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Role of *src*-family kinases in hypoxic vasoconstriction of rat pulmonary artery

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Aims We investigated the role of *src*-family kinases (*src*FKs) in hypoxic pulmonary vasoconstriction (HPV) and how this relates to Rho-kinase-mediated Ca^{2+} sensitization and changes in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$).

Methods and results Intra-pulmonary arteries (IPAs) were obtained from male Wistar rats. HPV was induced in myograph-mounted IPAs. Auto-phosphorylation of *src*FKs and phosphorylation of the regulatory subunit of myosin phosphatase (MYPT-1) and myosin light-chain (MLC_{20}) in response to hypoxia were determined by western blotting. Translocation of Rho-kinase and effects of siRNA knockdown of *src* and *fyn* were examined in cultured pulmonary artery smooth muscle cells (PASMCs). $[Ca^{2+}]_i$ was estimated in Fura-PE3-loaded IPA. HPV was inhibited by two blockers of *src*FKs, SU6656 and PP2. Hypoxia enhanced phosphorylation of three *src*FK proteins at Tyr-416 (60, 59, and 54 kDa, corresponding to *src*, *fyn*, and *yes*, respectively) and enhanced *src*FK-dependent tyrosine phosphorylation of multiple target proteins. Hypoxia caused a complex, time-dependent enhancement of MYPT-1 and MLC_{20} phosphorylation, both in the absence and presence of pre-constriction. The sustained component of this enhancement was blocked by SU6656 and the Rho-kinase inhibitor Y27632. In PASMCs, hypoxia caused translocation of Rho-kinase from the nucleus to the cytoplasm, and this was prevented by anti-*src* siRNA and to a lesser extent by anti-*fyn* siRNA. The biphasic increases in $[Ca^{2+}]_i$ that accompany HPV were also inhibited by PP2.

Conclusion Hypoxia activates *src*FKs and triggers protein tyrosine phosphorylation in IPA. Hypoxia-mediated Rho-kinase activation, Ca^{2+} sensitization, and $[Ca^{2+}]_i$ responses are depressed by *src*FK inhibitors and/or siRNA knockdown, suggesting a central role of *src*FKs in HPV.

1. Introduction

Hypoxic pulmonary vasoconstriction (HPV) is the acute localized adaptive process whereby blood flow is directed away from poorly ventilated areas of the lung in order to maximize ventilation–perfusion matching. In isolated pulmonary arteries, HPV is typically biphasic, characterized by a large but transient first phase and a sustained and often gradually developing second phase.[1,2] The first phase is associated with a sizeable transient elevation in intracellular Ca^{2+} ($[Ca^{2+}]_i$), whereas during the second phase, there is a relatively small and rapidly stabilizing increase in $[Ca^{2+}]_i$,[2] the effect of which is greatly enhanced by a Rho-kinase-dependent Ca^{2+} sensitization pathway.[3]

src-family kinases (*src*FKs) are a group of closely related non-receptor tyrosine kinases, of which three (*src*, *fyn*, and *yes*) are highly expressed in pulmonary artery.[4] Although tyrosine kinases are essential components of cell growth and proliferation signalling, they have also been implicated in vascular smooth muscle contraction.[5,6] We have recently shown in rat pulmonary artery, as have others in porcine coronary artery, that agonist-induced Rho-kinase-mediated Ca^{2+} sensitization and force generation is dependent on *src*FKs.[4,7]

Ca^{2+} sensitization of the contractile apparatus occurs through inhibition of myosin light-chain phosphatase

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(MLCP), leading to increased phosphorylation of the 20 kDa myosin light-chain (MLC_{20}), independently of changes in $[Ca^{2+}]_i$.[8] MLCP inhibition occurs through phosphorylation of the myosin-binding subunit MYPT-1[9] via Rho-kinase.[10,11] We have shown that the second phase of HPV is selectively inhibited by the Rho-kinase blocker Y27632 but not by the PKC blocker Ro31-8220.[2,3] In addition, hypoxia enhances phosphorylation of MYPT-1 and MLC_{20} in cultured pulmonary artery smooth muscle cells (PASMCs).[12,13] Rho-kinase-mediated Ca^{2+} sensitization is also a key factor in the pathogenesis of chronic hypoxia-induced pulmonary hypertension.[14,15]

Two studies using broad-spectrum tyrosine kinase blockers (genistein and tyrphostin) have suggested the involvement of tyrosine kinases in HPV.[16,17] *src* and *fyn* are both activated by hypoxia in cardiac myocytes,[18] and *src* plays a crucial role in hypoxia-inducible factor (HIF-1) induction in vascular smooth muscle cells.[19] However, a specific role of *src*FKs in HPV and the contractile pathways affected remains to be determined. In the present study, therefore, we evaluated the role of *src*FKs in HPV of rat small distal pulmonary arteries and determined how this relates to the hypoxia-mediated Rho-kinase-dependent Ca^{2+} sensitization and $[Ca^{2+}]_i$ responses.

2. Methods

2.1 Animals, solutions, drugs, and chemicals

This study conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Male Wistar rats (200–250 g) were killed by lethal overdose of pentobarbital (ip), with approval from local Ethics Review Board. Lungs were excised, then first- to third-order branches of the intra-pulmonary artery (IPA) were dissected free of surrounding parenchyma and placed in physiological salt solution (PSS), containing in mM: NaCl 118; $NaHCO_3$ 24; KCl 4; $CaCl_2$ 1.8; $MgSO_4$ 1; NaH_2PO_4 0.43; and glucose 5.56. A normal pH of 7.4 was maintained by gassing samples with air containing 5% CO_2. For hypoxia, gassing in different test chambers was adjusted in each case to achieve a pO_2 of ~15 Torr. SU6656, PP2, PP3, and Y27632 were all obtained from Calbiochem (Merck Biosciences Nottingham, UK). Fura-PE3/AM was purchased from Sigma (Poole, UK). $PGF_{2\alpha}$ (tromethamine salt) was purchased from Biomol (Exeter, UK). All other reagents were obtained from Calbiochem, Sigma, Invitrogen (Paisley, UK), or Fisher (Loughborough, UK).

2.2 Measurement of force and intracellular Ca^{2+}

Intra-pulmonary arteries (second- to third-order; 150–400 μm diameter) were mounted on a Mulvany–Halpern wire myograph (DMT A/S, Aarhus, Denmark), bathed in PSS at 37°C, pH 7.4, and stretched to give a wall tension equivalent to a transmural pressure of 30 mmHg.[1] All tension experiments were preceded by equilibration with three 3 min contractions to 80 mM K^+-substituted PSS (K^+PSS). In order to elicit a full contractile response to hypoxia, IPAs were pre-constricted with a concentration of $PGF_{2\alpha}$ necessary to produce tension equivalent to 10–15% of that produced by 80 mM K^+PSS (typically 5 μM), and then made hypoxic for 40 min. Two HPV responses were elicited in each artery; the second of which in treated arteries was preceded by a 10 min pre-incubation with inhibitor. In the presence of inhibitor, the concentration of $PGF_{2\alpha}$ required to achieve a 10–15% pre-constriction was adjusted accordingly.[20]

Isolated IPAs were mounted either on a modified Cambustion AM-10 myograph (Cambustion Ltd, Cambridge, UK) or on a confocal wire myograph (Danish Myo Technology). After stretching and equilibration as described above, IPAs were incubated for 1 h at 37°C in PSS containing 4 μM Fura PE-3/AM, and for a further 30 min to allow for de-esterification. Myograph modules were mounted on an inverted microscope (Nikon Diaphot, Nikon UK Ltd, Kingston-upon-Thames, UK) with a ×10 or ×20 Fluor objective combined with a double-excitation microfluorimeter (CairnResearch Ltd, Faversham, UK). Tension was recorded simultaneously with light emitted by the whole artery at >510 nm at excitation wavelengths 340 and 380 nm. The ratio of the emission intensities ($R_{340/380}$) was taken as a measure of $[Ca^{2+}]_i$.

2.3 Western blot

Intra-pulmonary artery samples (half main IPA plus 2–3 second-order side-branches per sample) were subjected to a 15 min equilibration period in normoxic PSS at 37°C, followed by a 10 min pre-incubation with pharmacological agents where appropriate. IPAs were then made hypoxic for 1, 2 5, 10, or 30 min and snap frozen in liquid N_2, prior to homogenization. Protein was extracted in 50 μL of Tris–SDS sample buffer containing phosphatase and protease inhibitor cocktails (Sigma). Protein extracts (12–15 μL per lane) were run on SDS–PAGE gels (4–12% gradient, Invitrogen), transferred to nitrocellulose membrane and blocked with 5% skimmed milk for 1 h at room temperature. Membranes were probed with primary antibody overnight at 4°C (1:500–1:5000 dilution where appropriate) in Tris-buffered saline containing 0.05% Tween 20 and 1% skimmed milk, followed by horseradish-peroxidase-conjugated anti-IgG secondary antibody for 1 h at room temperature (1:5000 dilution with 1% milk). Protein bands were visualized with chemiluminescence reagent (Pierce, Cramlington, UK, or Amersham, Bucks., UK) and then exposed to photographic film. Membranes were first probed with anti-phospho-antibodies, stripped for 1 h (Pierce stripping buffer), re-blocked, and re-probed with corresponding anti-total antibodies. Band intensity was expressed as a ratio of phospho/total for each protein, and values from each treated sample were normalized to those from control (untreated) samples run on the same gel. Two to three control samples were run on each gel and averaged. Antibodies were obtained from Cell Signalling, Upstate (UK), Santa Cruz Biotechnology (CA, USA), or Sigma.

2.4 siRNA design and transfection

siRNAs were designed as described previously.[21,22] The 19 nucleotide target sequences (*src*-siRNA: position 614–632, GenBank accession no. AF157016; and *fyn*-siRNA: position 1096–1114, GenBank accession no. U35365) were synthesized into 64–65 mer oligonucleotides with *Bam*HI/*Hin*dIII overhangs (Eurofins MWG Operon) and cloned into the expression vector pSilencer 3.0-H1 (Ambion Inc.). All clones were purified using an EndoFree Plasmid Maxi Kit (Qiagen Ltd) and sequenced (Geneservice Ltd) (see Supplementary material online for more details).

Pulmonary artery smooth muscle cells were dispersed enzymatically and grown in DMEM with 10% FCS to passage 3 or 4. Cells were plated on 13 mm coverslips and then growth arrested for 24 h. Identification of cells as smooth muscle was verified by positive staining with anti-smooth muscle α-actin and anti-calponin antibodies (as described previously[4]). Cells were transfected using the Basic Nucleofector® Kit for primary smooth muscle cells and a nucleofector device (Amaxa Biosystems). After 72 h, the transfection efficiency was >90%, determined using pmaxGFP (green fluorescent protein expressing vector) provided in the kit and confirmed by fluorescence microscopy. Efficiency and selectivity of knockdown was confirmed by western blot (see Supplementary material online).

2.5 Rho-kinase translocation

Pulmonary artery smooth muscle cells were treated in DMEM at 37°C under either normoxic or hypoxic conditions for 40 min. Reactions were terminated by addition of paraformaldehyde fixative. Fixed cells were permeabilized with Triton-100 and stained overnight

with anti-ROCK-2 primary antibody (1:100) at 4°C following with incubation with Alexa Fluor 488-labelled secondary antibody (1:300) for 2 h at room temperature. Specificity of anti-ROCK-2 was confirmed by western blot (single band at 160 kDa), and staining was negative with secondary antibody alone (as described previously[4]).

2.6 Data analysis and statistics

Western blot images were quantified by ImageJ software (rsb.info.nih.gov). Stained cells were photographed and analysed with Zeiss Axiovert-200 microscope with CARV II confocal imager (BD Biosciences) and MetaMorph software (Molecular Devices). Statistical analysis was performed with SigmaStat (Systat Software Inc., San Jose, CA, USA). Time-dependent effects of hypoxia on tension or $[Ca^{2+}]_i$ or of hypoxia and/or $PGF_{2\alpha}$ on phosphorylation were examined by two-way repeated measures ANOVA with appropriate *post hoc* tests. Comparisons of the effects of different drugs against hypoxia or $PGF_{2\alpha}$ were performed by one-way ANOVA with Holm–Sidak *post hoc* tests or by Kruskal–Wallis one-way ANOVA on ranks, where appropriate.

3. Results

3.1 *src*FK inhibitors block hypoxic pulmonary vasoconstriction

Hypoxic pulmonary vasoconstriction was induced by pre-constricting IPA with a low concentration of $PGF_{2\alpha}$ (typically 5–10 μM) then making the chamber hypoxic for 40 min. Hypoxia induced a biphasic contractile response comprising a rapid transient phase followed by a slower sustained phase which was fully reversed upon re-oxygenation, as described previously.[1–3] Two hypoxic exposures were performed in each artery. Drugs were applied during the second exposure and their effects were compared with the second exposure in untreated time controls. In time control experiments, the second HPV response was consistently larger than the first ($n = 16$, data not shown). HPV was inhibited by pre-incubation with the *src*FK inhibitors SU6656[23] and PP2[24,25] (*Figure 1A* and *B*). We used concentrations of SU6656 and PP2 previously shown to be required for inhibition of *src*FK auto-phosphorylation and protein tyrosine phosphorylation in IPAs.[4] The sustained phase was diminished by both

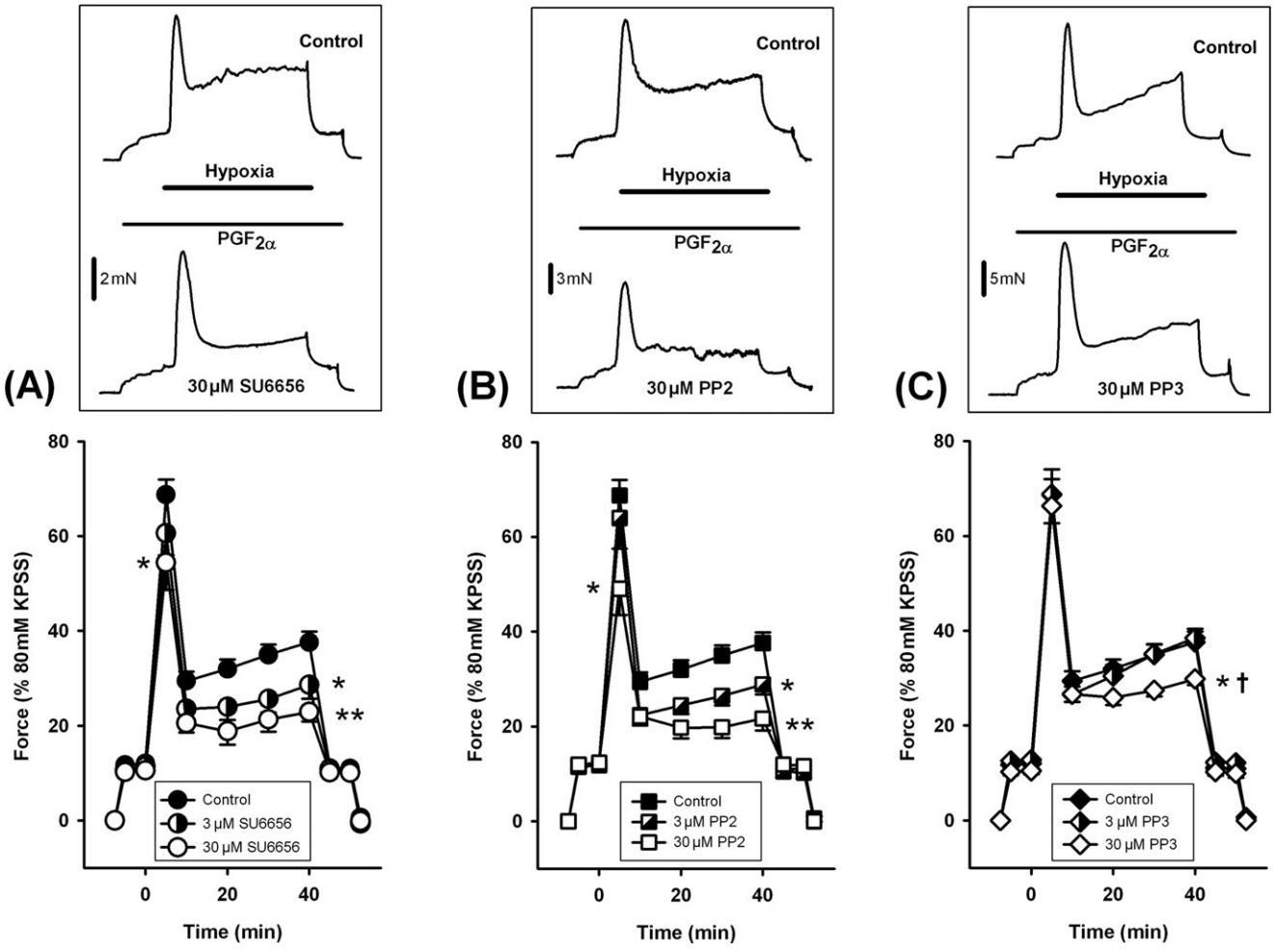

Figure 1 Effects of *src*-family kinase (*src*FK) inhibitors on hypoxic pulmonary vasoconstriction in intra-pulmonary arteries. Intra-pulmonary arteries were pre-constricted with $PGF_{2\alpha}$ before being made hypoxic. Example traces (top panels) show the effects of hypoxia first in the absence (upper trace), and then in the presence (lower trace) of inhibitors. The sustained phase contraction was inhibited by the *src*FK inhibitors SU6656 [(*A*) 3 μM, 33 ± 10% block, $^*P < 0.05$, $n = 8$ arteries; 30 μM, 56 ± 6% block, $^{**}P < 0.001$, $n = 8$ arteries] and PP2 [(*B*) 3 μM, 37 ± 6% block, $^*P < 0.01$, $n = 10$ arteries; 30 μM, 64 ± 9% block, $^{**}P < 0.001$, $n = 10$ arteries]. The inactive analogue PP3 also partially inhibited the sustained phase at 30 μM [(*C*) 28 ± 6% block, $^*P < 0.05$, $n = 8$ arteries], but this effect was significantly less than for PP2 ($^\dagger P < 0.01$ vs. 30 μM PP2); PP3 had no effect at 3 μM. The transient phase was partially inhibited by SU6656 (30 μM, 23 ± 10% block, $^*P < 0.01$) and PP2 (30 μM, 35 ± 9% block, $^*P < 0.01$).

3 and 30 μM of each drug, whereas the transient phase was only partially blocked by 30 μM. PP3, the inactive analogue of PP2, had no effect at 3 μM and caused only a slight inhibition of the sustained phase at 30 μM (*Figure 1C*).

3.2 Hypoxia enhances srcFK auto-phosphorylation and PP2-sensitive protein tyrosine phosphorylation

To determine whether *src*FKs were activated by hypoxia or whether the effects of inhibitors reflected suppressed constitutive activity, we first examined *src*FK auto-phosphorylation (an indication of *src*FK activation[26]) and protein-tyrosine phosphorylation in response to hypoxia in IPAs. As shown previously,[4] three protein bands were visualized with anti-*src*FK antibodies: a doublet at 59/60 kDa and another at ~54 kDa, corresponding to the *src*-family members *src*, *fyn*, and *yes*, respectively. *Figure 2A* shows that hypoxia caused a significant and transient increase in *src*FK (tyr-416) phosphorylation at the 59/60 kDa band (upper panel) and 54 kDa band (lower panel). Several protein bands or collections of bands were visualized with anti-phospho-tyrosine in IPAs. Four of these, at ~120, ~95, ~75, and 65 kDa are shown in *Figure 2B*. Hypoxia enhanced immunoreactivity at three of these bands, and this enhancement was reversed by prior incubation with 30 μM PP2 or 30 μM SU6656.

3.3 Hypoxia enhances MYPT-1 and MLC_{20} phosphorylation

Since a small degree of pre-constriction is required for a full HPV response, we evaluated the effects of hypoxia on MYPT-1 and MLC_{20} phosphorylation in IPAs both in the presence and absence of $PGF_{2\alpha}$. Five micromolar $PGF_{2\alpha}$ was used throughout as this approximates the concentration required to achieve the correct level of pre-constriction. The effects of 5 μM $PGF_{2\alpha}$ itself were also evaluated. Five micromolar $PGF_{2\alpha}$ alone caused a small but sustained increase in phosphorylation at both sites (*Figure 3A* and *B*). As is also shown in *Figure 3A*, hypoxia in the presence of $PGF_{2\alpha}$ caused a large sustained enhancement of MYPT-1 phosphorylation (at thr-855, equivalent to thr-850 in human) that was substantially greater than that caused by $PGF_{2\alpha}$ alone. Hypoxia in the presence of $PGF_{2\alpha}$ also caused significant enhancement of MLC_{20} phosphorylation (at ser-19, target of myosin light-chain kinase) above that of $PGF_{2\alpha}$ alone. This response was biphasic, with a peak at 5 min and another increase towards 30 min (*Figure 3B*).

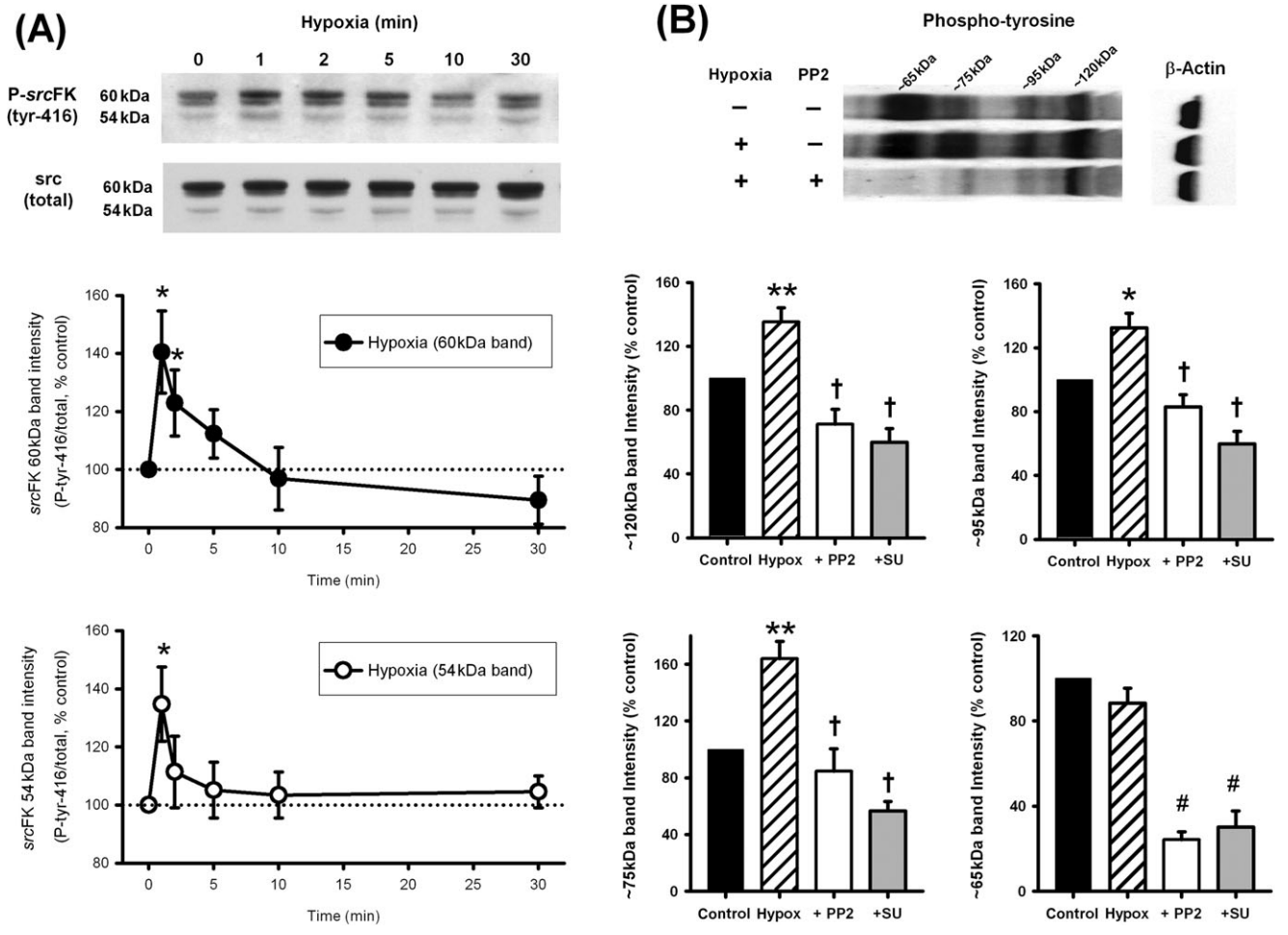

Figure 2 Effects of hypoxia on *src*-family kinase tyr-416 auto-phosphorylation and protein tyrosine phosphorylation in intra-pulmonary artery. (*A*) Hypoxia enhanced phospho-*src* (tyr-416) immunoreactivity at 60 and 54 kDa in a time-dependent manner ($^{*}P < 0.05$ vs. control, $n = 9$–11 rats). (*B*) Hypoxia (30 min) also enhanced phospho-tyrosine immunoreactivity at several protein bands ($^{*}P < 0.05$, $^{**}P < 0.01$ vs. control, $n = 20$ rats). At ~120, ~95, and ~75 kDa, this enhancement was reversed by either PP2 or SU6656 (30 μM, $^{\dagger}P < 0.001$ vs. hypoxia alone, $n = 8$–11 rats). At ~65 kDa, although not enhanced by hypoxia, basal immunoreactivity was also greatly inhibited by both inhibitors ($^{\#}P < 0.0001$ vs. control).

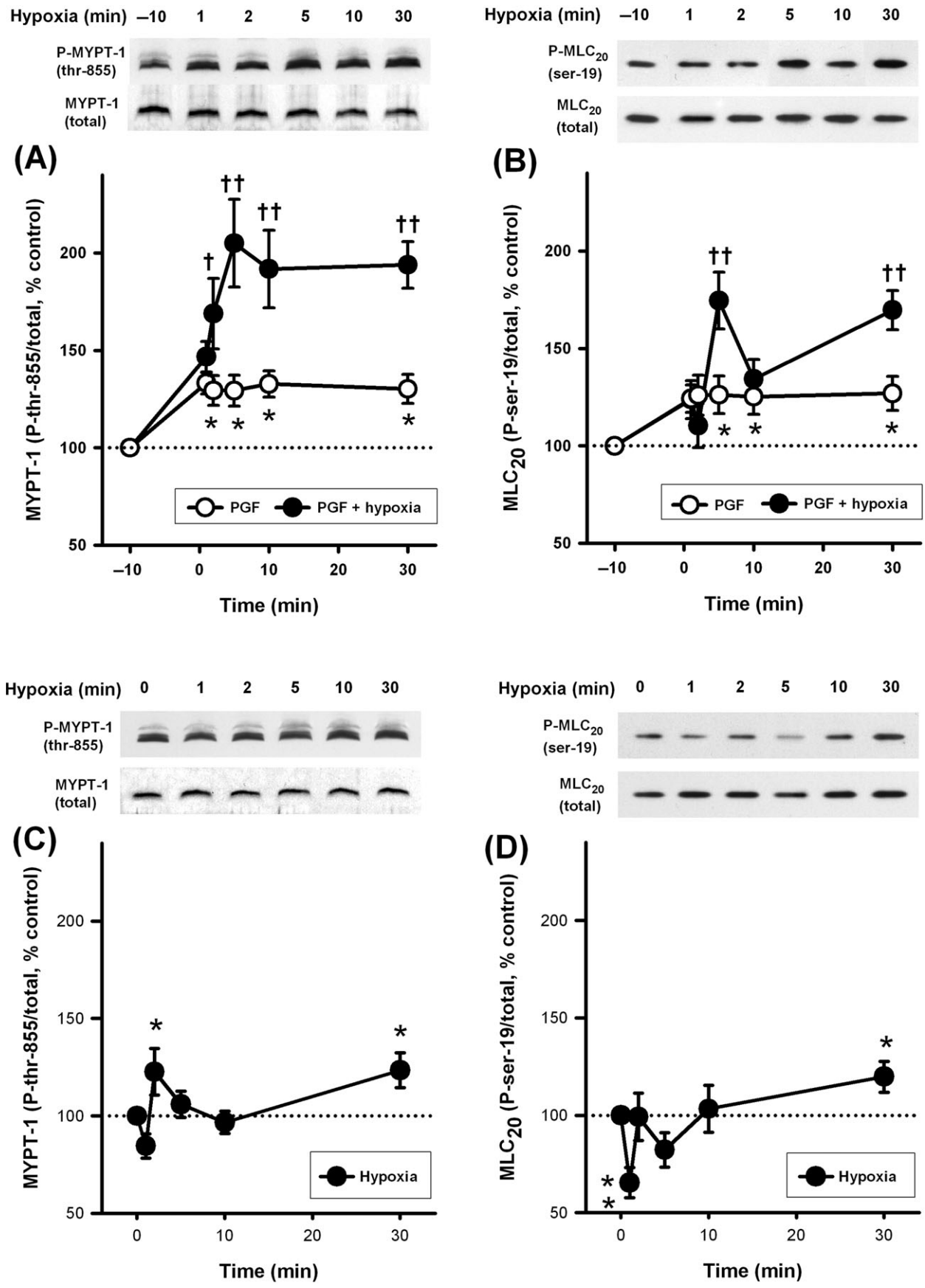

Figure 3 Effects of hypoxia and $PGF_{2\alpha}$ on phosphorylation of MYPT-1 (120–130 kDa) and MLC_{20} (18 kDa) in intra-pulmonary artery. (*A* and *B*) Phosphorylation at both sites was enhanced by 5 μM $PGF_{2\alpha}$ (open circles, $^{*}P < 0.05$ vs. control, $n = 12$–13 rats). In the continued presence of 5 μM $PGF_{2\alpha}$, hypoxia caused substantial further enhancement at both sites (filled circles, $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$ vs. $PGF_{2\alpha}$ alone, $n = 12$–17 rats). (*C* and *D*) In the absence of $PGF_{2\alpha}$, hypoxia caused a small sustained increase in phosphorylation ($^{*}P < 0.05$, $^{**}P < 0.01$ vs. control, $n = 12$–17 rats).

Hypoxia alone, however, produced a complex response in both MYPT-1 and MLC_{20} phosphorylation. This was characterized by a reduction at 1 min, a small peak at 2 min, another dip at 5–10 min, and a final small increase towards 30 min, which rose significantly above control levels for both phosphorylation sites (*Figure 3C* and *D*).

3.4 srcFK and Rho-kinase inhibitors block hypoxia-mediated MYPT-1 and MLC_{20} phosphorylation

In order to determine the contribution of Rho-kinase to phosphorylation of MYPT-1 and MLC_{20} during hypoxia and the possible upstream involvement of *src*FK, we performed additional 30 min exposures to hypoxia and 5 μM $PGF_{2\alpha}$, both together and individually, with the addition of the Rho-kinase inhibitor Y27632 and the *src*FK blocker SU6656. Both in the presence (*Figure 4A*) and absence (*Figure 4B*) of $PGF_{2\alpha}$, the hypoxia-induced enhancement of phosphorylation at both sites was inhibited by both SU6656 and Y27632. The small enhancement mediated by 5 μM $PGF_{2\alpha}$ was inhibited by Y27632 at both phosphorylation sites, but only the phosphorylation of MYPT-1 was significantly reduced by SU6656 (*Figure 4C*). We have previously shown that basal phosphorylation of MYPT-1 at thr-855 and of MLC_{20} at ser-19 is unaffected by SU6656 but that basal MYPT-1 phosphorylation at thr-855 is almost abolished by Y27632.[4] Inhibition of MYPT-1 phosphorylation at thr-855 by SU6656, therefore, suggests that *src*FK may be regulating the activity of Rho-kinase.

3.5 Hypoxia triggers srcFK-dependent translocation of Rho-kinase in pulmonary artery smooth muscle cells

In order to confirm that *src*FKs were acting upstream of Rho-kinase activation, we examined hypoxia-mediated translocation of Rho-kinase (ROCK-2) in PASMCs transfected with siRNAs directed against *src*, *fyn*, and a combination thereof, compared with sham-transfected cells. Basally, ROCK-2 was localized to the nucleus, and this was unaffected by either siRNA. Upon exposure to hypoxia, ROCK-2 translocated to the cytosol and/or cytoskeleton (*Figure 5A* and *D*). In cells transfected with *src*-siRNA and *fyn*-siRNA, however, translocation was completely and partially prevented, respectively (*Figure 5B–D*). Double transfection with both *src*- and *fyn*-siRNAs also abolished translocation (*Figure 5D*).

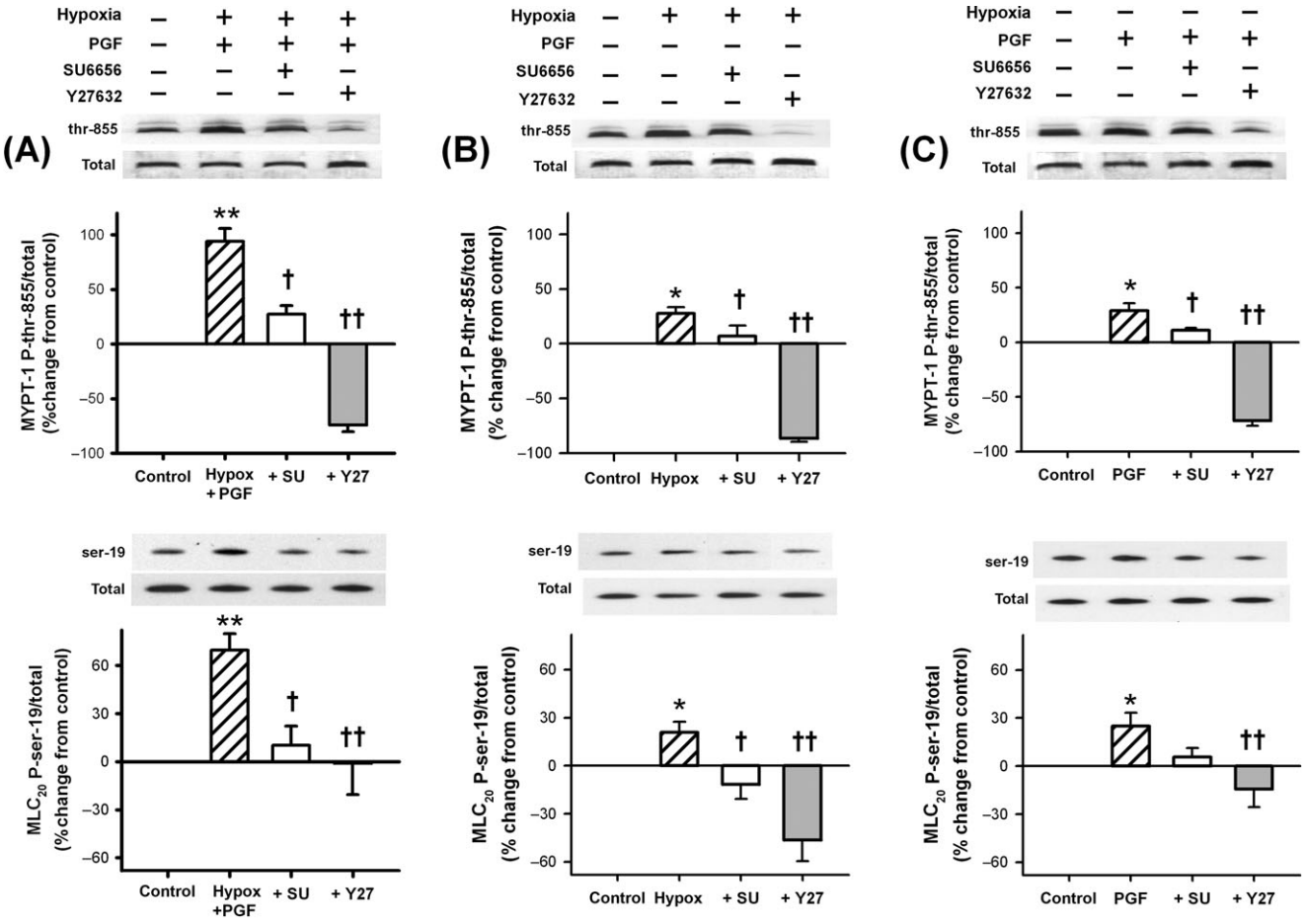

Figure 4 Effects of inhibition of *src*-family kinase and Rho-kinase on MYPT-1 (120–130 kDa) and MLC_{20} (18 kDa) phosphorylation. (*A*) In the presence of $PGF_{2\alpha}$, hypoxia greatly enhanced phosphorylation (**$P < 0.001$ vs. control, $n = 17$ rats for both sites), and this enhancement was reversed by SU6656 ($^{\dagger}P < 0.01$, $n = 10$ rats for both sites) and Y27632 ($^{\dagger\dagger}P < 0.001$, $n = 6$ rats for both sites). (*B*) In the absence of $PGF_{2\alpha}$, a smaller but still significant increase was apparent (*$P < 0.01$, $n = 17$ rats for both sites), and this increase too was inhibited by SU6656 ($^{\dagger}P < 0.01$, $n = 10$ rats for both sites) and Y27632 ($^{\dagger\dagger}P < 0.001$, $n = 6$ rats for both sites). (*C*) Five micromolar $PGF_{2\alpha}$ also enhanced phosphorylation (*$P < 0.01$, $n = 13$ rats for both sites). This increase was significantly reduced by both SU6656 [$^{\dagger}P < 0.01$ (MYPT-1 only), $n = 9$ rats for both sites] and Y27632 ($^{\dagger\dagger}P < 0.001$, $n = 6$ rats for both sites).

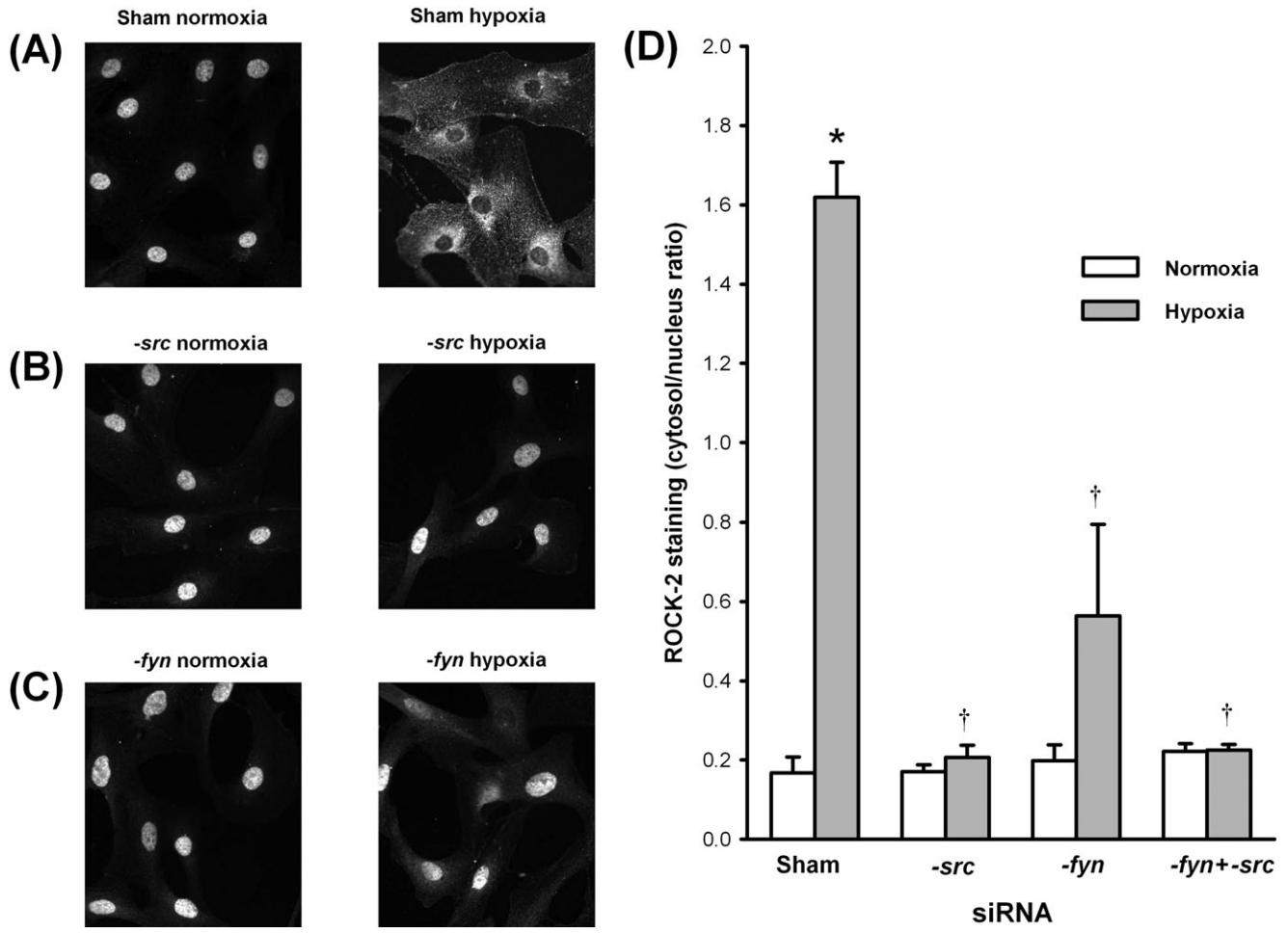

Figure 5 Hypoxia-mediated translocation of Rho-kinase in pulmonary artery smooth muscle cells: effects of *src* and *fyn* knockdown. (*A–C*) Examples of ROCK-2 staining in pulmonary artery smooth muscle cells either sham-transfected (*A*) or transfected with *src*-siRNA (*B*) or *fyn*-siRNA (*C*), under either normoxic (left panels) or hypoxic conditions (right panels). (*D*) Hypoxia-induced ROCK-2 translocation, as determined by the ratio of cytosol/nuclear staining intensity. $^{*}P < 0.01$ vs. normoxia; $^{\dagger}P < 0.01$ vs. sham-transfected. $n = 4$ experiments (in cells from four different rats).

3.6 Hypoxia-induced $[Ca^{2+}]_i$ response is PP2 sensitive

In addition to Ca^{2+} sensitization pathways, a rise in $[Ca^{2+}]_i$ is almost certainly also essential for a full HPV response.[20] In order to determine whether *src*FKs are also involved in changes in $[Ca^{2+}]_i$ during hypoxia, we examined the effects of the *src*FK inhibitor PP2 in Fura-PE3-loaded IPA. As shown in *Figure 6*, hypoxia induced a biphasic $[Ca^{2+}]_i$ response, comprising a large rapid transient phase followed by a small sustained phase, superimposed on the smaller $PGF_{2\alpha}$-mediated response. PP2 suppressed both the transient and sustained phases of this response. These experiments were not repeated with the other *src*FK blocker SU6656 because of the overlap of this drug's yellow colour with the emission spectra of Fura-PE3.

4. Discussion

This study provides the first direct evidence for a role or roles of *src*FKs in HPV and firm supportive evidence for the importance of Ca^{2+} sensitization in the sustained phase of the contraction. Our observations also forge a clear link between the actions of Y27632 on hypoxic force generation[3,12] and on hypoxia-mediated enhancement of Rho-kinase-dependent MYPT-1 and MLC_{20} phosphorylation. Our findings that hypoxia both activates *src*FK and enhances PP2-sensitive tyrosine phosphorylation of multiple protein targets, coupled with the inhibition of HPV by both SU6656 and PP2, are suggestive of an active role of these kinases in HPV. Hypoxia has been previously shown to activate *src* and the closely related kinase *fyn* in cardiac myocytes,[18] and to enhance *src*FK-specific tyrosine phosphorylation and activation of focal adhesion kinases $p125^{FAK}$ and paxillin.[27,28] $p125^{FAK}$ may be the ~120 kDa protein whose tyrosine phosphorylation was enhanced by hypoxia and inhibited by PP2 in the present study. HPV is believed to involve a change in the generation of reactive oxygen species (ROS) in the mitochondria.[29,30] Many protein kinases, including *src*FK, are activated by ROS,[31,32] indeed *src* appears to play an essential role in many of the intracellular pathways activated by hypoxia and ROS in the systemic vasculature.[19] Exactly how *src*FK activity may be induced by ROS, assuming ROS production is enhanced during hypoxia in IPAs, remains to be determined.

The time course of the phosphorylation responses to hypoxia was complex. In the absence of $PGF_{2\alpha}$, hypoxia caused a multi-phasic phosphorylation response ending in sustained enhancement at the maximum time point of 30 min. The initial drop at 1 min was large enough to become significant with regards to MLC_{20} at ser-19. A

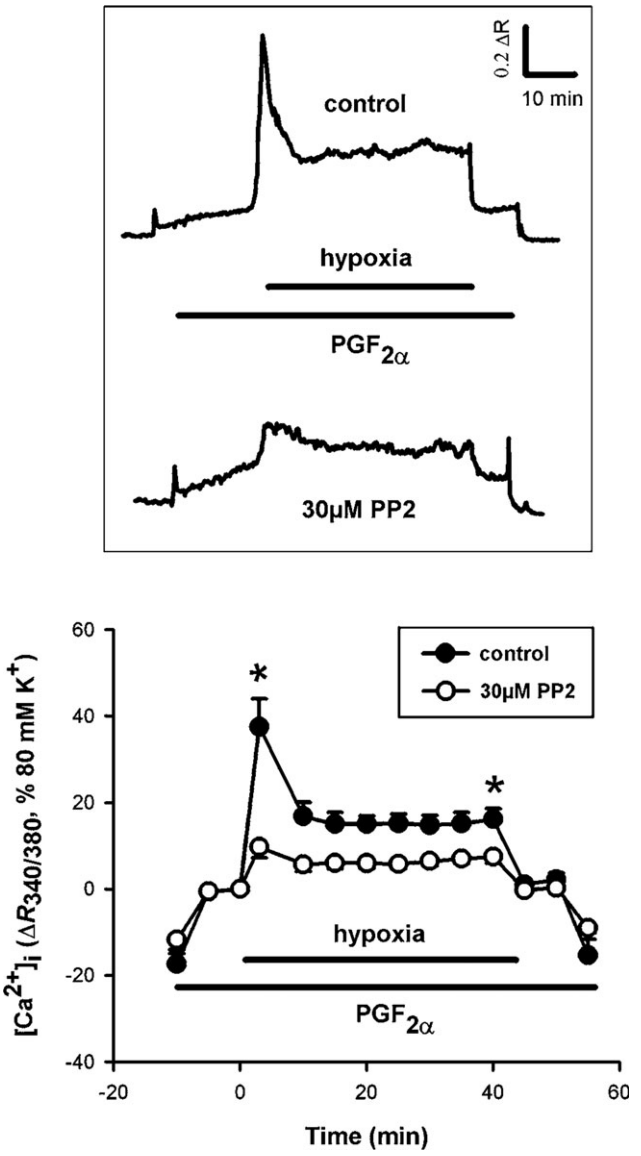

Figure 6 Effects of hypoxia and *src*FK inhibition on $[Ca^{2+}]_i$ in intra-pulmonary arteries (IPAs). Example traces (upper panel) of the ratio of light emitted at >510 nm from excitation at 340 nm over that at 380 nm ($R_{340/380}$) against time, and average effects with time (lower panel): 30 μM PP2 inhibited both the first phase (taken at 5 min; 58 ± 6% block, $^*P < 0.01$ vs. control, $n = 7$ arteries) and the second phase (taken at 40 min; 43 ± 7% block, $^*P < 0.01$ vs. control, $n = 7$ arteries). Note: the level of $[Ca^{2+}]_i$ immediately before IPAs were made hypoxic was set to zero in order to distinguish the effect of PP2 on the underlying $PGF_{2\alpha}$-induced response from that of hypoxia *per se*.

similar drop (although not significant) was also apparent with MLC_{20} in the presence of $PGF_{2\alpha}$, and indeed, in isolated IPAs, we sometimes observe a small very transient relaxation immediately after inducing hypoxia that precedes the first-phase contraction (unpublished observations). The causes of these fluctuations are unknown. In the presence of $PGF_{2\alpha}$, hypoxia caused a large sustained increase in MYPT-1 phosphorylation. This, however, was not exactly mirrored by MLC_{20} phosphorylation, the time course of which resembled the time course of contraction, with a distinct transient first phase at 5 min followed by a sustained phase of up to 30 min. This is not surprising since the first phase of HPV is clearly associated with a large transient increase in $[Ca^{2+}]_i$, whereas the sustained phase of HPV is primarily associated with a Ca^{2+} sensitization pathway, as shown previously.[2,3,20]

Prostanoids cause vascular smooth muscle contraction in part via Rho-kinase-mediated phosphorylation of MYPT-1.[33] In addition, we have recently demonstrated a direct involvement of *src*FK in $PGF_{2\alpha}$-mediated Ca^{2+} sensitization and contraction in rat IPAs, in which we showed that *src*FKs were activated by $PGF_{2\alpha}$ and provided evidence that *src*FKs were upstream mediators of Rho-kinase translocation, and phosphorylation of MYPT-1 and MLC_{20}.[4] Similarly, in this study, Rho-kinase-mediated MYPT-1 phosphorylation was inhibited by SU6656, and hypoxia-induced translocation of Rho-kinase in PASMCs was completely prevented by siRNA knockdown of *src* and strongly inhibited by knockdown of *fyn*. Therefore, *src*FKs are clearly involved in the upstream regulation of hypoxia-mediated Rho-kinase activity.

Since both hypoxia and $PGF^4_{2\alpha}$ activate *src*FKs and enhance MYPT-1 and MLC_{20} phosphorylation, we confirmed that the action of hypoxia on this phosphorylation or of *src*FK blockers on HPV was independent of any action on the underlying pre-constriction. Hypoxia caused enhancement of Rho-kinase-mediated phosphorylation of MYPT-1 in the absence as well in the presence of $PGF_{2\alpha}$, and this enhancement was sensitive to an inhibitor of *src*FKs (*Figure 4B*). However, the relative effectiveness of hypoxia in the presence of $PGF_{2\alpha}$ at enhancing phosphorylation (MYPT-1 at 30 min: 94%; and MLC_{20} at 30 min: 70%) was apparently greater than the sum of the individual effects of hypoxia alone and $PGF_{2\alpha}$ alone (MYPT-1 at 30 min: 28 + 29 = 57%; and MLC_{20} at 30 min: 21 + 25 = 46%), suggesting a synergistic action of the two stimuli. This is mirrored by force generation, where hypoxia in the absence of pre-constriction produced very small contractions of only 3–4% the size of that caused by 80 mM K^+PSS,[1] and yet in the presence of 5 μM $PGF_{2\alpha}$ which produces contractions of 10–15% of K^+PSS, hypoxia caused additional sustained contractions of up to 30% of K^+PSS. Thus, stimulation of *src*FKs by both $PGF_{2\alpha}$ and hypoxia may explain at least part of the enhancement of HPV seen in the presence of this agonist.

In addition to an involvement in Ca^{2+} sensitization, we have also found that the *src*FK inhibitor PP2 blocks the hypoxia-mediated $[Ca^{2+}]_i$ response in IPAs. It is well established that the net phosphorylation status of MLC_{20} at ser-19 involves a balance between Ca^{2+}-calmodulin-dependent phosphorylation by MLCK and constitutive dephosphorylation by MLCP.[8] Concomitant phosphorylation of MYPT-1 by Rho-kinase together with even a small elevation of $[Ca^{2+}]_i$ will, therefore, greatly enhance the phosphorylation of MLC_{20}. Inhibition of either MYPT-1 phosphorylation or $[Ca^{2+}]_i$-mediated MLCK activation, or both will thus inhibit force generation. In addition to triggering Rho-kinase-mediated Ca^{2+} sensitization, both hypoxia and $PGF_{2\alpha}$ cause an elevation of $[Ca^{2+}]_i$, which in both cases is comprised of transient and sustained components.[20,34–36] In our hands, the transient component of the hypoxic $[Ca^{2+}]_i$ response is partly due to voltage-dependent Ca^{2+} influx and partly due to capacitative Ca^{2+}-influx triggered by release from thapsigargin-sensitive stores, whereas the sustained component is probably due to influx through another as yet uncharacterized voltage-independent pathway.[20] Other studies propound the importance of influx through voltage-gated Ca^{2+} channels.[37,38] In any case, *src*FKs have been implicated in both voltage-

dependent and -independent mechanisms of Ca^{2+} mobilization as triggered by either agonists or ROS.[39,40]

In summary, our results suggest a direct role of *src*FKs in the acute contractile response to hypoxia in small pulmonary arteries of rat. Since *src*FK inhibitors block both hypoxia-mediated Rho-kinase-dependent MYPT-1 phosphorylation and the hypoxic $[Ca^{2+}]_i$ response in IPAs, we may speculate that *src*FKs may act upstream of both key pathways to MLC_{20} phosphorylation and contraction. The specific phosphorylation targets of *src*FKs as activated by hypoxia, whether they be guanine exchange factors[41] or guanine dissociation inhibitors[42] leading to RhoA/Rho-kinase activation, or L-type Ca^{2+} channels[40] or phospholipase-Cγ,[43] resulting in $[Ca^{2+}]_i$ mobilization, remain to be determined.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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