

## RESEARCH PAPER

# Mice lacking the Raf-1 kinase inhibitor protein exhibit exaggerated hypoxia-induced pulmonary hypertension

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## BACKGROUND AND PURPOSE

Increased pulmonary vascular remodelling, pulmonary arterial pressure and pulmonary vascular resistance characterize the development of pulmonary arterial hypertension (PAH). Activation of the Raf/mitogen-activated protein kinase/extracellular signal-regulated kinase (ERK)1/2 is thought to play an important role in PAH and Raf-1 kinase inhibitor protein (RKIP), negatively regulates this pathway. This study investigated whether genetic deletion of RKIP (and hence ERK1/2 up-regulation) resulted in a pulmonary hypertensive phenotype in mice and investigated a role for RKIP in mitogen-regulated proliferative responses in lung fibroblasts.

## EXPERIMENTAL APPROACH

Pulmonary vascular haemodynamics and remodelling were assessed in mice genetically deficient in RKIP (RKIP<sup>-/-</sup>) after 2 weeks of either normoxia or hypoxia. Immunoblotting and immunohistochemistry were used to examine phosphorylation of Raf-1, RKIP and ERK1/2 in mouse pulmonary arteries. *In vitro*, RKIP inhibition of mitogen signalling was analysed in CCL39 hamster lung fibroblasts.

## KEY RESULTS

RKIP<sup>-/-</sup> mice demonstrated elevated indices of PAH and ERK1/2 phosphorylation compared with wild-type (WT) mice. Hypoxic RKIP<sup>-/-</sup> mice exhibited exaggerated PAH indices. Hypoxia increased phosphorylation of Raf-1, RKIP and ERK1/2 in WT mouse pulmonary arteries and Raf-1 phosphorylation in RKIP<sup>-/-</sup> mouse pulmonary arteries. In CCL39 cells, inhibition of RKIP potentiated mitogen-induced proliferation and phosphorylation of RKIP, and Raf-1.

## CONCLUSIONS AND IMPLICATIONS

The lack of RKIP protein resulted in a pulmonary hypertensive phenotype, exaggerated in hypoxia. Hypoxia induced phosphorylation of RKIP signalling elements in WT pulmonary arteries. RKIP inhibition potentiated mitogen-induced proliferation in lung fibroblasts. These results provide evidence for the involvement of RKIP in suppressing the development of hypoxia-induced PAH in mice.

## Abbreviations

ALDA, aldehyde dehydrogenase; BMP, bone morphogenetic protein; BMPR2, bone morphogenetic protein receptor type II; ERK1/2, extracellular signal-regulated kinase; GSTO1-1, glutathione S-transferase omega 1-1; LV + S, left ventricle plus septum; MEK, mitogen activated protein kinase/ERK kinase; PAH, pulmonary arterial hypertension; PASMCs, pulmonary artery smooth muscle cells; PCNA, proliferating cell nuclear antigen; PDGF, platelet derived growth factor; PDGFR, platelet derived growth factor receptor; PKC $\zeta$ , atypical protein kinase C $\zeta$ ; Raf-1/MEK/ERK1/2, Raf-1/mitogen-activated protein kinase (MAPK) kinase 1/2 (MEK1/2)/extracellular signal-regulated kinase 1/2; RKIP, Raf-1 kinase inhibitor protein; RV, right ventricle; RVH, right ventricular hypertrophy; SERT, 5-HT transporter; sRVP, systolic right ventricular pressure; WT, wild-type

## Introduction

Pulmonary arterial hypertension (PAH) is a lethal disease characterized by increased pulmonary arterial pressure, increased pulmonary vascular resistance and pulmonary vascular remodelling leading to right ventricular failure and death (Chin and Rubin, 2008). Mutations in the gene-encoding bone morphogenetic protein (BMP) receptor type II (BMPR2) have been identified in about 70% of patients with the heritable form of PAH (Lane *et al.*, 2000). However, as only about 20% of individuals with a BMPR2 mutation develop PAH, additional genetic and environmental factors likely contribute to the development of PAH. For example, numerous studies have implicated *de novo* synthesized 5-HT (Eddahibi *et al.*, 2006; Morecroft *et al.*, 2007), the 5-HT transporter (SERT) (MacLean *et al.*, 2004; Morecroft *et al.*, 2010) and the 5-HT<sub>1B</sub> receptor (Keegan *et al.*, 2001) in the pathobiology of PAH and platelet derived growth factor receptor (PDGFR) signalling-associated cellular proliferation has been suggested as an important contributor to the development and progression of PAH (Perros *et al.*, 2008).

Activation of the Raf-1/mitogen-activated protein kinase (MAPK) kinase 1/2 (MEK1/2)/extracellular signal-regulated kinase 1/2 (ERK1/2) (Raf-1/MEK/ERK1/2) signal transduction pathway plays an important role in the induction of pulmonary artery smooth muscle cell (PASMC) proliferation by numerous mitogens including 5-HT and platelet derived growth factor (PDGF) (Lee *et al.*, 1999; Liu *et al.*, 2004; Hansmann *et al.*, 2008). Furthermore, this pathway is dysregulated in PAH. For example, unopposed ERK1/2 signalling, coupled with defective Smad signalling, is associated with the abnormal pulmonary cell proliferation observed in hereditary PAH (Yang *et al.*, 2005; 2008). Hypoxia activates ERK1/2, MAPK in rat (Murray *et al.*, 2003) and mouse pulmonary arteries (Yu *et al.*, 2006) and human PASMCs (Murray *et al.*, 2003) and hypoxia-induced proliferation of bovine pulmonary arterial fibroblasts is dependent on phosphorylation of ERK1/2 (Das *et al.*, 2001). Sorafenib, an inhibitor of serine/threonine kinases, including Raf-1, prevents pulmonary vascular remodelling, elevated right ventricular pressure (RVP) and improves right ventricular hypertrophy (RVH) in monocrotaline- and chronic hypoxia-induced pulmonary hypertension partially via inhibition of the downstream ERK1/2 signalling pathway activation (Klein *et al.*, 2008; Moreno-Vinasco *et al.*, 2008).

The novel metastasis suppressor, Raf-1 kinase inhibitor protein (RKIP), is a highly conserved and widely expressed protein which negatively regulates the Raf-1/MEK/ERK1/2 pathway by interfering with Raf-1-mediated phosphorylation of MEK1/2 (Yeung *et al.*, 1999) by disrupting the interaction between the two kinases (Yeung *et al.*, 2000). Pathophysiologically, RKIP inactivation is associated with various human diseases, including cancer and although RKIP has been extensively studied as a metastasis suppressor gene, PAH and cancer pathology share several MAPK, as well as growth factor, signalling pathways that lead to proliferative phenotypes. RKIP has also been implicated as a negative regulator of the NF- $\kappa$ B pathway (Tang *et al.*, 2010), an important inflammatory transcription factor. Enhanced NF- $\kappa$ B activity has been observed in patients with idiopathic PAH (Kimura *et al.*, 2009) and nuclear expression of an active subunit of NF- $\kappa$ B is

associated with the development of PAH in rats (Sawada *et al.*, 2007).

However, no previous study has examined the possible role of RKIP in the development of PAH. As the Raf-1/MEK/ERK1/2 pathway plays an important role in proliferation and PAH, we hypothesized that its endogenous inhibitor RKIP would be important in the development of PAH. Here, we have tested this hypothesis using mice deficient in RKIP through genetic deletion (Theroux *et al.*, 2007). 5-HT and PDGF are important contributors in the development and progression of PAH (MacLean *et al.*, 2004; Morecroft *et al.*, 2007; Perros *et al.*, 2008) and important pulmonary vascular mitogens (Lee *et al.*, 1999; Liu *et al.*, 2004; Hansmann *et al.*, 2008). As 5-HT and PDGF-induced proliferation is dependent on activation of the Raf-1/MEK/ERK1/2 pathway, we wished to assess whether 5-HT and PDGF-induced signalling is influenced by RKIP. Also, the atypical protein kinase C $\zeta$  (PKC $\zeta$ ) regulates activation of Raf-1 signalling through RKIP inactivation by phosphorylation of RKIP at serine-153 by protein kinase C (Corbit *et al.*, 2003). As hypoxia induces PKC $\zeta$  activation in pulmonary arterial fibroblasts (Das *et al.*, 2001; Short *et al.*, 2005), we investigated the effects of hypoxia, 5-HT and PDGF on PKC $\zeta$  activation in pulmonary fibroblasts. We found that mice deficient in RKIP had elevated indices of PAH which were further exaggerated by chronic hypoxia. Furthermore, RKIP inhibition potentiates mitogen-induced signalling in pulmonary fibroblasts, collectively suggesting an important role for RKIP in the development of PAH.

## Methods

### In vivo experiments

All animal care and experimental procedures were in accordance with the UK Animals (Scientific Procedures) Act 1986 and conformed to institutional regulations at the University of Glasgow. The generation and characterization of the RKIP knockout (RKIP<sup>-/-</sup>) mice have been described previously (Theroux *et al.*, 2007). The mice were maintained on a C57BL6 background. All mice were bred in the University of Glasgow and the genotype of each mouse was confirmed by PCR. The PCR primers were:

- (1a) RKIP Forward CTG ACT GGC TGG CTG GTA CT
- (1b) RKIP Reverse TCT GGA GGA AGA AAC GAC AG  
(Theroux *et al.*, 2007)

### Exposure to hypoxia

Female wild-type (WT) control mice (C57BL6 strain) or RKIP<sup>-/-</sup> (3–4 months old; *n* = 8–10 mice per group) were maintained in normoxic conditions or hypobaric/hypoxic conditions for 14 days as previously described (MacLean *et al.*, 2004; Morecroft *et al.*, 2007). The hypobaric chamber was depressurized over the course of 2 days to 550 mbar (equivalent to 10% O<sub>2</sub>). Temperature was maintained at 21°C to 22°C, and the chamber was ventilated with air at 45 L·min<sup>-1</sup>.

### Characterization of PAH

**Pressure measurements.** Under isoflurane (1.5% in O<sub>2</sub>) anaesthesia, systolic right ventricular pressure (sRVP) was measured

via a 25-gauge needle advanced into the right ventricle *trans*-diaphragmatically (Keegan *et al.*, 2001; MacLean *et al.*, 2004) RVP and heart rate (derived from the RVP) were recorded on a data acquisition system (MP 100, Biopac Systems). Systemic arterial pressure was recorded via a cannula placed in the carotid artery. Depth of anaesthesia was confirmed by lack of a pinch withdrawal reflex applied to the hind paw.

### Indices of pulmonary hypertension

The ratio of right ventricular weight to left ventricular weight plus septum [RV/(LV + S)] was used as an index of RVH (MacLean *et al.*, 2004; Morecroft *et al.*, 2007). Sagittal sections were obtained from left lungs, stained with Elastica Van Gieson stain and microscopically assessed for muscularization of pulmonary arteries (<80 µm external diameter) as described previously (MacLean *et al.*, 2004; Morecroft *et al.*, 2007). Briefly, sections were microscopically assessed for muscularization of small pulmonary arteries (<80 µm external diameter) associated with an airway distal to the respiratory bronchiole. Arteries were considered muscularized if they possessed a distinct double-elastic lamina visible for at least half the diameter of the vessel in cross-section. The percentage of vessels containing double-elastic lamina (and hence deemed remodelled) was calculated as the number of muscularized vessels/total number of vessels counted per section × 100. Two to three sections from each left lung were assessed for every mouse. Lung sections from four to six mice from each group were studied.

### Immunohistochemistry

To determine cell proliferation in the pulmonary arteries, lungs sections were stained for proliferating cell nuclear antigen (PCNA), an antigen that is expressed in cell nuclei during the DNA synthesis phase of the cell cycle (Hall *et al.*, 1990). Immunohistochemistry was performed in each group to identify any changes in expression of PCNA, phosphorylated RKIP (p-RKIP) and phosphorylated Raf-1 (p-Raf-1), as described previously (Morecroft *et al.*, 2005) using rabbit polyclonal anti-PCNA, anti-p-RKIP or anti-p-Raf-1 antibodies diluted 1:200, 1:50 or 1:100, respectively, in PBS containing 0.5% bovine serum albumin, 15% normal goat serum. Peroxidase staining was carried out using 3',3'-diaminobenzidine tetrahydrochloride dihydrate and hydrogen peroxide with enhanced nickel staining. Immunohistochemistry image analysis was performed on images (×40 magnification) of lung tissue sections using Metamorph software (version 6.1, Molecular devices, Downingtown, PA, USA). The average pixel intensity of each image correlated to a greyscale range of 0 (black) to 255 (white), with intermediate intensities being assigned an appropriate grey level. The vessel wall of small pulmonary arteries within the lung sections was selected and specifically analysed. In order to determine PCNA, p-Raf-1 and RKIP immunoreactivity the colour threshold was set to detect pixel intensity between 0–137, 0–92 and 0–130 respectively. The percentage threshold area detected was then expressed as the percentage of PCNA, p-Raf-1 or p-RKIP immunoreactivity within the vessel wall. Three to four sections per experimental group were analysed and the results expressed as mean ± SE.

### Cell signalling and proliferation

We have shown that CCL39 Chinese hamster lung fibroblast cells exhibit a very similar pharmacological profile to mouse pulmonary arterial fibroblasts (Mair *et al.*, 2008). Mouse pulmonary arterial fibroblasts cannot be harvested in sufficient quantity for extensive study. The CCL39 cells were therefore used to assess the effects of RKIP inhibition on cell signalling and proliferation *in vitro* (Mair *et al.*, 2008). For proliferation studies, CCL39 cells were plated out in 24-well plates at a density of ~20 000 cells per well and grown in full medium (Dulbecco's modified Eagle's medium) (supplemented with 10% v/v fetal bovine serum, penicillin, streptomycin, 1 µg·mL<sup>-1</sup> fungizone and 50 µg·mL<sup>-1</sup> gentamycin) at 37°C for 24 h before serum starvation for 24 h. Cells were then serum starved for a further 24 h in either normoxic or hypoxic (5% O<sub>2</sub>) conditions. Hypoxic conditions were achieved by maintaining cells in a humidified, temperature controlled and nitrogen-supplemented, Galaxy R incubator (Wolf Laboratories, York, UK). This allowed control of internal O<sub>2</sub> levels at 5%, and CO<sub>2</sub> levels were maintained at 5%. This achieves, for the hypoxic conditions, a tissue culture supernatant PO<sub>2</sub> of 35 mmHg. Media was then replaced with fresh media lacking serum and containing the appropriate stimuli. CCL39 cells were stimulated with either 5-HT (1 µM) or PDGF (10 ng·mL<sup>-1</sup>) in the presence or absence of the RKIP inhibitor locostatin (5 µM). (Ma *et al.*, 2009) Serum, PDGF and 5-HT-stimulated (24 h) CCL39 cell proliferation was evaluated by cell counting using a haemocytometer and key proliferation experiments confirmed using a [<sup>3</sup>H]-thymidine incorporation assay. Assays were carried out in duplicate. In order to normalize data, cell count mean of the basal control wells of each assay, carried out in duplicate, was calculated and each well of the same assay normalized to this value. For the [<sup>3</sup>H]-thymidine assay, CCL39 cells were plated out into 24-well plates and grown in full media for 24 h before serum starving for a further 48 h. The media was then replaced with fresh serum-free media and the appropriate stimuli. After 18 h, [<sup>3</sup>H]-thymidine was added and proliferation stopped after a further 6 h. Cells were washed with phosphate buffered saline followed by 10% trichloroacetic acid and then sodium hydroxide (0.5 M) and by liquid scintillation counting. Stimulation indices were calculated as the fold increase from the basal rate of proliferation as determined by [<sup>3</sup>H]-thymidine incorporation.

For cell signalling studies, CCL39 cells were plated out in six-well plates and grown to confluence in full medium for 24 h before serum starvation for 24 h. Cells were then serum starved for a further 24 h in either normoxic or hypoxic (5% O<sub>2</sub>) conditions before stimulation with either 5-HT (1 µM) or PDGF (10 ng·mL<sup>-1</sup>) for 5 min in the presence/absence of the RKIP inhibitor locostatin (5 µM) (Ma *et al.*, 2009). In separate experiments, the effects of the selective 5-HT receptor antagonists ketanserin (5-HT<sub>2A</sub>; 100 nM), SB224289 (1'-methyl-5-[[2'-methyl-4'-(5-methyl-1,2,4-oxadiazol-3-yl)biphenyl-4-yl]carbonyl]-2,3,6,7-tetrahydro-spiro-[furo] 2, 3-f]indole-3, 4'-piperidine) (5-HT<sub>1B</sub>; 200 nM), and citalopram (SERT inhibitor; 10 µM) on 5-HT stimulation were examined. Cells were treated with antagonists/inhibitors 45 min prior to the addition of the relevant mitogen. Treated cells were then solubilized in detergent lysis buffer before fractionation by

gel electrophoresis on 4–12% (w/v) Nupage Novex Bis-Tris resolving gels for immunoblotting.

### Western blotting

The RKIP, prolylendopeptidase, aldehyde dehydrogenase (ALDH1A1) and glutathione S-transferase omega 1-1 (GSTO1-1) expression as well as phosphorylation of RKIP, ERK1/2, Raf-1 and PKC $\zeta$  were evaluated by Western blot analysis. Phosphorylation of Raf-1, RKIP and ERK1/2 was evaluated in normoxic and hypoxic WT mouse pulmonary arteries by Western blot analysis on equal of protein extracts resolved by fractionation by gel electrophoresis on 4–12% (w/v) Nupage Novex Bis-Tris resolving gels. Phosphorylation of ERK1/2 was also evaluated in normoxic RKIP (–/–) mouse lungs to confirm that genetic knockout of RKIP results in enhanced ERK1/2 signalling. Immunoblot images were scanned and quantified using the TotalLab (Nonlinear dynamics, Newcastle-Upon-Tyne, UK) software program and samples were adjusted for loading errors, using the tubulin loading control band densitometry to standardize, prior to protein phosphorylation being normalized relative to control (unstimulated) values.

### Data analysis and statistical methods

Data are presented as mean  $\pm$  SE. Individual comparisons were made using Student's unpaired *t*-test, as appropriate. Multiple comparisons were made using one-way ANOVA, followed by Neuman Keuls *post hoc* test. GraphPad Prism (version 4; La Jolla, CA, USA) was used to perform all statistical analyses and values of *P* < 0.05 were considered to be significant.

### Materials

Phospho-ERK1/2 (p-ERK1/2) (Thr202/Tyr204) antibody was from Cell Signalling Technology (Beverly, MA, USA), rabbit monoclonal RKIP, p-RKIP (S153), rabbit polyclonal Raf-1 (phospho S338), rabbit monoclonal PKC $\zeta$  (phospho T560), rabbit polyclonal ALDH1A1, prolylendopeptidase, mouse polyclonal GSTO1, rabbit polyclonal PCNA and mouse monoclonal tubulin antibodies were from Abcam (Cambridge, UK). Rabbit monoclonal p-RKIP (S153), rabbit polyclonal Raf-1 (phospho S338) antibodies for the immunohistochemistry were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). 5-HT hydrochloride and RKIP inhibitor locostatin [(S)-(+)-4-benzyl-3-crotonyl-2-oxazolidinone] was from Sigma-Aldrich (Poole, UK). PDGF was from Peprotech (London, UK) and ketanserin, SB224289 and citalopram were all from Tocris Bioscience (Bristol, UK). Penicillin, streptomycin, fungizone, gentamycin and Nupage Novex Bis-Tris gels were from Invitrogen Ltd (Paisley, UK).

## Results

### Effects of RKIP knockout on indices of PAH

To test the hypothesis that decreased RKIP may cause increased PAH and pulmonary vascular remodelling, we measured the sRVP in RKIP–/– and WT mice. Neither deletion of RKIP nor chronic hypoxia had any effect on systemic arterial pressure (Figure 1A) or on heart rate (WT, 410  $\pm$  26 bpm;

RKIP, 407  $\pm$  18 bpm for normoxia: WT, 400  $\pm$  17 bpm; RKIP, 403  $\pm$  23 bpm for hypoxia). Under normoxic conditions, RKIP–/– mice had elevated sRVP compared with WT mice (*P* < 0.05; Figure 1B). In RKIP–/– mice, 2 weeks of chronic hypoxia increased sRVP compared with normoxic controls (*P* < 0.01; Figure 1B), and this value was significantly higher than the sRVP in hypoxic WT mice (*P* < 0.05; Figure 1B). Pulmonary vascular remodelling was greater in RKIP–/– mice than in WT mice under normoxic conditions (*P* < 0.05; Figures 1C and 2A) and hypoxia resulted in even greater remodelling in the RKIP–/– mice (*P* < 0.001; Figures 1C and 2A). Remodelling in small pulmonary arteries of the mice studied is illustrated in Figure 2A. RVH was markedly higher in the RKIP–/– mice than in WT mice (*P* < 0.01; Figure 1D) and was further exaggerated by chronic hypoxia in both genotypes (Figure 1D). However, RVH was significantly greater in the hypoxia-exposed RKIP–/– mice than in their WT counterparts (*P* < 0.05; Figure 1D). LV + S weight was unaffected by either deletion of RKIP (WT, 90.4  $\pm$  4.7 mg; RKIP (–/–), 85.5  $\pm$  2.3 mg) or by chronic hypoxia (WT, 81.3  $\pm$  4.2 mg; RKIP (–/–), 85.0  $\pm$  3.6 mg). Body weight was also unaffected by either deletion of RKIP [WT, 20.5  $\pm$  0.7 g; RKIP (–/–), 21.5  $\pm$  0.5 g] or by chronic hypoxia [WT, 19.6  $\pm$  0.3 g; RKIP (–/–), 21.0  $\pm$  0.6 g]. Thus, the altered RV/(LV + S) was not a result of changes in LV + S size or body weight.

### Phosphorylation of ERK1/2 in RKIP (–/–) lungs

To test the hypothesis that the absence of RKIP in the RKIP (–/–) mice would enhance the phosphorylation of ERK1/2 we examined the expression of p-ERK1/2 in the lungs of WT and RKIP (–/–) mice using Western blotting. The RKIP (–/–) mice had significantly increased levels of p-ERK1/2 compared with WT counterparts (Figure 1E,F).

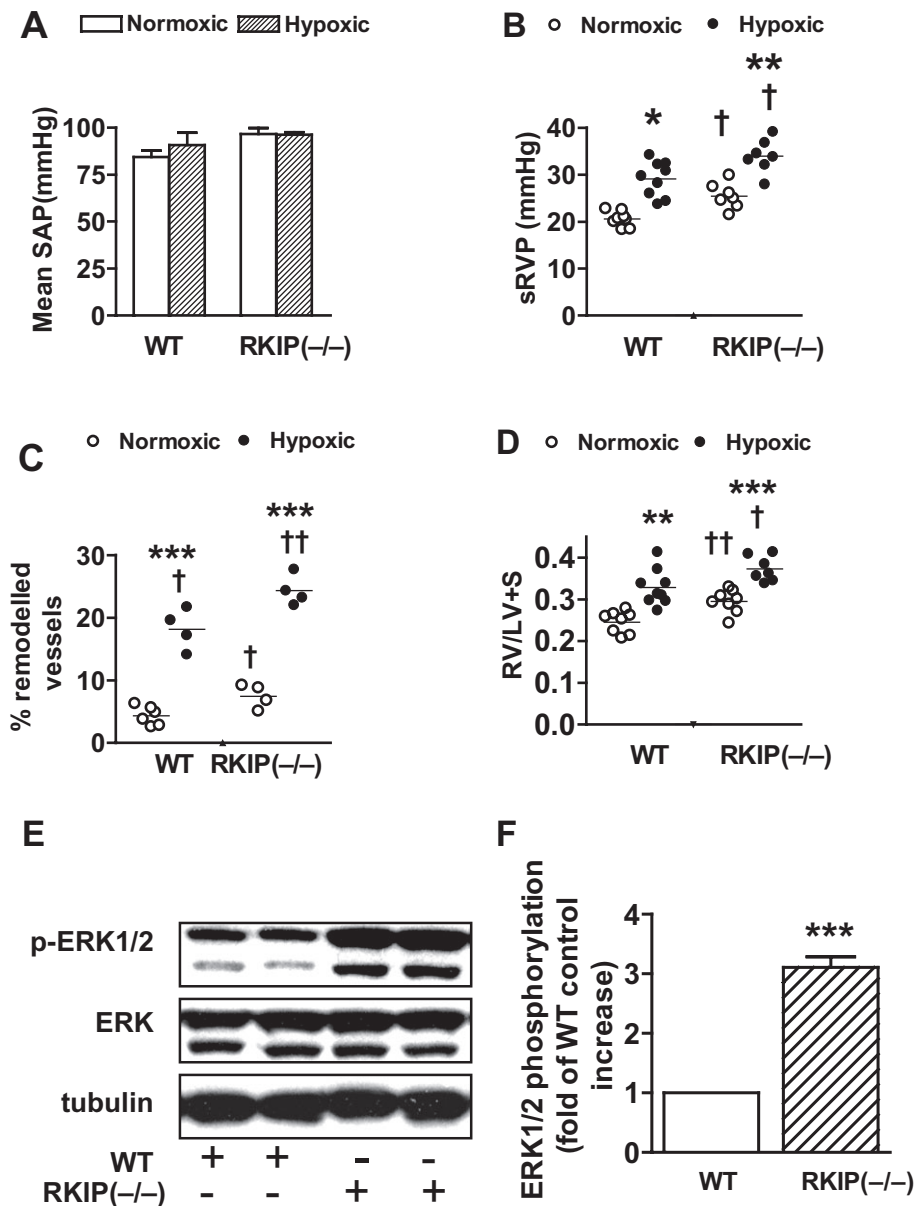
### Immunohistochemistry

Normoxic WT lungs demonstrated very little, faint staining for PCNA (Figure 2B) indicating few proliferating cells. However, RKIP (–/–) mouse pulmonary arteries had more proliferating cells (detected by PCNA), compared with pulmonary arteries from WT mice (Figure 2B). Two weeks exposure to hypoxia resulted in increased expression of PCNA which was further exaggerated in the RKIP (–/–) mice (Figure 2B), where proliferating cells were primarily detected in the smooth muscle and adventitial layers of the pulmonary arteries. Immunohistochemistry also demonstrated that the pulmonary arteries of hypoxic WT mice had significantly increased levels of p-Raf-1 (Figure 2C) and p-RKIP (Figure 2D) compared with normoxic counterparts. In addition, pulmonary arteries from normoxic RKIP (–/–) mice had increased p-Raf-1 compared with their WT controls (Figure 2C) that was further increased after chronic hypoxia (Figure 2C). Consistent with genetic deletion of RKIP, the pulmonary arteries of RKIP (–/–) mice had no detectable levels of p-RKIP (Figure 2D).

### Phosphorylation of Raf-1, RKIP and ERK in WT pulmonary arteries

As RKIP is a negative modulator of the Raf-1/MEK/ERK1/2 signalling pathway and dysregulated activation of ERK1/2 is





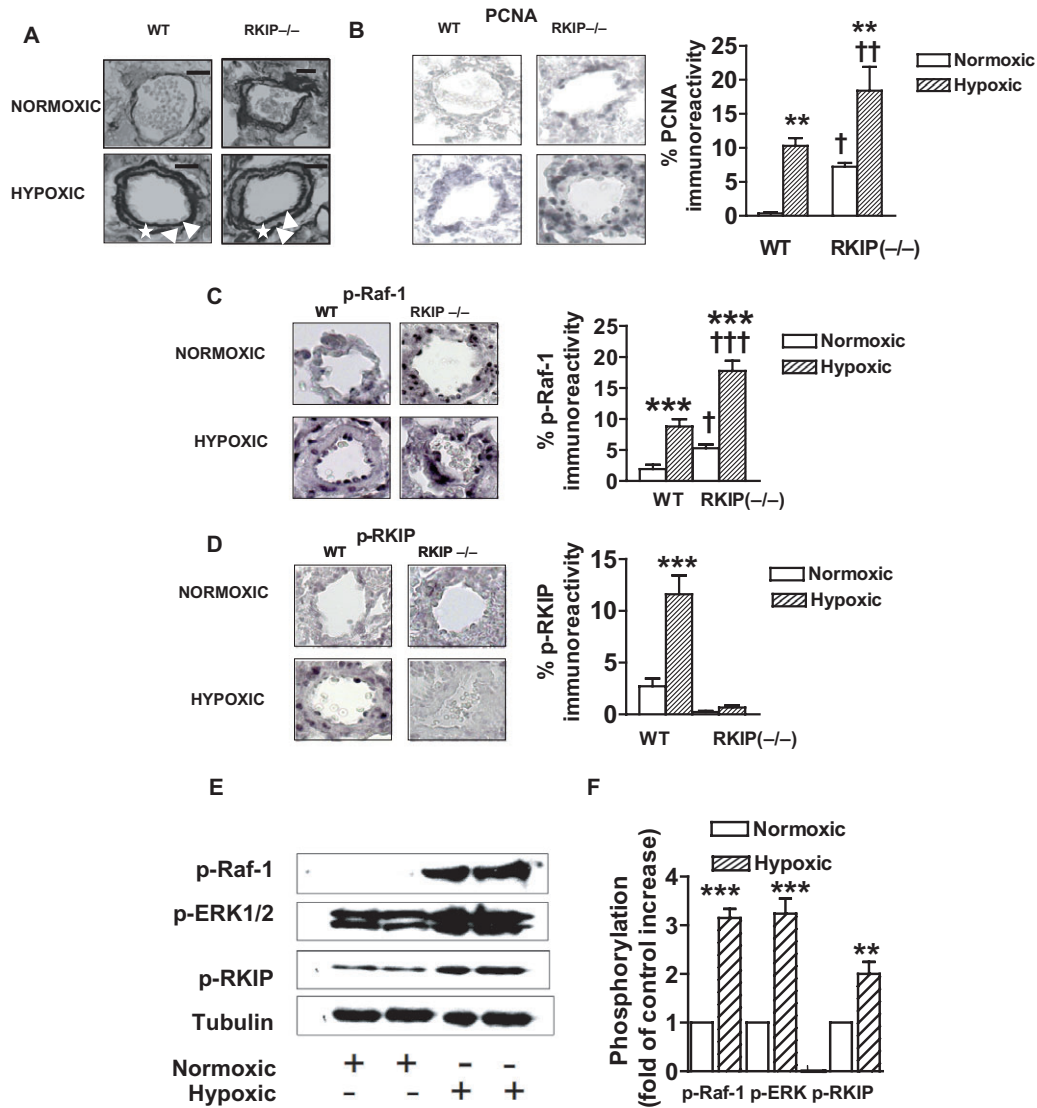
**Figure 1**

Effect of chronic hypoxia and genetic deletion of Raf-1 kinase inhibitor protein (RKIP) on (A) mean systemic arterial pressure (SAP), (B) systolic right ventricular pressure (sRVP), (C) pulmonary vascular remodelling, (D) right ventricular hypertrophy RV/(LV + S) in mice and (E,F) the effect of RKIP deletion on phosphorylation of ERK1/2 (p-ERK1/2) in murine lungs ( $n = 4$  to 10 mice in each group). \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , significantly greater than corresponding value in normoxic mice. † $P < 0.05$ , †† $P < 0.01$ , ††† $P < 0.001$ , significantly greater than corresponding value in wild-type (WT) mice. Data are shown as mean  $\pm$  SEM.

important in the pathophysiology of PAH, we investigated the effects of chronic hypoxia on the phosphorylation of Raf-1, RKIP and ERK in pulmonary arteries of WT mice using Western blotting. After 2 weeks of hypoxia, WT mice had significantly increased levels of p-Raf-1, p-RKIP (Figure 2E,F) consistent with the immunohistochemistry results. Hypoxic WT pulmonary arteries also had increased levels of p-ERK compared with normoxic counterparts (Figure 2E,F).

### Effects of RKIP inhibition on proliferation of CCL39 cells

To analyse whether RKIP inhibition directly affected mitogen-induced pulmonary cell proliferation, we assessed, *in vitro*, the effect of an RKIP inhibitor on CCL39 cell proliferation induced by 5-HT or PDGF in normoxic and hypoxic conditions. 5-HT (1  $\mu$ M) induced proliferation of CCL39 cells in normoxia with  $\sim 3.5$ -fold increase in cell number from

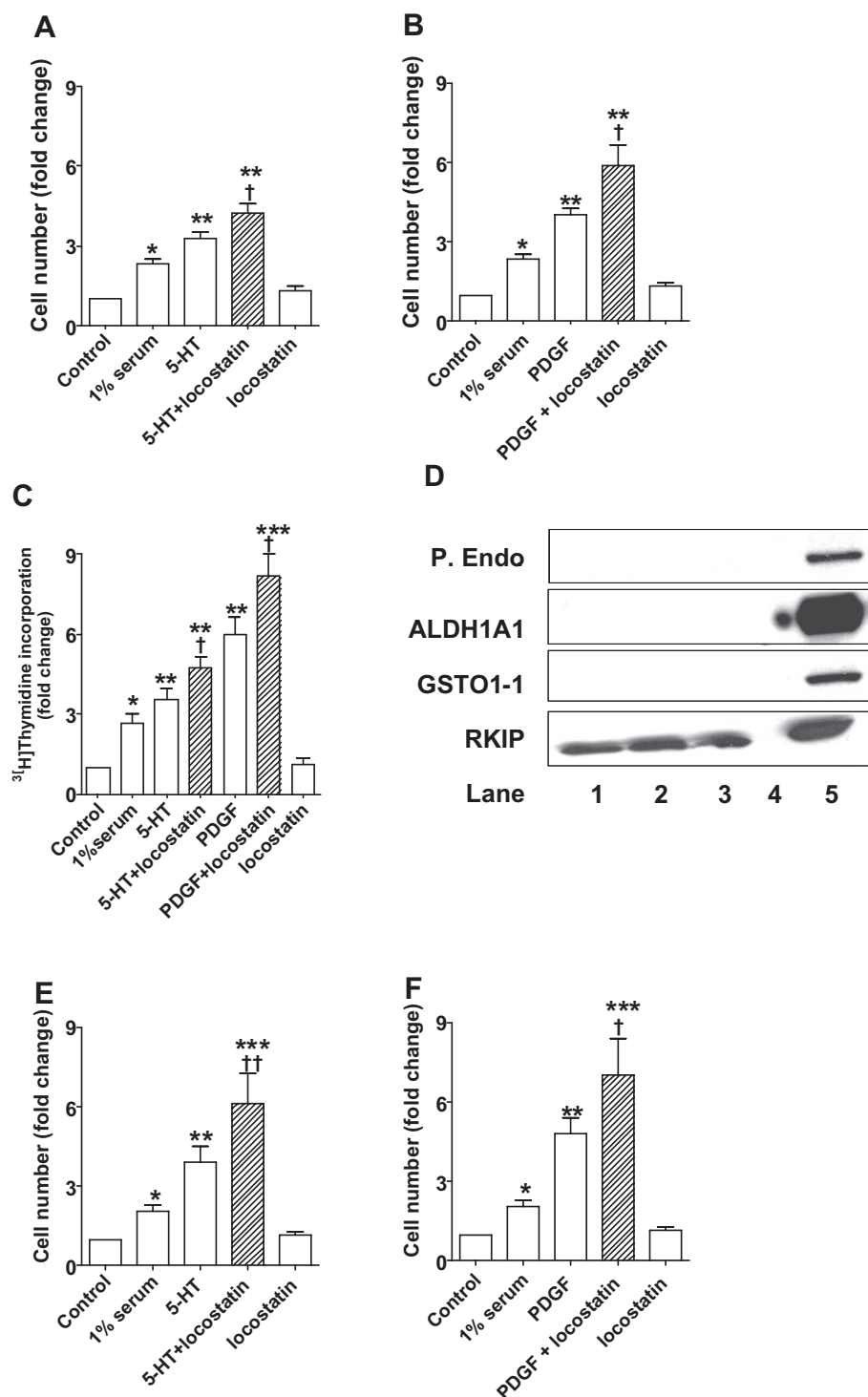


## Figure 2

Representative photomicrographs showing the effects of chronic hypoxia and deletion of the Raf-1 kinase inhibitor protein (RKIP) gene (A–D) on: (A) pulmonary vascular remodelling and (B) expression of proliferating cell nuclear antigen (PCNA), (C) expression of phospho-Raf-1, (D) phospho-RKIP in small pulmonary arteries from mice and the effect of chronic hypoxia on (E) and (F) the RKIP signalling elements in small pulmonary arteries from wild-type (WT) mice. (A) The figure demonstrates that remodelling occurs in the normoxic RKIP<sup>-/-</sup> group and is exaggerated in hypoxia when compared with WT mice. Note the readily identifiable double elastic lamina (white arrow) and newly formed smooth muscle layer (asterisk) in the hypoxic groups. (B) The figure demonstrates that expression of PCNA is increased in the pulmonary arteries of the mice lacking RKIP [RKIP<sup>-/-</sup>], indicative of increased proliferation. Hypoxia-induced proliferation is also observed in the WT mice which is further exaggerated by hypoxia in the RKIP<sup>-/-</sup> mice. (C) The figure demonstrates hypoxia-induced phosphorylation of Raf-1 (p-Raf-1; shown as black staining) in the WT mice which is further exaggerated by hypoxia in the RKIP<sup>-/-</sup> mice. (D) The figure demonstrates hypoxia-induced phosphorylation of RKIP [shown as black staining; absent in the RKIP<sup>-/-</sup> mice] in the WT mice. (E,F) The figure demonstrates that chronic hypoxia induces phosphorylation of the RKIP signalling elements Raf-1, RKIP and ERK1/2. Scale bars = 25  $\mu$ m. \* $P$  < 0.05, \*\* $P$  < 0.01, \*\*\* $P$  < 0.001 significantly greater than corresponding value in normoxic mice. † $P$  < 0.01, †† $P$  < 0.001, significantly greater than corresponding value in WT mice. Data are shown as mean  $\pm$  SEM.

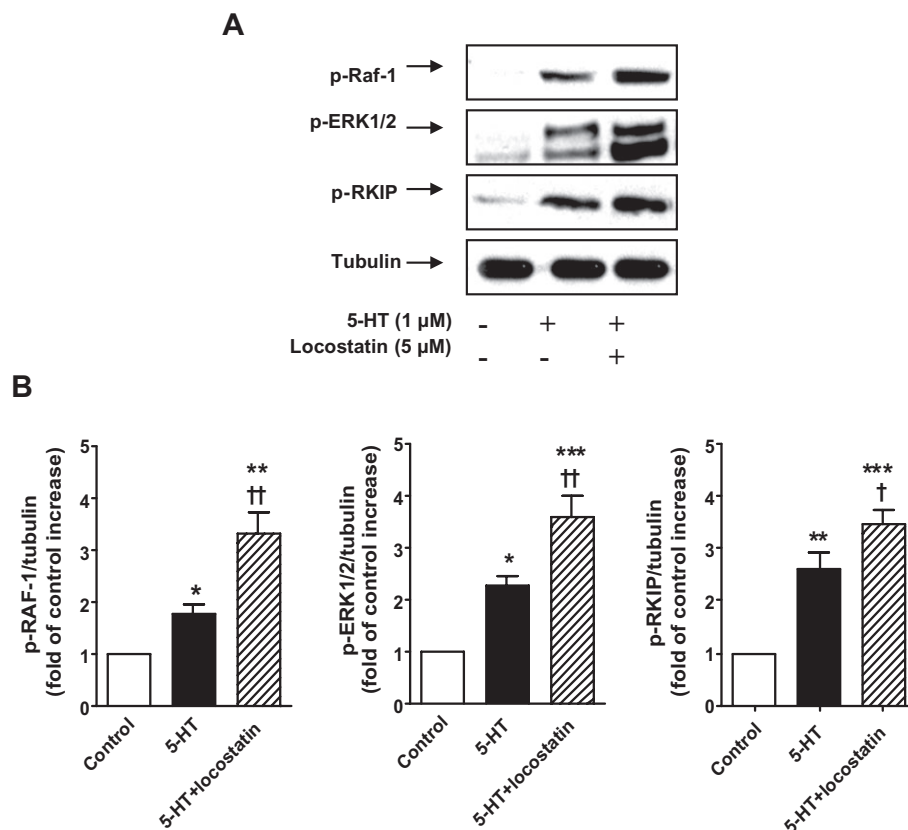
control, basal levels ( $P$  < 0.01; Figure 3A,C) and in hypoxia ( $P$  < 0.01; Figure 3E). The 5-HT-induced proliferation observed in the CCL39 cells was further increased by the RKIP inhibitor locostatin (5  $\mu$ M) both under normoxic ( $P$  < 0.05; Figure 3A,C) and hypoxic ( $P$  < 0.01; Figure 3E) conditions, with the greater increase demonstrated in hypoxia

(Figure 3E). PDGF (10 ng·mL<sup>-1</sup>) markedly induced a proliferative response in the CCL39 cells in normoxia (Figure 3B,C) and hypoxia (Figure 3F). This was further potentiated by locostatin (Figure 3B,F). To confirm that the observed effects of locostatin was due to selective inhibition of RKIP, we demonstrated that the expression of prolyl endopeptidase,



**Figure 3**

Effects of Raf-1 kinase inhibitor protein (RKIP) inhibition on 5-HT-stimulated (A,C,E) and platelet derived growth factor (PDGF)-stimulated (B,C,F) CCL39 fibroblast proliferation in normoxic (A,B,C) and hypoxic [5% O<sub>2</sub> (E,F)] conditions. Proliferation of CCL39 cells stimulated by 5-HT (1  $\mu$ M; 24 h) or PDGF (10 ng·mL<sup>-1</sup>; 24 h) was potentiated by the RKIP inhibitor locostatin (5  $\mu$ M; added 45 min prior to the addition of mitogen). (D) RKIP is expressed in CCL39 cells but prolyl endopeptidase (P. Endo.), glutathione S-transferase omega 1-1 (GSTO1-1), and aldehyde dehydrogenase (ALDH1A1) were all absent; Lanes 1–3: CCL39 cell lysates from three different experiments, lane 4: empty, lane 5: rat liver tissue lysate positive control. \* $P$  < 0.01, \*\* $P$  < 0.001, significantly greater than control (unstimulated) value. † $P$  < 0.01, †† $P$  < 0.001, significantly greater than corresponding value in the absence of RKIP inhibition. Data are combined from three to four experiments and are shown as mean  $\pm$  SEM.



**Figure 4**

(A) Increased phosphorylation of Raf-1 (p-Raf-1), ERK1/2 MAPK (p-ERK1/2) and Raf-1 kinase inhibitor protein (RKIP) (p-RKIP) in normoxic CCL39 cells stimulated by 5-HT (1 μM; 5 min) was potentiated by the RKIP inhibitor locostatin (5 μM; added 45 min prior to the addition of 5-HT). (B) Quantification of data is also shown representing three independent experiments with comparable outcomes [samples were adjusted for loading errors, using the tubulin blot density to standardize, prior to normalization relative to control (unstimulated) values]. Quantitative data are shown as mean ± SEM. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , significantly greater than control (unstimulated) value. † $P < 0.05$ , †† $P < 0.01$ , significantly greater than corresponding value in the absence of RKIP inhibition.

ALDH1A1 and GSTO1-1 was absent but that of RKIP was present in CCL39 cells (Figure 3D). Thus the effects of locostatin (potentiated mitogen-induced proliferation) are mediated solely via inhibition of RKIP in the CCL39 hamster lung fibroblast cell line.

### Effects of RKIP inhibition on phosphorylation of Raf-1, ERK1/2 and RKIP

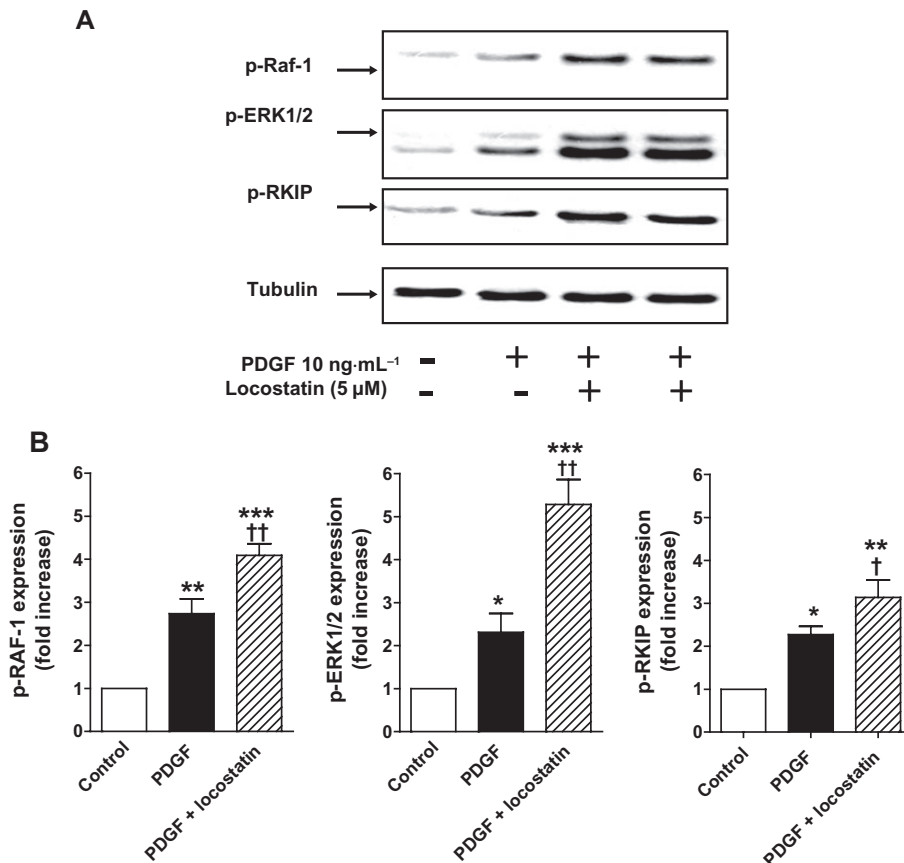
As previously described (Mair *et al.*, 2008) under normoxic conditions, 5-HT (1 μM) induced an increase in ERK1/2 phosphorylation in CCL39 cells by ~120% (Figure 4); here we show this is accompanied by a concomitant increase in phosphorylation of Raf-1 and RKIP by ~80% and 170%, respectively (Figure 4), indicative of 5-HT-induced activation of the Raf-1/MEK/ERK1/2 pathway. This increased phosphorylation was further potentiated by the RKIP inhibitor locostatin by ~68%, 83% and 22% respectively (Figures 4A and 3B). PDGF (10 ng·mL<sup>-1</sup>) induced a marked increase (~130%) in ERK1/2 phosphorylation accompanied by increased Raf-1 (~180%) and RKIP (~129%) phosphorylation, which was further increased in the presence of locostatin by ~135%, 48% and 38% respectively (Figure 5). Hypoxia increased the phospho-

rylation of Raf-1, RKIP and ERK1/2 in CCL39 cells by ~110%, 125% and 130% respectively (Figure 6), and this was accompanied by a concomitant increase in phosphorylation of PKCζ of ~80% (Figure 6B). Both 5-HT (1 μM) and PDGF (10 ng·mL<sup>-1</sup>), in the presence of hypoxia, potentiated the hypoxia-induced phosphorylation of PKCζ (~94% and 56% respectively; Figures 6B and 7B), Raf-1 (~90% and 97% respectively; Figures 6C and 7C), ERK1/2 (~77% and 109% respectively; Figures 6D and 7D) and RKIP (~69% and 79% respectively; Figures 6E and 7E).

### Effects of selective 5-HT receptor and SERT antagonists on phosphorylation of Raf-1, ERK1/2, RKIP and PKCζ

Selective inhibition of SERT and the 5-HT<sub>2A</sub> receptor with citalopram (10 μM) or ketanserin (100 nM), respectively, resulted in similar inhibitory effects on the phosphorylation of PKCζ ( $P < 0.001$ ; Figure 8B), Raf-1 ( $P < 0.05$ ; Figure 8C), ERK1/2 ( $P < 0.05$ ; Figure 8D) and RKIP ( $P < 0.05$ ; Figure 8E). In contrast, the selective 5-HT<sub>1B</sub> receptor antagonist SB224289 (200 nM) did not inhibit the 5-HT-induced phosphorylation of PKCζ (Figure 8B), RKIP (Figure 8E) or Raf-1 (Figure 8C).





**Figure 5**

A. Increased phosphorylation of Raf-1 (p-Raf-1), ERK1/2 MAPK (p-ERK1/2) and Raf-1 kinase inhibitor protein (RKIP) (p-RKIP) in normoxic CCL39 cells stimulated by platelet derived growth factor (PDGF) (10 ng·mL<sup>-1</sup>; 5 min) was potentiated by the RKIP inhibitor locostatin (5 μM; shown in duplicate lanes and added 45 min prior to the addition of PDGF). (B) Quantification of data is also shown representing three independent experiments with comparable outcomes [samples were adjusted for loading errors, using the tubulin blot density to standardize, prior to normalization relative to control (unstimulated) values]. Quantitative data are shown as mean ± SEM. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , significantly greater than control (unstimulated) value. † $P < 0.05$ , †† $P < 0.01$ , significantly greater than corresponding value in the absence of RKIP inhibition.

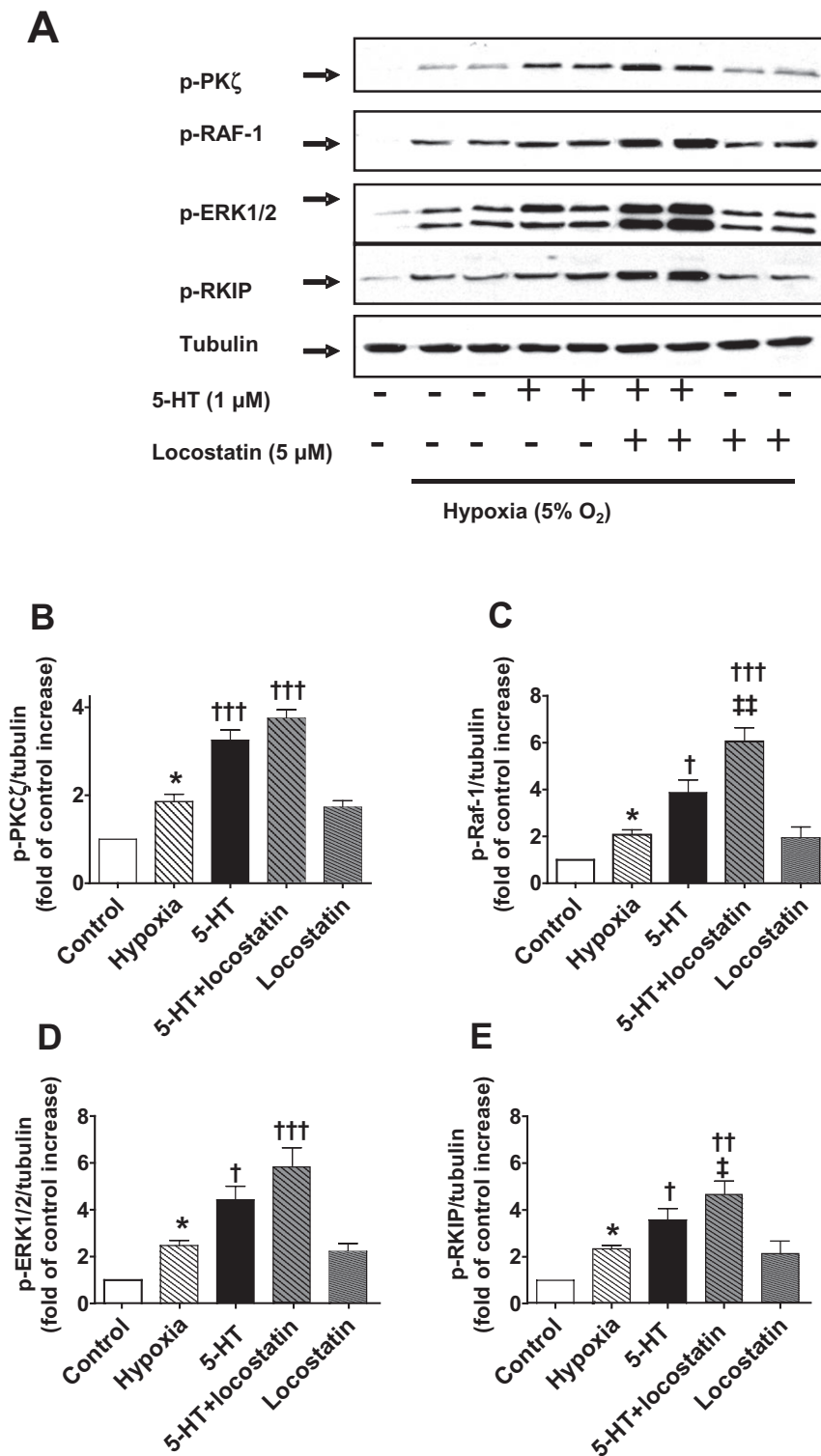
5-HT<sub>1B</sub> receptor blockade did however inhibit the phosphorylation of ERK1/2 ( $P < 0.05$ ; Figure 8D). As shown in Figure 9, ketanserin (100 nM) and citalopram (10 μM), but not SB224289 (200 nM) also inhibited the 5-HT-induced phosphorylation of PKCζ ( $P < 0.001$ ; Figure 9B), Raf-1 ( $P < 0.05$ ; Figure 9C) and RKIP ( $P < 0.05$ ; Figure 9E) in hypoxia. This finding implicates the involvement of the SERT and 5-HT<sub>2A</sub> receptor, but not the 5-HT<sub>1B</sub> receptor, both under normoxic and hypoxic conditions. In contrast, the 5-HT<sub>1B</sub> receptor in addition to the SERT and 5-HT<sub>2A</sub> receptor is responsible for 5-HT-induced phosphorylation of ERK1/2 in both normoxic and hypoxic conditions.

## Discussion

We report for the first time that RKIP suppresses the development of PAH. We demonstrate that deficiency of RKIP leads to elevated RVP and associated indices of PAH in mice. RKIP deficiency was also associated with exaggerated hypoxia-

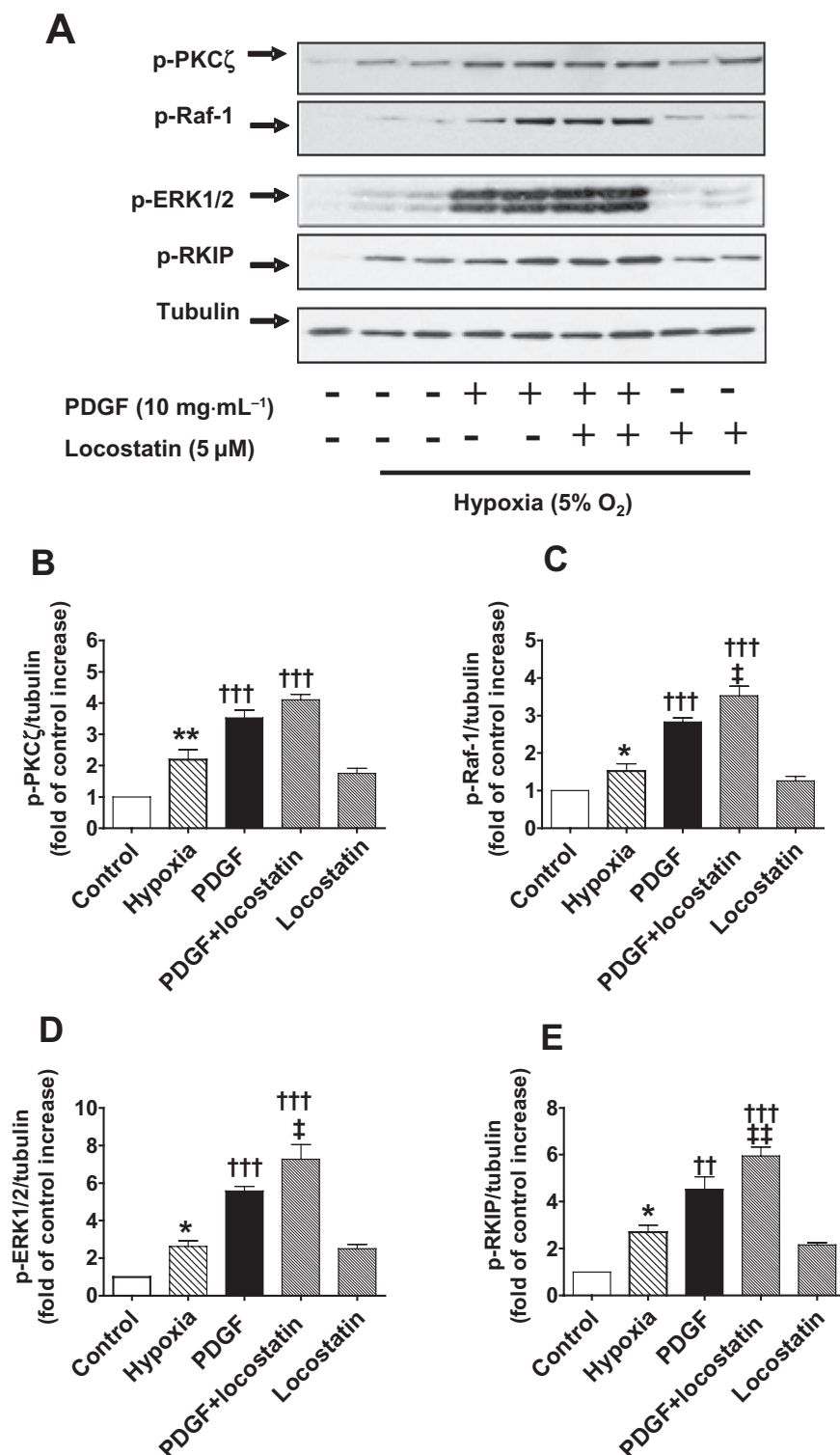
induced PAH in mice and phosphorylation of RKIP and its signalling elements (p-Raf-1 and p-ERK1/2) were elevated in mice exposed to chronic hypoxia. The data also provided evidence that 5-HT- and PDGF-mediated proliferation of CCL39 fibroblast cells involved Raf-1 kinase activation and RKIP phosphorylation. RKIP inhibition with locostatin potentiated this proliferative response.

The data suggest endogenous RKIP suppresses pulmonary vascular remodelling associated with the development of hypoxia-induced PAH. Deletion of the RKIP gene elevated sRVP, pulmonary vascular remodelling and markedly increased RVH while systemic haemodynamics were unaffected. This suggests that RKIP is present in the normal murine pulmonary vasculature and myocardium and the absence or reduction of RKIP can enhance hypoxia-induced PAH. As a consequence of unopposed Raf-1/MEK/ERK1/2 intracellular signalling (Yeung *et al.*, 1999) RKIP deficiency may predispose to proliferative pulmonary cellular phenotypes, resulting in pulmonary vascular remodelling, and this effect is increased further following chronic hypoxic



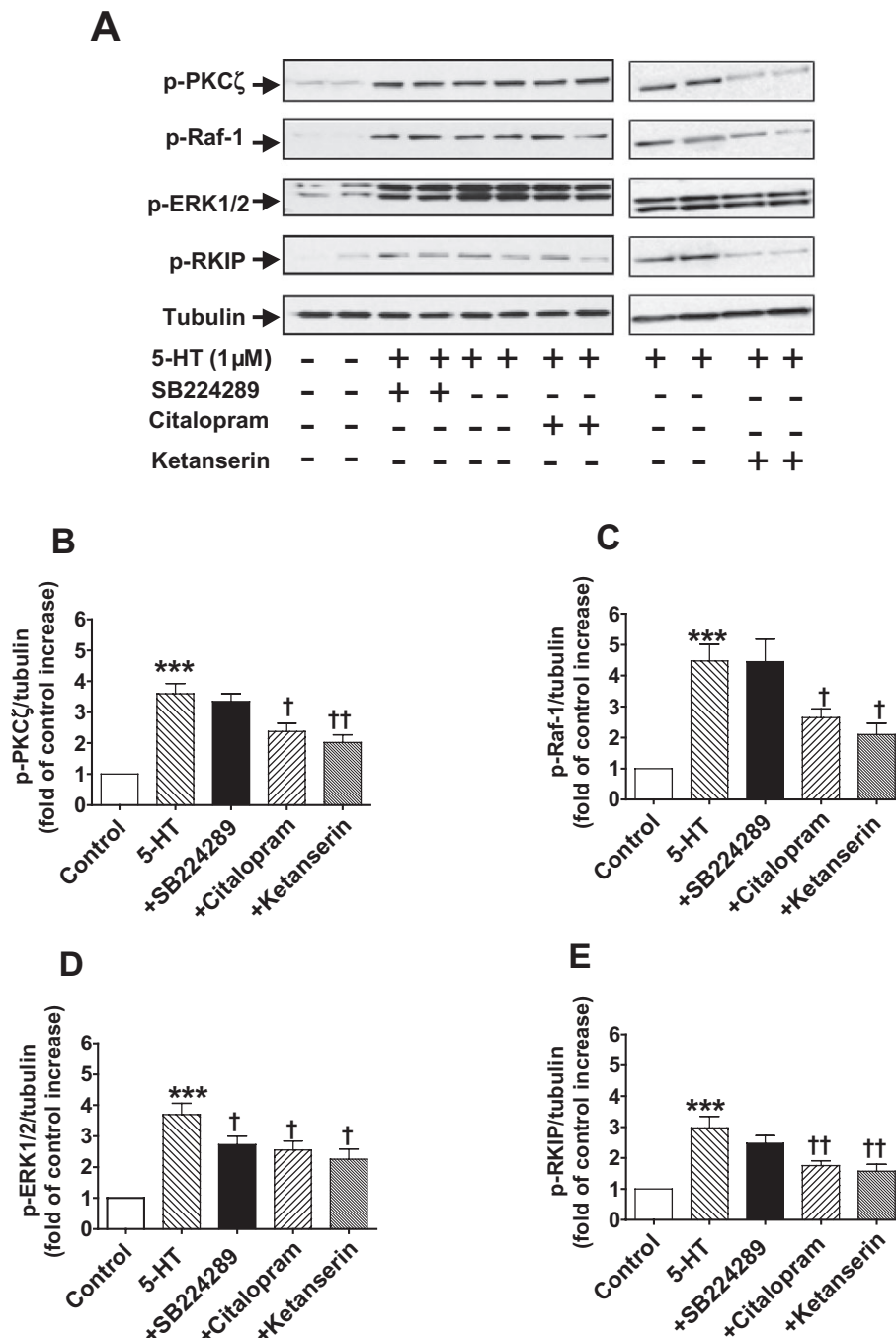
**Figure 6**

(A) Increased phosphorylation (shown in duplicate lanes) of Raf-1 (p-Raf-1), ERK1/2 MAPK (p-ERK1/2), Raf-1 kinase inhibitor protein (RKIP) (p-RKIP) and atypical protein kinase C $\zeta$  (PKC $\zeta$ ) (p-PKC $\zeta$ ) in hypoxic CCL39 cells stimulated by 5-HT (1  $\mu$ M; 5 min) which, except for p-PKC $\zeta$ , was potentiated by the RKIP inhibitor locostatin (5  $\mu$ M; added 45 min prior to the addition of 5-HT). (B–E) Quantification of data is also shown representing four independent experiments with comparable outcomes [samples were adjusted for loading errors, using the tubulin blot density to standardize, prior to normalization relative to control (unstimulated) values]. Quantitative data are shown as mean  $\pm$  SEM. \* $P$  < 0.05, significantly greater than normoxic control (unstimulated) value. † $P$  < 0.05, †† $P$  < 0.01, ††† $P$  < 0.001, significantly greater than hypoxic control value. ‡ $P$  < 0.05, ‡‡ $P$  < 0.01, significantly greater than 5-HT-treated cells.



**Figure 7**

(A) Increased phosphorylation (shown in duplicate lanes) of Raf-1 (p-Raf-1), ERK1/2 MAPK (p-ERK1/2), Raf-1 kinase inhibitor protein (RKIP) (p-RKIP) and atypical protein kinase C $\zeta$  (PKC $\zeta$ ) (p-PKC $\zeta$ ) in hypoxic CCL39 cells stimulated by platelet derived growth factor (PDGF) (10 ng·mL<sup>-1</sup>; 5 min) which, except for p-PKC $\zeta$ , was potentiated by the RKIP inhibitor locostatin (5  $\mu$ M; added 45 min prior to the addition of PDGF). (B–E) Quantification of data is also shown representing four independent experiments with comparable outcomes [samples were adjusted for loading errors, using the tubulin blot density to standardize, prior to normalization relative to control (unstimulated) values]. Quantitative data are shown as mean  $\pm$  SEM. \* $P$  < 0.05, \*\* $P$  < 0.01, significantly greater than normoxic control (unstimulated) value. † $P$  < 0.05, †† $P$  < 0.01, ††† $P$  < 0.001, significantly greater than hypoxic control value. ‡ $P$  < 0.05, ‡‡ $P$  < 0.01, significantly greater than PDGF treated cells.

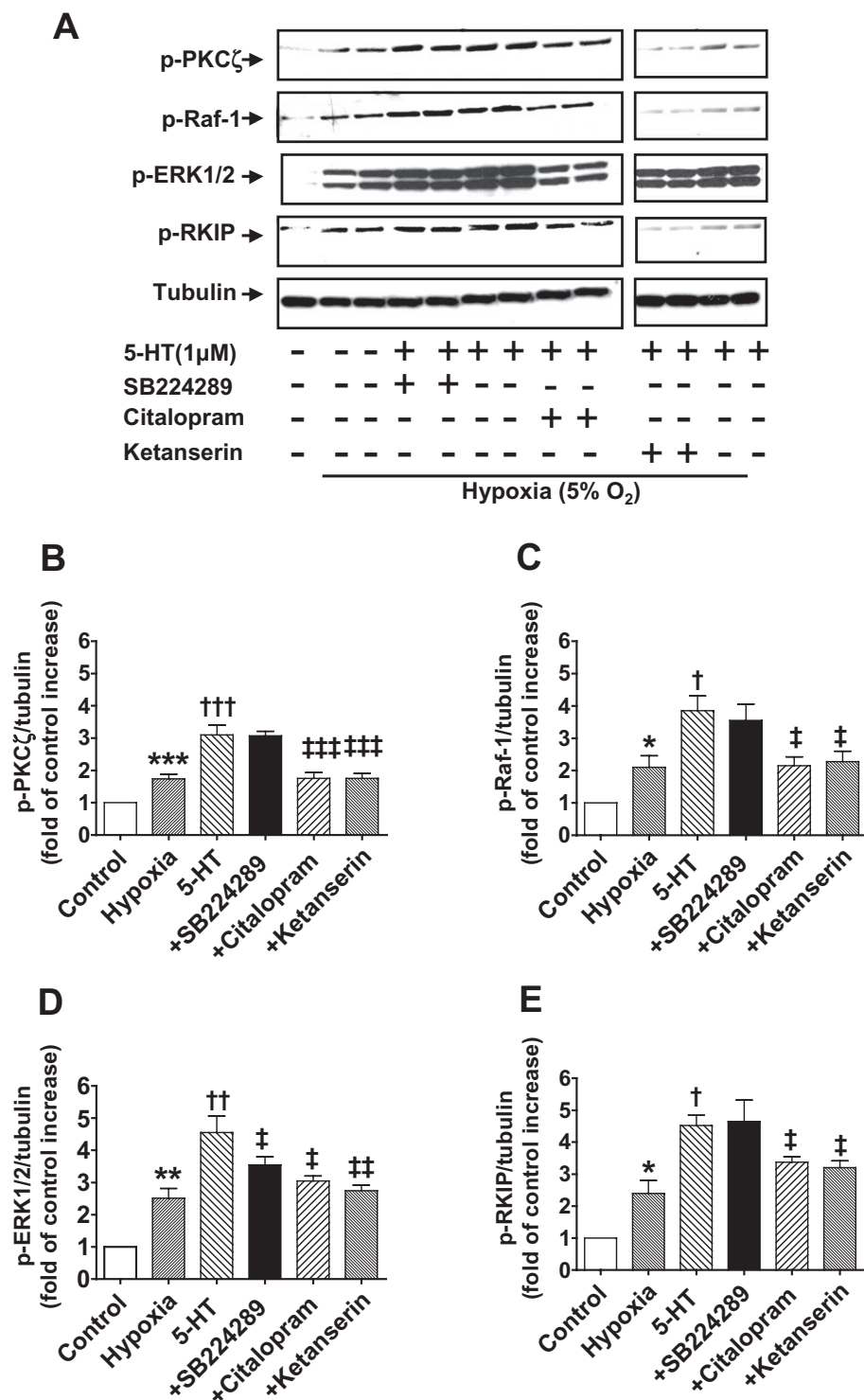


**Figure 8**

(A) Activation of 5-HT transporter (SERT) and the 5-HT<sub>2A</sub> but not the 5-HT<sub>1B</sub> receptor mediates 5-HT-activated phospho-Raf-1 (p-Raf-1), phospho-Raf-1 kinase inhibitor protein (p-RKIP) and phospho-atypical protein kinase C $\zeta$  (p-PKC $\zeta$ ) in normoxic CCL39 cells stimulated by 5-HT (1  $\mu$ M; 5 min) but 5-HT activates phospho-ERK1/2 MAPK (p-ERK1/2) via SERT and 5-HT<sub>1B</sub> receptor. Antagonists added 45 min prior to the addition of 5-HT. 5-HT-induced phosphorylation of the respective signalling elements is shown in duplicate lanes. (B–E) Quantification of data is also shown representing four independent experiments with comparable outcomes [samples were adjusted for loading errors, using the tubulin blot density to standardize, prior to normalization relative to control (unstimulated) values]. Quantitative data are shown as mean  $\pm$  SEM. \* $P$  < 0.05, \*\* $P$  < 0.01, \*\*\* $P$  < 0.001, significantly greater than control (unstimulated) value. † $P$  < 0.05, †† $P$  < 0.01, significantly less than 5-HT-treated cells.

exposure. Consistent with this, pulmonary arteries from normoxic RKIP (–/–) mice had increased levels of p-Raf-1 and p-ERK1/2 compared with those in WT mice. Pulmonary arteries removed from the RKIP (–/–) mouse also had increased

levels of the marker of proliferation, PCNA. This is indicative of increased pulmonary arterial cellular proliferation (contributing to pulmonary vascular remodelling). This marker was further increased after exposure to hypoxia consistent



**Figure 9**

(A) Activation of 5-HT transporter (SERT) and the 5-HT<sub>2A</sub> but not the 5-HT<sub>1B</sub> receptor mediates 5-HT-activated phospho-Raf-1 (p-Raf-1), phospho-Raf-1 kinase inhibitor protein (p-RKIP) and phosphor-atypical protein kinase C $\zeta$  (p-PKC $\zeta$ ) in hypoxic CCL39 cells stimulated by 5-HT (1  $\mu$ M; minutes) but 5-HT activates phospho-ERK1/2 MAPK (p-ERK1/2) via SERT, 5-HT<sub>2A</sub> and 5-HT<sub>1B</sub> receptor. Antagonists added 45 min prior to the addition of 5-HT. 5-HT-induced phosphorylation of the respective signalling elements is shown in duplicate lanes. (B–E) Quantification of data is also shown representing four independent experiments with comparable outcomes [samples were adjusted for loading errors, using the tubulin blot density to standardize, prior to normalization relative to control (unstimulated) values]. Quantitative data are shown as mean  $\pm$  SEM. \* $P$  < 0.05, \*\* $P$  < 0.01, \*\*\* $P$  < 0.001, significantly greater than normoxic control (unstimulated) value. † $P$  < 0.05, †† $P$  < 0.01, ††† $P$  < 0.001, significantly greater than hypoxic control value. ‡ $P$  < 0.05, ‡‡ $P$  < 0.01, ‡‡‡ $P$  < 0.001, significantly less than 5HT-treated cells.



with hypoxia-induced pulmonary vascular remodelling. In arteries from WT mice, hypoxia also increased the phosphorylation of various signalling elements in the Raf-1/MEK/ERK1/2 signalling pathway including RKIP. This is consistent with hypoxia-induced hyperactivation of the Raf-1/MEK/ERK1/2 pathway in the PASMCs which would contribute to the increased pulmonary vascular remodelling.

Previous studies have demonstrated that the ability of RKIP to suppress Raf-1/MEK/ERK1/2 signalling is inhibited upon PKC-stimulated phosphorylation of Ser<sup>153</sup> (Corbit *et al.*, 2003). Upon phosphorylation, RKIP dissociates from Raf-1 to associate with G-protein coupled receptor kinase 2 (GRK-2) and blocks its activity (Lorenz *et al.*, 2003). GRK-2 is responsible for the phosphorylation and desensitization of many G-protein coupled receptors (GPCRs) including 5-HT receptors (Lorenz *et al.*, 2003; Huang *et al.*, 2007). Many GPCRs, including 5-HT receptors, play a role in pulmonary vascular remodelling and contribute to the development of PAH (Keegan *et al.*, 2001; Welsh *et al.*, 2004; Lawrie *et al.*, 2005). The ability of RKIP to switch binding partner specificity from Raf-1 to GRK-2 upon phosphorylation by PKC under hypoxia is therefore of particular relevance to the pathogenesis of PAH.

Hypoxic exposure increases ERK1/2 phosphorylation in rat pulmonary arteries and human PASMCs (Jin *et al.*, 2000; Murray *et al.*, 2003), and ERK1/2 phosphorylation is necessary for hypoxia-induced proliferation of bovine pulmonary arterial fibroblasts (Das *et al.*, 2001). We showed that hypoxia increased ERK1/2 and Raf-1 phosphorylation in the WT pulmonary arteries consistent with RKIP phosphorylation (p-RKIP) and its subsequent uncoupling from Raf-1 and MEK1/2, which would allow their interaction and subsequent ERK1/2 activation. This was confirmed in the CCL39 cell cultures where hypoxia enhanced p-RKIP, and p-ERK1/2 signalling, which was further enhanced in the presence of 5-HT and PDGF.

The RKIP deletion also markedly increased RVH in normoxic and hypoxic mice indicating that RKIP may protect the myocardium from stress-induced hypertrophy. Consistent with this, serine/threonine kinases such as Raf-1 and its downstream signalling pathways are expressed in the myocardium and associated with myocardial hypertrophy (Harris *et al.*, 2004). The Raf-1/MEK/ERK1/2 pathway is one of the main cardiac hypertrophy signalling pathways (Harris *et al.*, 2004) and is up-regulated in the right ventricular myocardium in experimental PAH (Klein *et al.*, 2008). Furthermore, sorafenib, an inhibitor of several kinases, including Raf-1, reduces Raf-1 and ERK1/2 phosphorylation in the RV myocardium of rats with monocrotaline-induced PAH. This mediates its beneficial action via inhibition of the Raf-1/MEK/ERK1/2 pathway (Klein *et al.*, 2008).

5-HT induced proliferation in CCL39 cells, consistent with previous reports that also implicated 5-HT<sub>1B</sub> and 5-HT<sub>2A</sub> receptors as well as the SERT (Lee *et al.*, 1999; Mair *et al.*, 2008). Here we show that 5-HT- and PDGF-induced proliferation of CCL39 cells was increased in the presence of the RKIP inhibitor locostatin. This suggests that endogenous RKIP can attenuate 5-HT- and PDGF-induced proliferation in pulmonary fibroblast cells and that both 5-HT and PDGF mediate proliferation, at least partly, through the Raf-1/MEK/ERK1/2 signal transduction pathway. Consistent with this, 5-HT and

PDGF induced expression of p-Raf-1, p-ERK1/2 and p-RKIP, which were further increased in the presence of RKIP inhibition. Pulmonary mitogens such as 5-HT may, therefore predispose the Raf-1/MEK/ERK1/2 to hyperactivation thereby increasing proliferation, by disabling the inhibitory effect of RKIP via its phosphorylation. Mitogen-induced uncoupling of RKIP from Raf-1 kinase to enhance Raf-1/MEK/ERK1/2 signalling may be an important component of the pulmonary hypertensive process.

Locostatin disrupts the ability of RKIP to bind and inhibit Raf-1 kinase *in vitro* through alkylation thereby restoring the kinase activity of Raf-1 and enhancing Raf-1/MEK/ERK1/2 signalling (Zhu *et al.*, 2005). Locostatin alone did not increase proliferation in CCL39 cells yet there was a hypertensive phenotype *in vivo* in mice lacking RKIP. The difference between the *in vivo* and *in vitro* effects of RKIP reduction may be the presence of endogenous mitogen synthesis within the pulmonary vasculature *in vivo* which is absent *in vitro*. PAH is associated with enhanced proliferative responses in the pulmonary vasculature and both PASMCs and pulmonary arterial fibroblasts play a role. In the normoxic WT mice, it is possible that endogenous RKIP, as a negative modulator of the Raf-1/MEK/ERK1/2 pathway, acts to suppress the proliferative effects of mitogens including 5-HT, which is known to be synthesized in the pulmonary arterial endothelial cells of the pulmonary vasculature. In the normoxic RKIP (–/–) mice, the suppressive action of RKIP is removed. This would allow a hyperactivation of the Raf-1/MEK/ERK1/2 signalling pathway by locally synthesized mitogens, leading to the increased cell proliferation contributing to pulmonary vascular remodelling. By contrast, in the absence of endogenous mitogen synthesis, the RKIP inhibitor locostatin potentiates the proliferation of the CCL39 cells only in response to exogenous mitogens.

As discussed above, hypoxia induced phosphorylation of RKIP and Raf-1, and one explanation for this observation is through increased activation of the PKC $\zeta$  by hypoxia. The present studies clearly demonstrated the ability of hypoxia, 5-HT and PDGF, to activate PKC $\zeta$  in CCL39 pulmonary fibroblast cells. Increased PKC $\zeta$  activity would uncouple and phosphorylate RKIP and promote phosphorylated ERK1/2 status alongside GRK-2 inhibition to enhance and prolong GPCR signalling. This would contribute to the hypoxia-induced pulmonary vascular remodelling observed in the *in vivo* study.

Numerous studies have shown 5-HT to be important in the pathobiology of PAH (MacLean and Dempsie, 2009). In mice, hypoxia-induced PAH depends on peripheral 5-HT synthesis (Morecroft *et al.*, 2007). 5-HT-induced proliferation is increased in human PASMCs from idiopathic PAH patients due to increased SERT expression (Marcos *et al.*, 2004). The 5-HT<sub>1B</sub> receptor mediates 5-HT-induced constriction in human pulmonary arteries (Morecroft *et al.*, 1999) and human PASMC proliferation (Lawrie *et al.*, 2005). Female mice over-expressing SERT (SERT+) have elevated pulmonary pressures and are more susceptible to hypoxia-induced PAH (MacLean *et al.*, 2004). This is not observed in male SERT+ mice and the development of PAH in these female mice is dependent on 17 $\beta$ -oestradiol and involves increased expression of key genes including the oestrogen metabolizing enzyme CYP1B1 (White *et al.*, 2011a,b). Combined 5-HT<sub>1B</sub>

receptor and SERT inhibition are effective at preventing and reversing experimental PAH and 5-HT-induced proliferation of PSMCs derived from idiopathic PAH patients (Morecroft *et al.*, 2010).

Our data confirm that the SERT, 5-HT<sub>1B</sub> and 5-HT<sub>2A</sub> receptors are involved in 5-HT-induced ERK1/2 activation in CCL39 cells, PSMCs and pulmonary arterial fibroblasts. Moreover, the present study is the first to demonstrate that phosphorylation of PKC $\zeta$ , RKIP and Raf-1 activation also participate in 5-HT signalling in pulmonary fibroblasts via the SERT and 5-HT<sub>2A</sub> receptor, but not the 5-HT<sub>1B</sub> receptor. The present study also suggests that 5-HT, via the 5-HT<sub>2A</sub> receptor, initiates a signalling cascade whereby PKC $\zeta$  stimulation leads to RKIP phosphorylation, and Raf-1 and ERK1/2 activation, resulting in proliferation of CCL39 pulmonary fibroblasts. 5-HT can transactivate the PDGF receptor via SERT, in bovine PSMCs and 5-HT-induced proliferation is blocked by tyrosine kinase inhibition in these cells (Liu *et al.*, 2007). As Raf-1 activation, via PKC $\zeta$ , can occur in response to PDGF stimulation (van Dijk *et al.*, 1997) and is observed in this study, 5-HT may also mediate signalling through transactivation of the PDGF receptor.

In conclusion, RKIP deficiency results in elevated indices of PAH in mice, which are further enhanced following chronic hypoxia. Increased phosphorylation of RKIP related signalling elements (RKIP, Raf-1 and ERK1/2) are also increased in hypoxic WT pulmonary arteries. Following RKIP inhibition we have also demonstrated potentiation of mitogen-induced proliferation in CCL39 cells via stimulation of the Raf-1/MEK/ERK1/2 pathway. RKIP interacts with mitogens involved in the pathophysiology of PAH to influence pulmonary vascular remodelling and lack of RKIP may result in a PAH phenotype. Enhanced RKIP, coupled with inhibition of PKC $\zeta$  to prevent RKIP phosphorylation, may be a novel therapeutic strategy in the treatment of PAH.

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## Conflict of interest

None.

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