels, as their activity is not controlled by voltage and they may be considered as the main contributor to the resting membrane potential and input resistance in primary human PASMCs [2, 3].

Src family tyrosine kinase (SrcTK) is a member of the non-receptor tyrosine kinase family, participating in a wide range of cellular signaling and functions [4]. C-SrcTK is targeted to the plasma membrane due to a myristylated N-terminal region. The SH1 domain of the SrcTK contains the phosphorylation site tyr419 which is required for full C-SrcTK activation [5]. This mechanism allows C-SrcTK to interact with ion channels to modulate their properties [6, 7]. Inactivation of human C-SrcTK occurs when its C-terminal tyr530 is phosphorylated which then binds to the SH2 domain. Crystallographic studies have shown that interactions between the C-terminus and the SH2 domain, and between the kinase domain and the SH3 domain, cause the C-SRC molecule to assume a closed configuration that covers the kinase domain and reduces its potential for substrate interaction [5]. The C-terminus of the background TASK-1 potassium (K⁺) channel contains possible phosphorylation sites for tyrosine and serine kinases (for details see Supplementary Fig. 1A). If potassium channels are normally activated by SrcTK, reversal of this mechanism could explain the increased pulmonary vascular tone observed after SrcTK inhibition by dasatinib treatment [8].
In the present study we investigated the functional role of SrcTK on K⁺ channel function and membrane potential. We studied hypoxic inhibition of TASK-1 channels in hPASMC to define the specific role of SrcTK. We demonstrated that specific inhibition of SrcTK results in the inhibition of TASK-1 and other K⁺ channels and causes membrane depolarization. In addition, hypoxia decreases active phospho-SrcTK co-localization with TASK-1 channels in the membrane, while activation of SrcTK increases TASK-1 current and this activated current is inhibited by moderate hypoxia. This suggests that K⁺ channel function is critically dependent on SrcTK explaining why SrcTK inhibition might cause life-threatening pulmonary vasoconstriction. This is the first report demonstrating the functional role of SrcTK in the regulation of resting membrane potential, in the hypoxic inhibition of TASK-1 channels in primary human PASMC and finally in pulmonary vascular tone.
Methods

The study protocol for tissue donation was approved by the Institutional Review Board of the Medical University of Graz and is in accordance with the national law and the guidelines on Good Clinical Practice/International Conference on Harmonization. Written informed consent was obtained from each patient if appropriate. All animal experiments were approved by the Institutional Animal Care and Use Committee.

Preparation of Human Primary Pulmonary Artery Smooth Muscle Cells and Cell Culture (hPASMC)
Primary SMC were isolated from human resistance pulmonary arteries from patients (n = 30) undergoing lung surgery for lung cancer without a history of pulmonary vascular disease or arterial hypoxemia or unused donor lungs harvested for lung transplantation. In the case of obtaining pulmonary arteries from patients with lung cancer, only arteries were used which had a distance of at least 5 cm from the cancer tissue. For details see online supplement.

Electrophysiology
The whole-cell patch-clamp technique on hPASMC was used as previously described to measure the resting membrane potential under current clamp and macroscopic K⁺ currents under voltage clamp [3]. Detailed description of the protocols and solutions are given in the online supplement.

The data were stored and analyzed with commercially available pCLAMP 9.0 software (Axon Instruments, Foster City, CA, USA).

Calcium measurements
The fluorescent dye fluo-4-AM was used for detection for detection of changes in intracellular calcium in hPAMSCs. Detailed description of the protocols and solutions are given in the online supplement.
The acquired images were stored and subsequently processed offline with TillVision software (Till Photonics, Germany).

**Transfection of small interfering RNA against C-Src, Fyn and TASK-1**

Small interfering RNAs (siRNA) against C-Src (siC-Src), Fyn (siFyn) and Task-1 (siTASK-1) were commercially synthesized (Eurogentec, Seraing, Belgium). For details see Online supplement. As negative control, non-silencing RNA (nsRNA) which does not target any human gene product was used. The hPASMCs were grown on coverslips or in 6-well plates, to which annealed siRNA was transfected using Effectene transfection reagent (QIAGEN). Gene knock-down was checked by quantitative RT-PCR using the RNA extracted (RNeasy, QIAGEN) from the transfected cells. RNA levels, live cell calcium and electrophysiological measurements were performed 48–56h post transfection. To assess the efficiency of the siRNA-transfection FITC-conjugated siRNA were used. Only FITC-positive cells were used for electrophysiological studies. In addition, TASK-1-siRNA-transfection were functionally controlled by superfusion of the cells with a bath solution adjusted to the pH of 8.3.

**RT-PCR**

Detailed description is provided in the online data supplement.

**Quantitative RT-PCR**

Quantitative RT-PCR was made to check the expression of C-Src, Fyn, and TASK-1 in hPASMCs. For more details see the online supplement.

**Co-immunoprecipitation and Immunoblotting and Immuno co-localization**

For details see the online supplement.
Hypoxic treatment of hPASMCs
The effect of hypoxia in the patch–clamp and calcium imaging studies was studied by switching between normoxic and hypoxic perfusate reservoirs. Detailed description is provided in the online data supplement.

Isolated, perfused and ventilated mouse lungs
Lungs from adult C57BL/6 mice (Harlan Laboratories, Inc) were removed from the chest under deep anesthesia, artificially ventilated and perfused with Krebs Henseleit buffer. The lungs were mounted in a water-heated chamber that allows for negative pressure ventilation with a gas containing 5.3 % CO₂, 21.0 % O₂, balanced with N₂. An initial steady state period of 15 minutes (with Krebs Henseleit buffer (flow rate 1 ml/min) was taken as baseline. For detailed description see online supplements. PP2 (30 µM), PP3 (30 µM) or dasatinib (100 µM) was added into the buffer for a period of 15 minutes. Delta pulmonary arterial pressure (PAP) indicates the PAP-change after application of PP2, PP3 or dasatinib.

Solutions and Chemicals
4-Amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (PP2) and 4-Amino-7-phenylpyrazol[3,4-d]pyrimidine (PP3) were purchased from Sigma Chemical Company (St. Louis, MO). Src Activator peptide was from Santa Cruz Biotechnology Inc. All drugs were dissolved in the experimental (bath) solution, except PP2, PP3 and dasatinib. They were dissolved in DMSO. At this concentration, the vehicle alone had no effect on ion current, resting membrane potential or pulmonary artery pressure. The pH of solutions containing drugs was tested and corrected to eliminate potential pH-induced effects. Krebs Henseleit buffer (mmol/L): NaCl 120, KCl 4.3, KH₂PO₄ 1.1, CaCl₂ 2.4, MgCl₂ 1.3, and glucose 13.3, as well as 5% [wt/vol] hydroxyethylamyllopectin [molecular weight 200,000]). NaHCO₃ was adjusted to result in a constant pH of 7.37 - 7.40.
**Statistical analysis**

Numerical values are given as means ± SE of n cells or measurements. Intergroup differences were assessed by a factorial analysis of variance with post-hoc analysis with Tukey's significant difference test, or Student's unpaired and paired t-tests as appropriate. p values <0.05 were considered significant and shown as * in the figures (*<0.05, **<0.01; ***<0.001 compared to control).
Results

Expression of SrcTK in human lung and PASMC

To analyze the expression of SrcTK isoforms, C-Src, Lck, Lyn, Fyn, Yes, and Frg, RT-PCR was performed on human lung tissue, human pulmonary artery and human pulmonary artery smooth muscle cells (hPASMCs) (Fig. 1A). Our results demonstrated the presence of mRNA encoding all investigated isoforms in lung tissue. Human primary PASMCs expressed mRNA only for the isoforms C-Src, Fyn and Yes.

SrcTK is co-localized with TASK-1 channels in hPASMC

Co-immunoprecipitation of TASK-1 with SrcTK: Immunoprecipitation (IP) was performed with the anti-SrcTK antibody or isotype control (IgG) from the hPASMC lysate. Later immunoblot (IB) was performed, either with anti-TASK-1 (top panel) or with anti-ScrTK antibodies (bottom panel; Fig. 1B) showing the direct binding of TASK-1 to SrcTK. The localization of the background TASK-1 channel and SrcTK were also visualized by confocal laser scanning microscopy in hPASMC (Fig. 1C). Immunofluorescent staining of TASK-1 and SrcTK revealed that TASK-1 and SrcTK are co-localized in the cell membrane. Staining was absent in negative controls, when cells without exposure to primary antibody were imaged. The TASK-1 and SrcTK antibodies were evaluated by immunoblot (Online Supplementary Fig. 1B).

SrcTK modulates TASK-1 channel activity in hPASMCs

To further confirm the functional relevance of the SrcTK interaction with TASK-1 channels, we used pharmacological tools and siRNA techniques. Patch-clamp recordings of the non-inactivating TASK-1 current (I_{KN}) on hPASMCs were made as described previously[3]. SrcTK increases TASK-1 channel activity as confirmed by silencing of C-Src and Fyn in primary hPASMCs, resulting in decreased current compared to control (Fig. 2A and B). TASK-1 current density is significantly inhibited by treatments with the SrcTK inhibitor PP2 (0.23±0.06
Although PP2 is a potent and selective inhibitor of the Src family of protein tyrosine kinases, in order to investigate the role of the Src subtypes, siRNA against C-Src and Fyn was used. Transfection with siC-Src (0.17±0.01 pA/pF; n=4) or siFyn (0.20±0.02 pA/pF; n=8) significantly inhibited TASK-1 current density compared to control (0.67±0.1 pA/pF; n=13). As a control, PP3, the inactive analog of the C-Src inhibitor PP2 and transfection with non-silencing(ns)RNA was applied. The treatment with PP3 (0.58±0.02 pA/pF; n=4) or transfection with non-silencing(ns)RNA (0.62±01 pA/pF; n=8) does not significantly affect TASK-1 current (Fig. 2C). The relative degree of knock-down of C-Src and Fyn by their respective siRNA was confirmed by quantitative RT-PCR (Online Supplementary Fig. 2).

**SrcTK inhibition depolarizes hPASMCs**

TASK-1 channels are active at resting membrane potential and set the negative resting membrane potential in PASMCs, as we have previously demonstrated by siRNA treatment in primary human PASMCs. As the SrcTK inhibition also decreases the TASK-1 current (Fig. 2C), the physiological role of SrcTK for the resting membrane potential of primary hPASMCs was further investigated. Electrophysiological measurements carried out in hPASMCs showed a significant depolarization after treatment with PP2 (-24.1±1.3mV; n=39) or transfection with siC-Src (-19.6±1.5mV; n=14) or with siFyn (-20±1.1mV; n=14) compared to control (-44.3±1.6mV; n=36) or to cells treated with the inactive analog of PP2, PP3 (-43.4±1.5mV; n=4) or transfected with nsRNA (-43.6±1.5mV; n=18) (Fig. 2D). Taken together, these data further strengthen the role of TASK-1 and the importance of SrcTK in setting the negative resting membrane potential in primary PASMCs.

**Hypoxic regulation of SrcTK in hPASMCs**

Hypoxia causes membrane depolarization in hPASMCs, partly due to TASK-1 channel inhibition. Silencing of SrcTK decreases TASK-1 current leading to membrane depolarization. Activity of SrcTK is determined by the phosphorylation state of SrcTK at
tyr419. Therefore, we investigated the phosphorylated (active, Fig. 3A upper panel) and non-phosphorylated (inactive, Fig. 3A middle panel) state of SrcTK in hPASMCs at different time points under hypoxia (30 minutes of normoxia or 0, 1, 5, 10, 15, 20 and 30 minutes of hypoxia). Next, the co-localization of the background TASK-1 channel (green) and phospho-SrcTK (red) was examined in hPASMC under normoxia and in 15 minutes hypoxia and visualized by confocal laser scanning microscopy (Fig. 3B). The lower panel of Fig. 3B clearly shows the reduced phospho-SrcTK staining in hypoxia. Fig. 3C shows that hypoxia, as well as application of the SrcTK-inhibitor PP2, decrease the tyrosine-phosphorylation state of TASK-1 channels, whereas PP3, the inactive analog of PP2, did not change the TASK-1-phosphorylation. Lower panel shows the unchanged total level of TASK-1 under different experimental conditions.

Next, the functional role of SrcTK for the hypoxic inhibition of TASK-1 channels was examined. Intracellular dialysis with Src activator peptides (EPQYEEIPYLY) significantly increased the TASK-1 current density (0.72±0.07 pA/pF; n=8) compared to control (0.51±0.05 pA/pF; n=10, Fig. 4A and B). Hypoxia was able to inhibit both the control and the activated current. Fig. 4C summarizes the effects of SrcTK activator peptides and hypoxia. As the initial TASK-1 current was decreased by inhibiting SrcTK with blocker or siRNA (Fig. 4E), hypoxia did not have any further effect on cells treated with PP2 or transfected with siC-Src or siFyn. However, hypoxia still was able to inhibit the TASK-1 current in control, PP3-treated or nsRNA transfected hPASMCs (Fig. 4D). Relative TASK-1 currents under hypoxia in hPASMCs are presented in Fig. 4F.

TASK-1 channels can be modulated by different pathways, such as AMPK [9], PKA [3], PKC [10] and PLC [11], in response to different agonist stimulations. In order to exclude the involvement of these pathways, we investigated the hypoxic inhibition of TASK-1 current using different inhibitors. None of these treatments affected control TASK-1 current or its hypoxic inhibition (Online Supplementary Fig. 3 and Fig. 4G) showing that AMPK, PKA, PKC and PLC are not involved.
Impact of SrcTK on the hypoxia-induced increase of intracellular calcium (Ca\(^{2+}\)) in hPASMCs

Hypoxia-induced rise in Ca\(^{2+}\) is an integral and characteristic property of hPASMCs, which is directly linked to downstream signaling leading to vasoconstriction. The role of SrcTK in the hypoxia-induced Ca\(^{2+}\) rise was further analyzed in hPASMCs. Fluo4-loaded hPASMCs were continuously monitored for changes in Ca\(^{2+}\) under hypoxia (Fig. 5A). Hypoxia significantly increased Ca\(^{2+}\) in control cells (0.54±0.04, n=63), and in cells treated with PP3 (0.52±0.04, n=17) or transfected with nsRNA (0.45±0.06, n=16). In contrast, the hypoxia-induced increase in Ca\(^{2+}\) was markedly attenuated after treatment with PP2 (0.18±0.01, n=68), transfection with siC-Src (0.12±0.02, n=18) or with siFyn (0.12±0.02, n=14) or with siTASK-1 (0.25±0.02, n=22, respectively). Summarised results are presented in Fig. 5B.

Inhibition of SrcTK attenuates Kv and KCa current in hPASMCs

The impact of SrcTK inhibition on whole-cell potassium currents, (voltage-gated (K\(_v\)) and calcium-dependent (K\(_{Ca}\)) K\(^+\) currents), was investigated in primary human PASMCs. Representative Kv current recordings in control hPASMCs (Fig. 6A) and after treatment with siC-Src are presented in Fig. 6B, showing that silencing of C-Src significantly decreases Kv current. Similar results were obtained from experiments recording KCa current in control (Fig. 6D) and after silencing of C-Src (Fig. 6E) in primary human PASMCs.

To further assess the role of SrcTK for activation of Kv and KCa channels, the effect of PP2, PP3 and the treatment with siC-Src, siFyn or nsRNA was investigated and compared with the effect of hypoxia (Fig. 6C). Treatment of hPASMCs with PP2 (3.8±0.4 pA/pF; n=16), or siC-Src (2.9±0.3 pA/pF; n=5) or siFyn (3.1±0.3 pA/pF; n=5) significantly decreased the Kv current compared to control (8.9±0.8 pA/pF; n=24, respectively). Hypoxia showed a similar effect (Fig. 6C). Treatment with PP3 (9.3±0.7 pA/pF; n=4) or transfection with nsRNA (7.8±0.4 pA/pF; n=9) did not alter the current (Fig. 6C). Similar results were observed when KCa current was recorded (Fig. 6E and F). Only hypoxia (2.3±0.5 pA/pF; n=16), treatment with PP2 (4±0.3 pA/pF; n=17), siC-Src (2.35±0.6 pA/pF; n=5) or siFyn (1.81±0.4 pA/pF; n=5)
decreased the current, compared to control (5.7±0.6 pA/pF; n=12), whereas PP3 (5.58±0.3 pA/pF; n=5) or nsRNA (5.17±0.9 pA/pF; n=8) had no significant effects (Fig. 6F).

**Pulmonary vasoconstriction in response to SrcTK inhibitors**

To depict the role of SrcTK in pulmonary vascular tone, we used isolated perfused mouse lungs, where PP2 showed a significant increase in PA pressure (6.3±1.3 mmHg, n=4) compared to the inactive analog PP3 (0.6±0.2 mmHg, n=4) and, interestingly, the second-line treatment of chronic myeloid leukemia, dasatinib which is also a potent inhibitor for SrcTK, showed a similar increase (5.5±0.2 mmHg: n=3) in PA pressure compared to the solvent DMSO (0.4±0.4 mmHg, n=4). Fig. 6G shows the summarized delta PAP before and after PP2, PP3, DMSO and dasatinib.
Discussion

The main findings of this study are that 1) two members of SrcTK family, C-Src and Fyn, are highly expressed in primary human pulmonary artery smooth muscle cells (hPASMCs); 2) TASK-1 channels and SrcTK are co-localized in the plasma membrane of hPASMCs; 3) SrcTK is required for the activity of TASK-1 channels; 4) the inhibition of SrcTK depolarizes hPASMCs; 5) hypoxia reduces the tyrosin phosphorylation level of TASK-1; 6) hypoxia reduces the active phosphorylated state of SrcTK and inhibits TASK-1 current facilitated by SrcTK activator; 7) SrcTK inhibition markedly attenuates the hypoxia-induced intracellular calcium rise; 8) whole cell potassium current (Kv and KCa) is reduced by SrcTK inhibition and 9) inhibition of SrcTK by PP2 or dasatinib causes a substantial increase in the pulmonary arterial pressure of isolated perfused mouse lungs.

The membrane potential of smooth muscle cells is an important factor in controlling pulmonary vascular tone. At rest, the membrane potential of PASMCs is approximately –50mV [2, 3, 12]. This is maintained by K⁺ efflux from these cells through K⁺ channels. Agents that inhibit or activate pulmonary vascular smooth muscle cell K⁺ channels cause depolarization or hyperpolarization, respectively. The function, control and expression of K⁺ channels in pulmonary arteries is a matter of continued interest, because it is likely that a decrease in K⁺ channel expression e.g. Kv1.5 [13, 14] or dysfunction of K⁺ channels gives rise to membrane depolarization [15] and, together with an increase in the cytosolic calcium, results in increased proliferation and decreased apoptosis, ultimately contributing to the pathogenesis of pulmonary hypertension [16].

Depolarization in hPASMCs, induced by the inhibition of K⁺ channels, is followed by influx of calcium through voltage-gated (L-type) calcium channels and results in increased pulmonary vascular resistance. Acute hypoxia causes vasoconstriction by several mechanisms. It depolarizes PASMC by inhibiting K⁺ channels, leading to calcium influx as described above [17], causes the release of calcium from the sarcoplasmic reticulum (SR) and subsequent repletion through store-operated channels [18, 19], increases calcium influx
into PASMCs through L-type Ca channels, independent of the membrane potential [20] and also promotes calcium sensitization [21], thus increasing pulmonary vascular resistance [22]. Most of the calcium responsible for the increase in cytosolic calcium induced by hypoxia comes from outside the PASMC but some is released from internal stores such as the SR [1]. It is likely that the reduction in the hypoxia-induced increase in calcium that is caused by PP2, or siC-Src, or siFyn (Fig. 5), is secondary to the lack of hypoxic inhibition of K\(^+\) currents, because inhibition of K\(^+\) channels has already been caused by SrcTK inhibition. The hypoxic response is abolished in the presence of diminished SrcTK activity (Figs. 4 and 6).

Several studies have indicated that tyrosine kinases may act on K\(^+\) channels. However, these studies were carried out on cell lines or in heterologous expression systems using a broad range inhibitors but without showing the physiologic role of the findings [7, 23, 24]. We have previously shown that the background two pore domain TASK-1 channel sets the membrane potential in primary human PASMCs, and it can be modulated by PKA, PLC, PKC and AMPK pathways through serine-threonine phosphorylation by different agonists, such as endothelin [10], treprostinil [3] etc. Furthermore, hypoxia depolarizes the membrane potential by reversibly inhibiting TASK-1 channels in hPASMC [3]. In addition, K\(_{Ca}\) and K\(_{v}\) channels may also contribute to the membrane potential, particularly if they are stimulated by agents like cAMP and cGMP. Therefore, our results suggest that SrcTK activity is essential for the low physiologic tone of PASMC and thus for the low pulmonary vascular resistance.

The mechanism by which hypoxia inhibits these channels is currently unknown. However, in the present study, specific inhibition of endogenous SrcTK reduces TASK-1 current in hPASMC. Likewise, when hPASMC are dialysed with SrcTK activator, a significant increase in the TASK-1 current is observed, suggesting that TASK-1 current requires SrcTK activity, with dephosphorylation decreasing the current and phosphorylation increasing it. Several other potassium channels, including K\(_{v}\) and K\(_{Ca}\), have previously been shown to be modulated by SrcTK-mediated tyrosine phosphorylation [25]. Our observations confirm this but demonstrate the critical role of ScrTK activity for the function of these channels and show that TASK-1 activity requires SrcTK activity.
In the present study, we demonstrated that SrcTK inhibition reduces TASK-1 current and plays a crucial role in the hypoxic inhibition of TASK-1 channels, probably through a reduction in phosphorylation of SrcTK at tyr419 and dissociation of TASK-1 and SrcTK, although the molecular regulation of this link has yet to be determined (Fig. 7).

A recent study by Knock et al. describes that SrcTK inhibition by PP2 blunts hypoxia-induced pulmonary vasoconstriction and inhibits Rho kinase in rat pulmonary artery and isolated PASMC [26, 27]. Their finding that PP2 reduces the hypoxia-induced increase in PASMC cytosolic calcium is concordant with our observations. Given the inhibitory results of PP2 and the siRNAs on TASK-1, Kv and KCa currents that we describe here, and the similar effects of hypoxia on these currents, it makes sense that the SrcTK phosphorylation is inhibited in the sequence leading to K+ current inhibition. However, in their experiments hypoxia increased phosphorylation of SrcTK at tyr419 rather than decreased it, as we report here. The difference in these results may be due to differences in species, cell culture conditions, the number of cycles, and the severity, duration or time point of the study under hypoxia.

The observed increase in PAP following the inhibition of SrcTK by PP2 and dasatinib suggests a functional role for SrcTK in regulating pulmonary vascular tone. It is an open question what this means for patients with severe pulmonary hypertension. Tuder et al. describe decreased levels of c-Src in sixteen PPH (IPAH according to the current classification) patients [28]. This would correspond to decreased potassium channel activity and might contribute to vasoconstriction and other pathologic mechanisms leading to pulmonary hypertension. Here we report a reduced TASK-1, whole-cell K current and KCa current after c-Src inhibition in hPASMC. This is in line with observations showing that reduced Kv1.5 activity leads to pulmonary hypertension [13]. In contrast, a recent study by Courboulin et al. reports increased levels of phosphor-Src and total Src in lung samples from three PAH patients [29].

Recent clinical observations highlight the potential importance of tyrosine kinases in the pathophysiology of PAH. Chronic myeloid leukemia (CML) is caused by a constitutively
active BCR-ABL tyrosine kinase. Imatinib, which inhibits this kinase, is the first–line therapy for CML [30]. Imatinib is also an effective inhibitor of the platelet-derived growth factor receptor [31] and this is thought to be the reason why it may improve hemodynamics in some PAH patients [32]. Dasatinib is a tyrosine and a serine/threonine kinase inhibitor, which is 325 times as potent as imatinib in the inhibition of BCR-ABL kinase in vitro and induces higher and faster rates of cytogenic response in CML [33]. However, dasatinib potently inhibits the Src family, which is probably the single most prominent dasatinib-targeted family of protein kinases including C-Src and Fyn and it has been reported to cause pulmonary arterial hypertension [8, 34, 35]. The pulmonary hypertension tends to resolve rapidly after discontinuation of dasatinib [34], suggesting that it may not be primarily due to marked cellular proliferation, but to chronic vasoconstriction. One can speculate that dasatinib-initiated pulmonary hypertension may relate to our finding that siRNA against C-Src and Fyn reduces potassium channel current and causes depolarization of hPASMCs. It is clear that much work remains to be done to clarify the role of different kinases in the etiology and therapy of PAH.

Limitations of the study: Although the present findings suggest that TASK-1 channels and SrcTK are co-localized in the plasma membrane of hPASMCs, which is further supported by co-immunoprecipitation studies, we cannot exclude that Src could also act indirectly on potassium channels via other Src downstream molecules. In addition, mechanism(s) involved in acute hypoxia-induced inhibition of potassium channels could be different than those involved with chronic exposure to hypoxia and/or dasatinib. Finally, changes in pulmonary artery pressure in the presence of PP2 and/or dasatinib might not be related to this precise proposed mechanism.

In conclusion, we demonstrate that SrcTK has an important role in the control of TASK-1 and other potassium channels and that it sets the negative resting membrane potential in hPASMCs. The physiological relevance of this SrcTK and TASK-1 channel association is emphasized by the fact that the hypoxia-induced inhibition of TASK-1 current and the intracellular calcium rise is dependent on SrcTK. It is likely that a better description of
the multifunctional role of SrcTK in regard to K⁺ channel regulation will facilitate our understanding of the pathophysiology of pulmonary hypertension.
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Conflict of Interest Statement:

References


**Figure legends**

**Figure 1. SrcTK is co-localized with TASK-1 channels in hPASMC.**

(A) RT-PCR screening for SrcTK in RNA extracts from homogenized human lung tissue and human primary PASMC. Representative gels illustrate mRNA expression of C-Src (204 bp), Lck (398 bp), Lyn (213 bp), Fyn (206 bp), Yes (202 bp) and Frg (402 bp). The arrows indicate 200 bp. Identical results were obtained with at least 3 preparations of RNA from different donor lungs and primary hPASMCs. (B) Blot represents co-immunoprecipitation with anti-SrcTK, isotype control IgG and cell lysate, then immunoblotted for TASK-1 (upper panel) and SrcTK (lower panel). Star indicates the IgG. (C) Fluorescent immunostainings indicate DAPI nuclear staining (blue), Src family kinases (red), TASK-1 channel (green) and an overlayed image (merged) in a single–plane confocal image of hPASMC.
Figure 1.
Figure 2. SrcTK is crucial for TASK-1 channel activity in primary hPASMC.

(A) Representative recordings of TASK-1 current ($I_{\text{KN}}$) in control cells and (B) in cells transfected with siC-Src. (C) Histogram summarizing the TASK-1 current density in primary hPASMC. After treatment with Src family kinase inhibitor PP2 (1 µM) or transfection with siC-Src and siFyn, the current density is significantly reduced. There is no reduction of current density after transfection with non-silencing (ns)RNA or after treatment with PP3 (1 µM; inactive analog of PP2). (D) Histograms summarizing the effects of SrcTK inhibition on resting membrane potential of hPASMCs. Significant depolarization was observed when the cells were treated with PP2 (1 µM) or transfected with siC-Src, siFyn or siTASK-1, but not after transfection with nsRNA and treatment with PP3.
Figure 2.
Figure 3. Effect of hypoxia on SrcTK phosphorylation in primary hPASMCs.

(A) Hypoxia decreased phospho-SrcTK (tyr-419) immunoreactivity (60 kDa), whereas enhanced immunoreactivity was detected at the non-phospho SrcTK (60-65 kDa) in a time-dependent manner. Protein loading equivalence is shown by total SrcTK (n=4). (B) An evident decrease of phospho-SrcTK tyr-419 (red) is shown after 15 minutes of hypoxia compared to normoxia in single–plane confocal images of hPASMC. (C) Application of hypoxia or the SrcTK-inhibitor PP2 decreased the tyrosine-phosphorylation state of TASK-1 channel. Lower panel shows the unchanged total level of TASK-1 under different experimental conditions.
Figure 3.
Figure 4. SrcTK activator increases TASK-1 current and this effect is completely inhibited by hypoxia in primary hPASMC.

Representative recordings of TASK-1 current (I_{K\text{N}}) control and under hypoxia without (A) or with (B) SrcTK activator peptide, EPQYEEIPIYL (1 mM) in the patch pipette. (C) Bar graph summarizes the effect of SrcTK activator peptide which significantly increases the current density of TASK-1 current compared to control. This increased TASK-1 current is significantly inhibited by hypoxia. TASK-1 current recording from cells transfected with (D) nsRNA or (E) siC-Src. In the latter case, hypoxia could not further inhibit TASK-1 current. (F) Histogram summarizing the lack of further hypoxia-induced TASK-1 current inhibition after treatment with PP2, and siC-Src, siFyn, siTASK-1. I_0 indicates the current under normoxia in every cell. (G) Bar graphs showing the I_{K\text{N}} density of TASK-1 channel in the presence of Ro-31-8220 (PKC inhibitor), Gö6983 (PKC inhibitor), KT5720 (PKA inhibitor) and compound C (AMP-activated kinase inhibitor) under normoxia (control) and hypoxia in primary hPASMCs.
Figure 4.
Figure 5. Inhibition of SrcTK attenuates hypoxia-induced increase in intracellular calcium concentration in primary hPASMCs.

(A) Representative recordings of fluo-4 fluorescence after hypoxic challenge in a control hPASMC and in cells treated with PP2, PP3, siC-Src, or siFyn. (B) Hypoxia-induced intracellular calcium increase was decreased after treatment with PP2, siC-Src or siFyn, but not with nsRNA or PP3.
Figure 5.
Figure 6. Inhibition of SrcTK decreases whole cell $K_v$ and $K_{Ca}$ current in primary hPASMC and increases the pulmonary artery pressure in the isolated, perfused mouse lung model.

(A, D) Representative traces of voltage-gated ($K_v$) and calcium-activated ($K_{Ca}$) $K^+$ current in control cells and (B, E) after treatment with siC-Src. (C, F) Histograms summarizing the effects of hypoxia, PP3, PP2, siC-Src, siFyn or nsRNA for $K_v$ and $K_{Ca}$ current density.

(G) Histogram summarizing change in pulmonary artery pressure in the presence of PP2, PP3, DMSO and dasatinib expressed as delta PAP mmHg from isolated perfused mouse lungs.
Figure 6.
Figure 7. Scheme of the proposed interplay between TASK-1 channel and c-Src in human PASMCs. Under normoxia, the phospho-Src (active-Src, phosphorylated at Tyr419) binds to TASK-1 channels resulting in functional TASK-1 channels. Active TASK-1 channels maintain negative resting potential in hPASMCs. In hypoxia, the phospho-Src (active-Src) is decreased. Closed TASK-1 leads to depolarisation and increased intracellular calcium level. (+) indicate increase and (-) indicate decrease. Em: resting membrane potential, TASK-1: TWIK-related acid sensitive potassium channel-1.