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RESEARCH PAPER

Novel interactions between the 5-HT transporter, 5-HT$_{1B}$ receptors and Rho kinase *in vivo* and in pulmonary fibroblasts

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Background and purpose: While the 5-HT and Rho-kinase (ROCK) pathways have been implicated in the development of pulmonary arterial hypertension (PAH), the nature of any interactions between them remain unclear. This study investigated a role for ROCK in 5-HT-regulated proliferative responses in lung fibroblasts *in vivo* and *in vitro*.
Experimental approach: PAH was examined in mice over-expressing human 5-HT transporters (SERT+), from which pulmonary artery fibroblasts (PFs) were isolated to assess ROCK expression. *In vitro* analysis of 5-HT signalling employed CCL39 hamster lung fibroblasts.
Key results: ROCK inhibition ablated increased pulmonary remodelling and hypertension observed in SERT+ mice, and ROCK1/2 protein levels were elevated in SERT+ PFs. ROCK inhibition also reduced 5-HT-stimulated proliferation by suppressing MEK-stimulated ERK phosphorylation. While optimal 5-HT-stimulated proliferation required 5-HT$_{1B}$ and 5-HT$_{2A}$ receptors and SERT, receptor sensitivity to Y27632 was restricted to the 5-HT$_{1B}$ receptor. Also, while hypoxia-induced pulmonary vascular remodelling and hypertension were sensitive to Y27632 in WT and SERT+ animals, the proportions sensitive to ROCK inhibition were increased by SERT over-expression.
Conclusions and implications: SERT over-expression increased ROCK-dependent pulmonary remodelling in normoxia and hypoxia and SERT over-expression was associated with elevated ROCK1/2 levels. ROCK also potentiated 5-HT$_{1B}$ receptor-stimulated ERK activation and proliferation *in vitro* by facilitating MEK-ERK interaction.
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Keywords: 5-HT; pulmonary arterial hypertension; Rho kinase; fibroblast; proliferation

Abbreviations: BMPR, bone morphogenetic protein receptor; ERK, extracellular signal-regulated kinase; MEK, mitogen-activated protein kinase/ERK kinase; PAH, pulmonary arterial hypertension; PASMC, pulmonary arterial smooth muscle cell; PF, pulmonary arterial fibroblast; PMA, phorbol 12-myristate 13-acetate; ROCK, Rho kinase; SERT, 5-HT transporter; sRVP, systolic right ventricular pressure; WT, wild type

Introduction

Pulmonary arterial hypertension (PAH) is characterized by a sustained and progressive elevation in pulmonary arterial pressure, pulmonary vascular remodelling, right heart failure and death. Familial PAH has been shown to be related to heterozygous germline mutations in the gene encoding bone morphogenetic protein receptor-2 (BMPR2) (Lane *et al.*, 2000), and mutations in activin receptor-like kinase-1 (ALK-1) gene have also been reported (Harrison *et al.*, 2003). BMPR2 gene mutations have been identified in individuals who do not then develop the disease, and it is recognized that other 'risk factors' must be involved in the development of PAH. These may include polymorphisms in the gene encoding the 5-HT (serotonin) transporter, SERT (Eddahibi *et al.*, 2001; Willers *et al.*, 2006). Mice over-expressing SERT (SERT+ mice) demonstrate elevated pulmonary pressures and enhanced hypoxia-induced PAH (MacLean *et al.*, 2004; Guignabert *et al.*, 2006) while conversely, hypoxia-induced PAH is inhibited in SERT-deficient mice (Eddahibi *et al.*, 2000). 5-HT promotes pulmonary arterial smooth muscle cell (PASMC) proliferation, pulmonary arterial vasoconstriction, local microthrombosis and can facilitate the development of PAH (MacLean *et al.*, 2000). For example, exogenously administered 5-HT

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can potentiate the development of PAH in rats (Eddahibi *et al*., 1997) and can uncover a PAH phenotype in BMPR2$^{+/-}$ mice (Long *et al*., 2006).

Proliferation of PASMCs and pulmonary arterial fibroblasts (PFs) is an important component of pulmonary arterial remodelling in PAH, which accounts for the increased thickness of the medial muscular coat in normally muscularized arteries and extension of muscle into smaller and more peripheral arteries. 5-HT has been shown to induce PASMC and PF proliferation through both SERT and 5-HT receptor activation (Lee *et al*., 1998; Welsh *et al*., 2004; Lawrie *et al*., 2005). 5-HT induces pulmonary vasoconstriction in man mainly through the 5-HT$_{1B}$ receptor (MacLean *et al*., 1996a; Morecroft *et al*., 1999). Recently, SERT activity and 5-HT$_{1B}$ receptor activation have been shown to co-operate in both the mitogenic (Lawrie *et al*., 2005) and vasocontractile effects (Morecroft *et al*., 2005; MacLean *et al*., 1996b) of 5-HT.

The small G protein RhoA and its downstream Rho-kinase (ROCK) effectors play a central role in a variety of cellular functions, including smooth muscle contraction, cytoskeletal rearrangement, cell migration, proliferation and regulation of gene expression. There are two ROCK isoforms (ROCK1 and ROCK2) derived from distinct genes (Noma *et al*., 2006), though ROCK1 is thought to be expressed preferentially in the lung (Nakagawa *et al*., 1996). Activation of the RhoA/ROCK pathway has recently been shown to contribute to the vasoconstriction and pulmonary vascular remodelling associated with PAH (Guilluy *et al*., 2005; Hyvelin *et al*., 2005; Nagaoka *et al*., 2005, 2006; Oka *et al*., 2007; McNamara *et al*., 2008). Also, inhibitors of ROCK have been shown to reduce pulmonary pressures and pulmonary vascular remodelling in hypoxic mice (Fagan *et al*., 2004) and monocrotaline-treated rats (Abe *et al*., 2004), and preliminary studies suggest that these may be useful in the treatment of patients with PAH (Ishikura *et al*., 2006; Fukumoto *et al*., 2007). Recent evidence also suggests that in PASMCs, ROCK may be pivotal to the mitogenic effects of 5-HT downstream from 5-HT$_{1B}$ receptor activation and/or SERT not by enhancing extracellular signal-regulated kinase (ERK) phosphorylation, but instead by specifically promoting the nuclear translocation of phosphorylated active ERK (Liu *et al*., 2004; Liu and Fanburg, 2006).

As 5-HT-induced proliferation is dependent upon ERK activity (Liu *et al*., 2004), we wished to assess whether 5-HT-induced activation of ERK is dependent on ROCK activity and whether overexpression of SERT (and the resulting increase in intracellular 5-HT levels) would lead to an increase in ROCK-dependent pulmonary vascular remodelling. To test these novel hypotheses, we have studied 5-HT/ROCK interactions *in vitro* and also investigated whether ROCK plays a role in the exaggerated PAH observed in SERT+ mice *in vivo*. We selected PFs as the cell of choice as we have previously shown that it is the adventitial region of pulmonary arterioles from SERT+ mice that predominantly overexpresses SERT *in vivo* (MacLean *et al*., 2004). The importance of changes in PF phenotype is suggested by the observation that adventitial changes precede intimal and medial remodelling following hypoxic, high-flow and monocrotaline models of PAH, as well as in idiopathic forms of human PAH (Stenmark *et al*., 2006). In addition, PFs can differentiate in response to various stimuli into myofibroblasts, which have been implicated as key participants in tissue remodelling due to their ability to produce collagen, elastin and other extracellular matrix components (Gabbiani, 2003). Accumulation of myofibroblast cells in the intima of patients with pulmonary hypertension has been well documented and consistently observed (Smith *et al*., 1990; Yi *et al*., 2000). Finally, fibroblasts have been shown to release various mediators, including reactive oxygen species, in response to a variety of stresses, and these can elicit paracrine effects on neighbouring PASMCs, stimulating both proliferation and contractile responses (Stenmark *et al*., 2006). Thus, there is an overwhelming body of evidence indicating that PFs play a particularly crucial role in the pulmonary vascular remodelling processes.

As the preparation of PFs requires the use of very large numbers of mice, we chose to investigate potential mechanisms in more depth using CCL39 Chinese hamster lung fibroblasts, as these cells exhibit a very similar pharmacological profile to mouse PFs and have previously been shown to proliferate in response to 5-HT through SERT and 5-HT receptor activation (Lee *et al*., 1999). Specifically, we demonstrated that overexpression of SERT increased ROCK-sensitive PAH, both in normoxia and hypoxia, which was associated with elevated expression of ROCK isoforms. Furthermore, we describe a unique permissive role for ROCK in the control of lung fibroblast proliferation by facilitating productive interaction between mitogen-activated protein kinase/ERK kinase (MEK) and ERK.

Methods

Cell isolation and culture

Fibroblasts were prepared as described by Welsh *et al*. (2004) from pulmonary arteries that were excised from the lungs of wild-type (WT) and SERT+ mice, cut longitudinally and opened into a flat sheet. Exposure of PFs to hypoxia and densitometric scanning of immunoblots for quantification were performed as described previously (Welsh *et al*., 2004).

The preparation of PFs requires the use of very large numbers of mice. Thus, to comply with UK Home Office requirements for animal replacement strategies, we chose to investigate signalling mechanisms in more depth by studying CCL39 Chinese hamster lung fibroblasts. These cells exhibit a very similar pharmacological profile to mouse PFs and have previously been shown to proliferate in response to 5-HT through SERT and 5-HT receptor activation and were cultured as described by Lee *et al*. (1999).

Preparation of cell extracts and immunoblotting

Confluent CCL-39 cells seeded in six-well plates were treated as described in the appropriate figure legends before solubilization in detergent lysis buffer, equalization for protein content and fractionation by discontinuous SDS-polyacrylamide gel electrophoresis on 10 or 12% (w/v) resolving gels for immunoblotting.

[^{3}H]-Thymidine incorporation assay of DNA synthesis
CCL39 cells were plated out at a density of 2×10^4 cells per mL into 96-well plates and grown in full media for 24 h before serum starving for a further 24 h. The media was then replaced with fresh media containing no serum and the appropriate stimuli. After 18 h, 0.5 μCi per well [^{3}H]-thymidine was added and proliferation stopped after a further 6 h by harvesting cells onto glass fibre filter mats using a cell harvester and quantitation of incorporated [^{3}H]-thymidine by liquid scintillation counting. Where dimethyl sulphoxide was used to dissolve drugs, a parallel dimethyl sulphoxide vehicle control (<0.1% (v/v)) was also included. Stimulation indices were calculated as the fold increase from the basal rate of proliferation as determined by [^{3}H]-thymidine incorporation.

In vivo *experimental groups*
All animal procedures and experiments were conducted in accordance with the United Kingdom Animals (Scientific Procedures) Act 1986 and conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health. C57BL/6XCBA strain mice used were either WT or engineered to overexpress human SERT (SERT+). The transgene used was a 500-kb yeast artificial chromosome (YAC35D8) containing a modified human SERT open reading frame modified to include a haemagglutinin epitope tag at its C terminus and a *lacZ* reporter gene downstream of an internal ribosomal entry site, as described previously (MacLean *et al*., 2004). Previous studies have demonstrated that lungs from SERT+ animals display significant overexpression of human SERT, relative to that of the endogenous murine protein, as determined by immunohistochemistry, immunoblotting and reverse transcription-PCR (MacLean *et al*., 2004). For induction of hypoxia, female mice (5–6 months) were placed in a hypobaric chamber. This was depressurized over the course of 2 days to 550 mbar (equivalent to 10% (v/v) O_2). The temperature was maintained at 21–22 °C and the chamber was ventilated with air at approximately 45 L min^{-1}. The duration of hypoxia was 14 days, as previously described (Keegan *et al*., 2001). Female mice were used as SERT+ male mice do not develop PAH (MacLean *et al*., 2004). Preliminary experiments demonstrated that a 14-day exposure to hypoxia was optimal for observing pulmonary artery remodelling, with longer incubation times of up to 21 days producing no significant further increase (MR MacLean and Y Dempsie, unpublished observations). Mice were dosed by oral gavage with either Y27632 (30 mg kg^{-1} day^{-1}) or water alone. Age- and sex-matched Y27632- and water-treated control groups were maintained in room air. Right ventricular pressure (RVP) and pulmonary artery remodelling were assessed immediately after drug treatment.

In vivo *haemodynamic measurements*
Anaesthesia was induced by 3% halothane and subsequently maintained with halothane (1–1.5%) and a mixture of nitrous oxide and oxygen (1:6 ratio). Pressure and heart rate measurements were obtained and analysed as described previously (MacLean *et al*., 2004). Systemic arterial pressure was measured by insertion of a cannula (SIMS Portex, Hythe, Kent; 0.75 mm outer diameter) into a carotid artery. For measurement of systolic RVP (sRVP), a 25-gauge needle was advanced into the right ventricle using a transdiaphragmatic approach. The selection procedure for obtaining correct RVP was based on two criteria; primarily, after termination of the experiment, heart and lungs were removed and the heart examined macroscopically to verify needle puncture of the right ventricle. Second, the RVP waveform had to be arterial in nature, but with a characteristically low diastolic value compared with that observed for systemic arterial pressure. Typically, three different time-point measurements were analysed for each mouse.

Measurement of right ventricular hypertrophy
Right ventricular hypertrophy was assessed by measuring the thickness of the right ventricular free wall (RV) and left ventricle together with the septum (LV+S) to derive RV/LV+S ratios.

Lung histology for assessment of remodelling
Three sagittal sections were obtained from the left lungs. Sections were stained with ElasticaVan Gieson stain and microscopically assessed for muscularization of pulmonary arteries (<80 μm external diameter), as described previously (MacLean *et al*., 1996a). Lung sections from four to seven mice from each group were studied.

Statistical methods
Data are presented as mean ± s.e. unless stated otherwise and were analysed using one-way ANOVA with Newman–Keuls multiple comparison post tests. Where appropriate, Student's two-tailed unpaired *t*-tests were also employed.

Materials
Phospho-MEK1/2 (Ser217/221), total and phospho-ERK1,2 (Thr202/Tyr204) antibodies were from Cell Signalling Technology (Beverly, MA, USA), rabbit polyclonal anti-ROCK1 antibody (catalogue no. AB3885) was from Chemicon International (Hampshire, UK) and mouse monoclonal anti-ROCK2 antibody (catalogue no. 610623) was from BD Transduction Labs (Oxford, UK). Cell-permeable recombinant *Clostridium botulinum* C3 transferase was from Cytoskeleton Inc. (Denver, CO, USA). [Methyl-^{3}H]-thymidine (specific activity 2.0 Ci mmol^{-1}) was from GE Healthcare (Buckinghamshire, UK). ROCK inhibitor Y27632 was from Tocris Bioscience (Bristol, UK). Taqman probes specific for murine ROCK1 and ROCK2 were from Applied Biosystems (Warrington, UK).

Results

Effect of ROCK inhibition on development of PAH in WT and SERT+ mice
Wild-type and SERT+ animals were maintained under control or hypoxic conditions and treated with or without

the ROCK1/2 inhibitor Y27632 (oral, 30 mg kg^{-1} day^{-1}) or vehicle (water) before assessing pulmonary vascular remodelling, sRVP and right ventricular hypertrophy. This dose of Y27632 has previously been shown by others to be effective in reducing the development of PAH (Fagan *et al*., 2004; Nagaoka *et al*., 2005). Pulmonary vascular remodelling and sRVP were each elevated by SERT overexpression, and these changes were blocked by inhibition of ROCK (Figures 1a and b). In WT mice, hypoxia triggered remodelling of the pulmonary vasculature and increased sRVP, both of which were reduced by ~25% by Y27632 (Figures 1a and b). In SERT+ mice, hypoxia-induced remodelling and hypoxia-induced sRVP elevation were ~35% greater than those in WT mice, and Y27632 inhibited these effects by ~50% (Figures 1a and b). This suggests that the component of both hypoxia-induced pulmonary vascular remodelling and hypertension dependent on ROCK activity is increased when SERT is overexpressed and intracellular 5-HT levels are elevated. The development of right ventricular hypertrophy in response to chronic hypoxia was similar for WT and SERT+ animals. However, the inhibition of pulmonary vascular remodelling and pulmonary pressures by ROCK inhibitor Y27632 was reflected in a decrease in right ventricular hypertrophy in SERT+ hypoxic mice (Figure 1c). Neither hypoxia, SERT overexpression nor treatment with Y27632 produced any significant effects on mean systemic arterial pressure (Figure 1d) or heart rate (data not shown).

The *in vivo* results suggested that remodelling due to SERT overexpression was mediated in part by ROCK activity. Consistent with this hypothesis, both ROCK1 and ROCK2 protein levels were significantly elevated in PFs prepared from normoxic SERT+ mice compared with WT mice (Figure 1e). In addition, hypoxia increased the levels of ROCK1 and ROCK2 protein in WT mice (Figure 1e). Interestingly, levels of ROCK1 were significantly reduced in hypoxic SERT+ mice, compared with those in normoxic SERT+ mice (Figure 1e). In contrast, ROCK2 levels remained elevated and were not significantly reduced in hypoxic versus normoxic SERT+ animals (Figure 1e).

5-HT receptor-mediated ERK activation and lung fibroblast proliferation

Our *in vivo* results supported our hypothesis that overexpression of SERT (resulting in increased intracellular 5-HT levels) increases ROCK-dependent pulmonary vascular remodelling *in vivo* through 5-HT-dependent proliferation. We wished to test our other hypothesis that 5-HT-induced phosphorylation and activation of ERK is dependent on increased ROCK expression and chose to carry out these experiments in CCL39 cells, as explained above. As 5-HT-mediated proliferation is dependent on ERK phosphorylation (Liu *et al*., 2004), we first confirmed this phenomenon in our own experimental system. 5-HT induced a transient increase in ERK phosphorylation, which peaked between 1 and 2 min, before returning to basal levels by 30 min (Figure 2a). Exposure to 5-HT for up to 24 h failed to reveal a second phase of activation, like that often observed with mitogenic stimuli (data not shown). Interestingly, this transient activation of ERK was absolutely required for the mitogenic effect of 5-HT, as pretreatment with MEK inhibitor U0126 at a concentration that largely abolished 5-HT-stimulated ERK phosphorylation (Figure 2b) also blocked the ability of 5-HT to stimulate DNA synthesis, as determined by [^{3}H]-thymidine incorporation (Figure 2c).

Pharmacology of 5-HT-mediated ERK activation

Selective blockade of 5-HT$_{1B/1D}$ or 5-HT$_{2A}$ responses with GR55562 or ketanserin, respectively, produced equivalent inhibitory effects on [^{3}H]-thymidine incorporation and ERK phosphorylation (phosphorylation at 10 μM 5-HT reduced by 57 ± 12% in response to GR55562 versus vehicle-treated control, $P<0.001$, $n=3$; phosphorylation at 10 μM 5-HT reduced by 50 ± 15% in response to ketanserin versus vehicle-treated control, $P<0.05$, $n=3$) (Figures 3a and b). In addition, blockade of SERT by citalopram pretreatment also attenuated [^{3}H]-thymidine incorporation and ERK phosphorylation (phosphorylation at 10 μM 5-HT reduced by 44 ± 9% in response to citalopram versus vehicle-treated control, $P<0.05$, $n=3$) (Figure 3c). Thus, although 5-HT appears to stimulate ERK activation and proliferation by mechanisms involving multiple receptors and SERT, antagonism of any one of these pathways is sufficient to markedly inhibit ERK activation and block proliferation.

Involvement of the Rho/ROCK pathway in 5-HT-stimulated ERK activation and proliferation

Inhibition of ROCK with Y27632 *in vivo* reduced the exaggerated pulmonary vascular remodelling observed in both normoxic and hypoxic SERT+ mice. We therefore assessed directly the influence of the Rho/ROCK pathway on 5-HT-mediated mitogenic responses *in vitro*. At a concentration of 5 μM, the ROCK inhibitor Y27632 specifically inhibited 5-HT- but not phorbol 12-myristate 13-acetate-stimulated ERK activation and [^{3}H]-thymidine incorporation (Figures 4a and b). This supported and proved our hypothesis that 5-HT-induced phosphorylation of ERK specifically requires ROCK activity and, to our knowledge, reveals an interaction of ROCK with signalling events upstream of ERK phosphorylation that has not previously been described in pulmonary fibroblast cells. Y27632 also abolished the ability of 5-HT to promote the accumulation of cyclin D1 (Figure 4c). The importance of the Rho/ROCK pathway was further confirmed independently using a cell-permeable recombinant *Clostridium* C3 transferase that catalyses ADP ribosylation and inactivation of endogenous Rho proteins (Aktories *et al*., 2004). These experiments demonstrated that C3 transferase treatment of CCL39 cells inhibited 5-HT-stimulated ERK phosphorylation to a similar extent as pretreatment with a ROCK inhibitor (Figure 4d). Thus, optimal 5-HT-mediated activation of ERK, cyclin D1 accumulation and proliferation in lung fibroblasts appear to require proper functioning of the Rho/ROCK pathway.

Interestingly, under conditions in which ERK phosphorylation was reduced, Y27632 had no effect on the ability of 5-HT to stimulate the phosphorylation of MEK (Figure 5a), suggesting that ROCK either enhanced the ability of ERK to

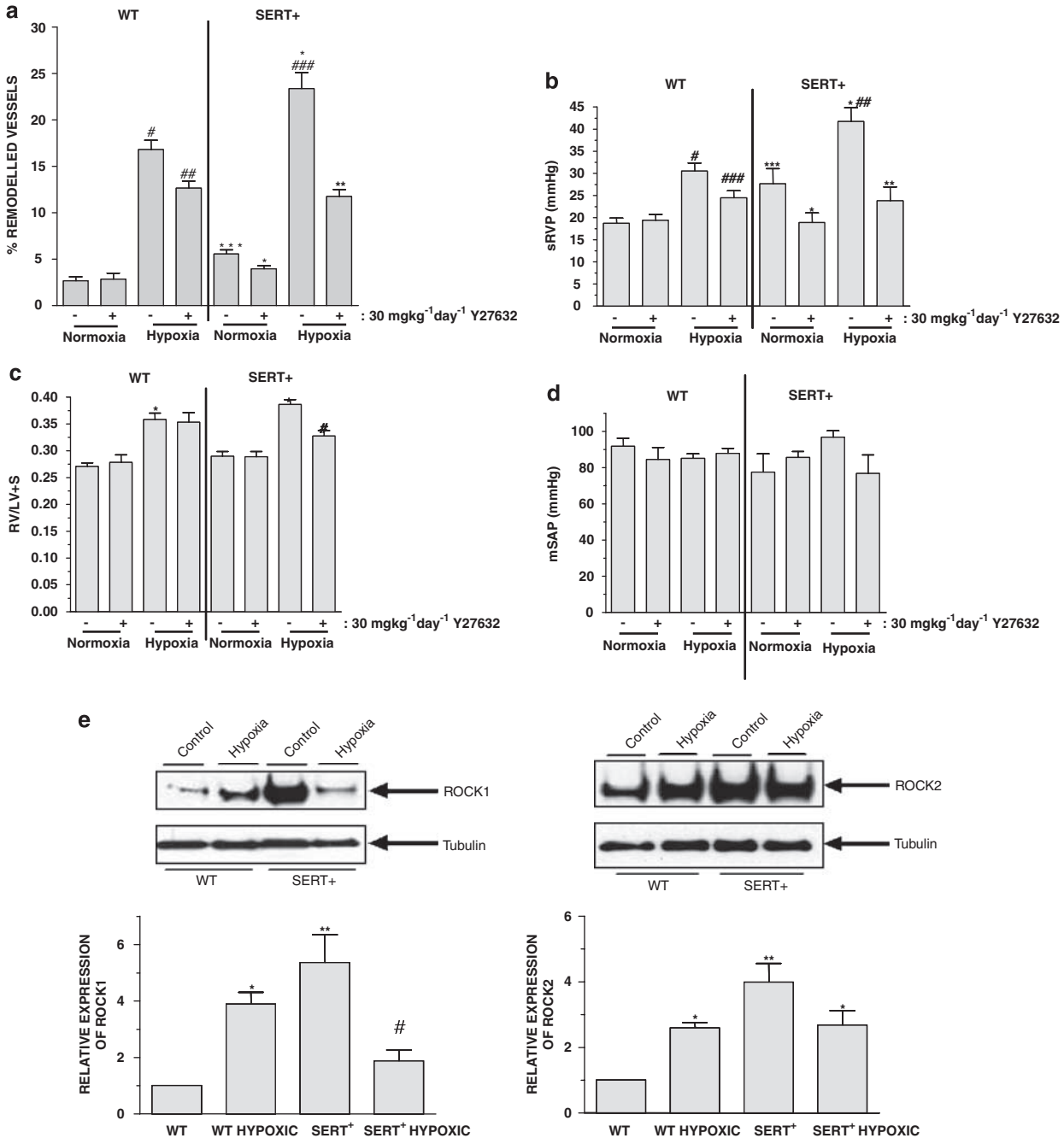

Figure 1 Effects of SERT overexpression and hypoxia on ROCK expression and pulmonary haemodynamics. Wild-type mice (WT) and mice overexpressing the serotonin transporter (SERT+) were treated with the ROCK inhibitor Y27632 (30 mg kg^{-1} day^{-1}) (+) or vehicle (−) and maintained in either normoxic or hypoxic conditions. (**a**) Pulmonary vascular remodelling. Data are from four to seven animals ($^{\#}P<0.001$ versus normoxic WT animals treated with vehicle, $^{\#\#}P<0.01$ versus hypoxic WT animals treated with vehicle, $^{\#\#\#}P<0.001$ versus hypoxic WT animals treated with vehicle, $^{*}P<0.001$ versus normoxic SERT+ animals treated with vehicle, $^{**}P<0.001$ versus hypoxic SERT+ animals treated with vehicle, $^{***}P<0.05$ versus normoxic WT animals treated with vehicle). (**b**) Systolic right ventricular pressures (RVPs). Data are from five to eight animals ($^{\#}P<0.01$ versus normoxic WT animals treated with vehicle, $^{\#\#}P<0.05$ versus hypoxic WT animals treated with vehicle, $^{\#\#\#}P<0.05$ versus hypoxic WT animals treated with vehicle, $^{*}P<0.001$ versus normoxic SERT+ animals treated with vehicle, $^{**}P<0.001$ versus hypoxic SERT+ animals treated with vehicle, $^{***}P<0.05$ versus normoxic WT animals treated with vehicle). (**c**) Right ventricular hypertrophy (RV/LV+S ratios). Data are from 7 to 15 animals ($^{*}P<0.001$ versus normoxic animals, $^{\#}P<0.01$ versus hypoxic SERT+ animals treated with vehicle). (**d**) Mean systemic arterial pressures (mSAPs). Data are from five to eight animals. No statistical differences were detectable between any of the groups. (**e**) ROCK1 and ROCK2 protein levels in pulmonary artery fibroblasts from WT and SERT+ mice: effect of 24 h hypoxia. The upper panels are representative blots and the lower panels show quantitative analysis of combined densitometric data from three experiments ($^{*}P<0.05$, $^{**}P<0.01$ versus WT normoxic animals; $^{\#}P<0.05$ versus SERT+ normoxic animals). ROCK, Rho kinase.

be phosphorylated by active MEK or suppressed the activity/expression of dual-specificity phosphatases such as MKP-3/Pyst1 (Dickinson and Keyse, 2006). However, cellular pretreatment with a concentration (200 μM) of a broad-specificity tyrosine phosphatase inhibitor, sodium orthovanadate, sufficient to increase basal ERK phosphorylation,

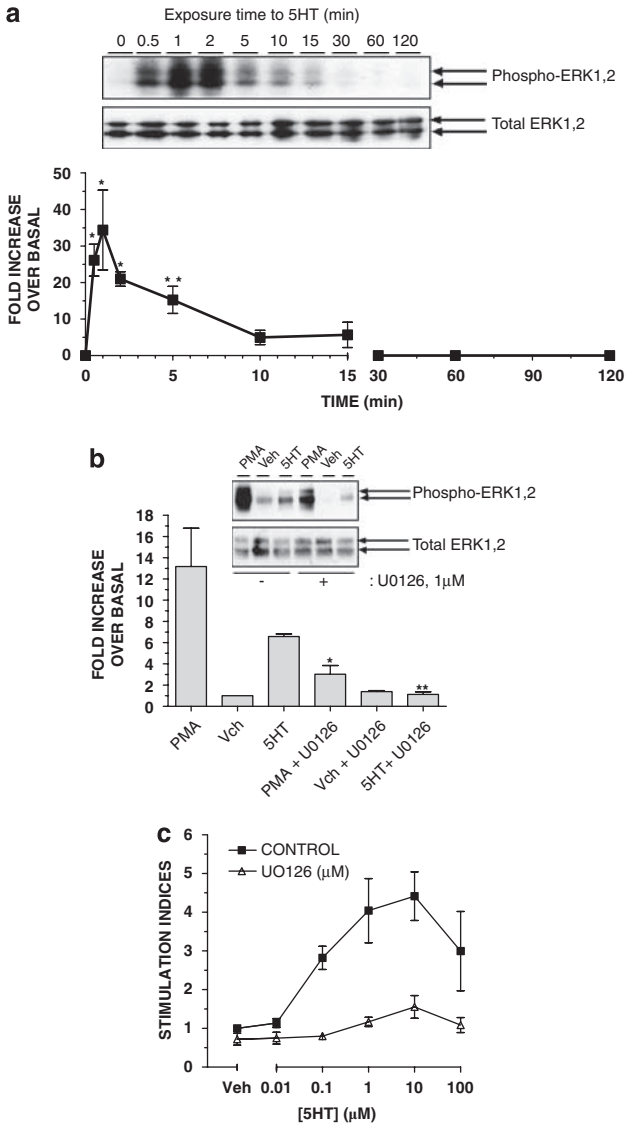

Figure 2 5-HT-stimulated proliferation of CCL39 hamster lung fibroblasts is ERK-dependent. (**a**) Time course of 5-HT (10 μM) effects on ERK phosphorylation in CCL39 cells. A representative blot and quantitation of densitometry data from ($n=3$) experiments are shown (*$P<0.05$, **$P<0.01$ versus vehicle-treated controls). (**b**) Upper: effect of pretreatment with the MEK inhibitor U0126 (1 μM) on ERK phosphorylation in CCL39 cells in response to either 1 μM PMA or 10 μM 5-HT. A representative blot and combined data from three experiments are shown (*$P<0.05$ versus PMA alone, **$P<0.001$ versus 5-HT alone). (**c**) Effect of 1 μM U0126 pretreatment on [^{3}H]-thymidine incorporation in CCL39 cells after stimulation with increasing concentrations of 5-HT for 18 h. Data are from three experiments. ERK, extracellular signal-regulated kinase; MEK, mitogen-activated protein kinase/ERK kinase; PMA, phorbol 12-myristate 13-acetate.

failed to block the inhibitory effect of Y27632 on 5-HT-stimulated ERK phosphorylation (Figure 5b). Thus, the Rho/ROCK pathway is able to support 5-HT-mediated stimulation of ERK downstream of Raf by allowing efficient phosphorylation of ERK by active MEK.

Having shown that 5-HT receptors couple positively to ERK in CCL39 cells, selective agonists were used to determine whether specific 5-HT receptor subtypes were under the control of ROCK. Interestingly, under conditions in which 5-HT-mediated ERK phosphorylation was inhibited by Y27632, the ERK response to the 5-HT$_{2A}$-selective agonist, α-methyl-5-HT, was unaffected. In contrast, ERK phosphorylation following treatment with 5-HT$_{1B}$-selective agonist CP93129 was largely abolished (Figure 5c). Together, these data suggest that although multiple 5-HT receptors are capable of activating ERK in CCL39 cells, it is the activation of this pathway through the 5-HT$_{1B}$ receptor that is selectively regulated by Rho/ROCK.

Discussion

Inhibition of ROCK has now been shown to be effective in attenuating PAH in several animal models including the hypoxic, fawn-hooded rat and monocrotaline models (Abe *et al*., 2004; Fagan *et al*., 2004; Nagaoka *et al*., 2006). In this study, we have tested and proved the novel hypothesis that optimal 5-HT-induced phosphorylation and activation of ERK requires ROCK activity and/or increased ROCK expression and that overexpression of SERT, and the resultant intracellular accumulation of 5-HT, increases ROCK-dependent pulmonary vascular remodelling *in vivo* and *in vitro* through potentiation of 5-HT-dependent proliferation. Furthermore, we have identified the 5-HT$_{1B}$ receptor as being responsible for mediating ROCK-sensitive ERK activation. This is the first description of an interaction of ROCK upstream of the ERK phosphorylation/activation step in pulmonary cells.

As SERT overexpression may be a risk factor for PAH (Willers *et al*., 2006), we wished to test the hypothesis that overexpression of SERT and the concomitant increase in intracellular 5-HT levels would potentiate ROCK-dependent pulmonary vascular remodelling *in vivo*. We have previously shown that SERT+ mice are prone to elevations in pulmonary pressure and hypoxia-induced PAH (MacLean *et al*., 2004). The results in the current study confirm that SERT overexpression triggered increases in sRVP and pulmonary vascular remodelling and, unique to this study, we have demonstrated that these changes were sensitive to ROCK inhibition. Consistent with these observations, both ROCK1 and ROCK2 protein levels were elevated in normoxic PFs derived from SERT+ mice compared with their WT controls. This is the first time that overexpression of SERT has been associated with an increase in ROCK expression under normoxic conditions. Previous studies have shown that monocrotaline-induced PAH can be inhibited by ROCK inhibition (Abe *et al*., 2004). It is of interest, therefore, that monocrotaline-induced PAH is also associated with increased pulmonary arterial SERT expression and increased ROCK activity (Jiang *et al*., 2007; Laudi *et al*., 2007), and these studies together suggest that increased SERT activity may contribute to PAH through increased ROCK activation.

ROCK controls pulmonary arterial tone largely by its ability to phosphorylate and inactivate myosin phosphatase, thereby increasing the levels of phosphorylated myosin light chain and inducing contraction (Guilluy *et al*., 2005; Hyvelin *et al*., 2005; Noma *et al*., 2006). It has also been recently reported that 5-HT receptors can activate the Rho/ROCK

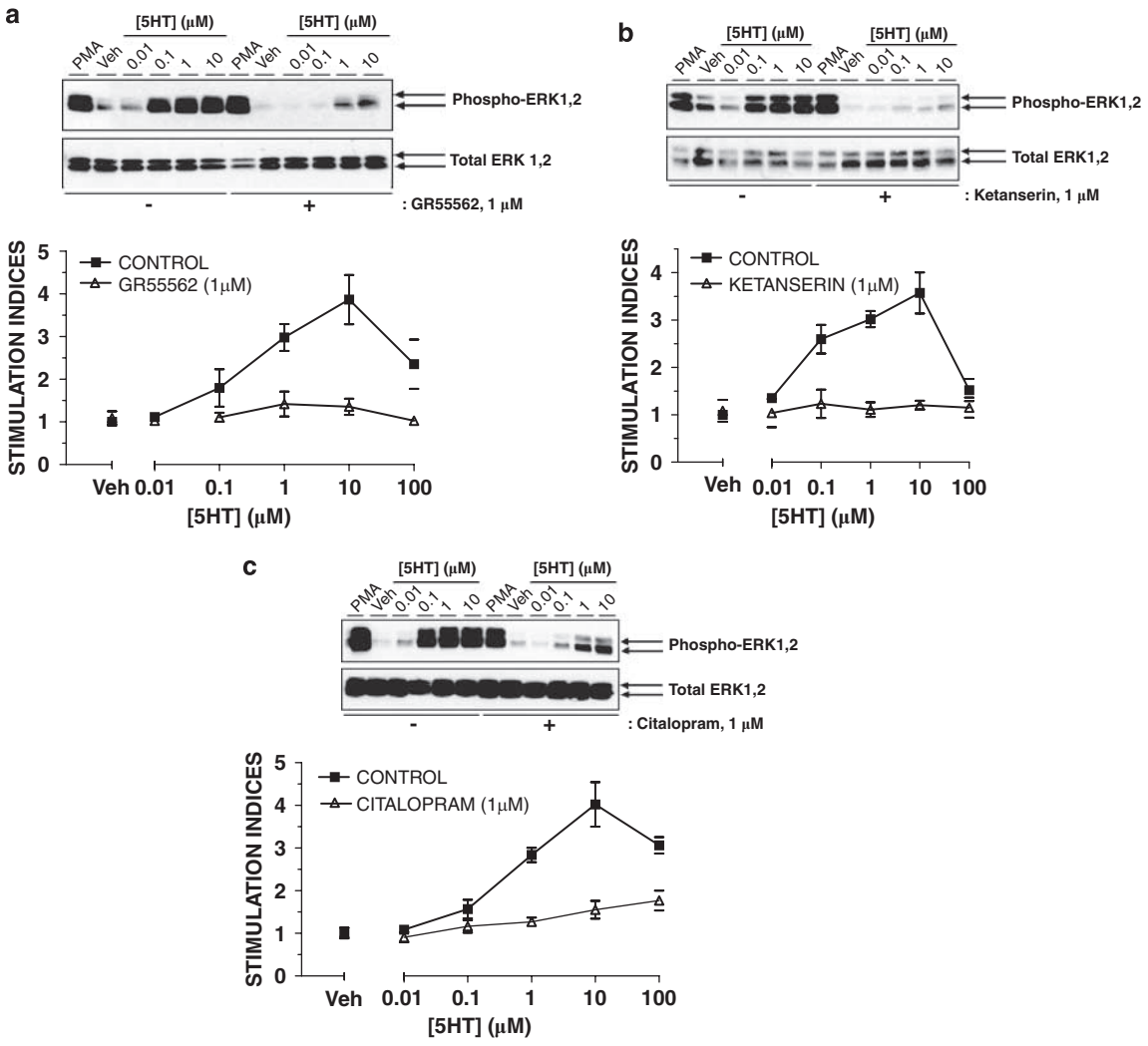

Figure 3 Pharmacology of 5-HT-mediated ERK activation and fibroblast proliferation. (**a**) Upper—effect of the 5-$HT_{1B/D}$-selective antagonist GR55562 (1 μM) on 5-HT-stimulated ERK phosphorylation in CCL39 cells. A representative blot from three experiments is shown. Lower—effect of 1 μM GR55562 on [^{3}H]-thymidine incorporation in CCL39 cells after stimulation with increasing concentrations of 5-HT for 18 h. Data are combined from three experiments. (**b**) Upper—effect of 1 μM of the 5-HT_{2A} receptor-selective antagonist ketanserin on 5-HT-stimulated ERK phosphorylation in CCL39 cells. A representative blot from three experiments is shown. Lower—effect of 1 μM ketanserin on [^{3}H]-thymidine incorporation in CCL39 cells after stimulation with increasing concentrations of 5-HT for 18 h. Data are combined from three experiments. (**c**) Upper—effect of 1 μM of the SERT inhibitor citalopram on ERK phosphorylation in CCL39 cells. Representative blot of three experiments. Lower—effect of the SERT inhibitor citalopram (1 μM) on [^{3}H]-thymidine-incorporation in CCL39 cells before stimulation with increasing concentrations of 5-HT for 18 h. Data are from three experiments. ERK, extracellular signal-regulated kinase.

pathway in hypertensive pulmonary arteries isolated from chronically hypoxic rats (Homma *et al*., 2007). However, our studies both confirm and extend a growing appreciation of the importance of Rho/ROCK signalling in the development of PAH, although the mechanism we describe by which Rho/ROCK influences cellular proliferation in response to 5-HT is unique. A previous study by Liu *et al* (2004) described how ROCK activation through the 5-HT_{1B} receptor might subsequently facilitate the nuclear translocation of phosphorylated active ERK, rather than facilitate ERK activation *per se*. Our results in pulmonary fibroblast cells differ from this PASMC model. Our results suggest that (1) 5-HT enters the cell through the transporter and this activates the Rho/ROCK pathway and (2) ERK activation is downstream from both the 5-HT_{1B} receptor and SERT. ROCK1 and ROCK2 play a role in the activation of ERK downstream from Raf by allowing efficient MEK phosphorylation of ERK. Thus, although Liu *et al*. (2004) and other studies (Croft and Olson, 2006; Im and Kazlauskas, 2007) have established that there is extensive cross talk between the Rho/ROCK and ERK pathways in different cell systems, the relationship between SERT, 5-HT and ROCK that we describe may be unique to pulmonary fibroblasts. Our suggestion that 5-HT enters pulmonary fibroblasts through SERT and subsequently activates the Rho/ROCK pathway is supported by the recent observation that 'serotonylation' of small GTPases (Walther *et al*., 2003) occurs in aortic smooth muscle cells in which 5-HT is transamidated to the small G protein RhoA by transglutaminase, a process that is dependent on SERT and 5-HT_{1B} receptor activity. The more recent observation that

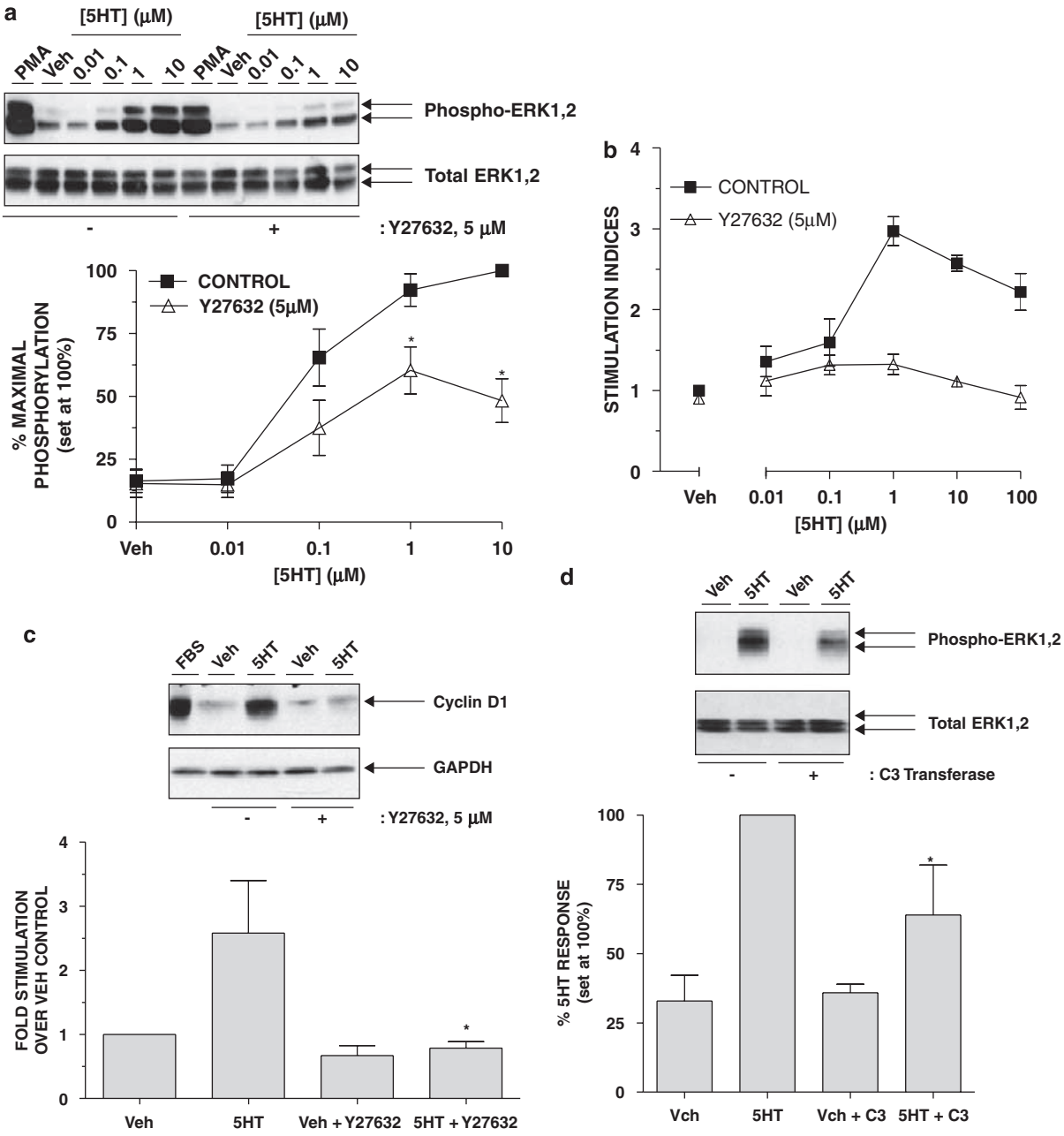

Figure 4 Effects of Rho/ROCK inhibition on 5-HT-stimulated ERK activation and fibroblast proliferation. (**a**) Upper—effect of 5 μM of the ROCK inhibitor Y27632 on ERK phosphorylation in CCL39 cells before or after stimulation with either 1 μM PMA or increasing concentrations of 5-HT and preparation of soluble cell extracts. A representative blot from four experiments is shown. Lower—quantitation of combined densitometric data from four experiments (*$P<0.05$ versus corresponding vehicle-treated controls). (**b**) Effect of 5 μM of the ROCK inhibitor Y27632 on [^{3}H]-thymidine incorporation in CCL39 cells before stimulation with increasing concentrations of 5-HT for 18 h. Data are combined from three experiments. (**c**) Effect of 5 μM of the ROCK inhibitor Y27632 on cyclin D1 expression after stimulation with or without 10 μM 5-HT for 6 h (FBS = cyclic D-positive extracts from foetal bovine serum-treated cells). A representative blot from three experiments is shown. Lower—quantitation of combined densitometric data from three experiments (*$P<0.05$ versus 5-HT alone). (**d**) Effects of 2 μg mL^{-1} cell-permeable C3 transferase on ERK phosphorylation in CCL39 cells before or after stimulation with or without 10 μM 5-HT. A representative blot from three experiments is shown. Lower—quantitation of combined densitometric data from three experiments (*$P<0.05$ versus 5-HT alone). ERK, extracellular signal-regulated kinase; PMA, phorbol 12-myristate 13-acetate; ROCK, Rho kinase.

transamidation of 5-HT to RhoA can occur in the pulmonary artery of hypoxic rats supports the suggestion that this process may participate in pulmonary artery remodelling and hypertension (Guilluy *et al.*, 2007). However, future studies will need to examine the relative contributions of transamidation versus more straightforward mechanisms involving guanine nucleotide exchange factors in controlling activation of the Rho/ROCK pathway in pulmonary fibroblasts.

Previous studies in mice have already demonstrated that inhibition of SERT or the 5-HT$_{1B}$ receptor attenuates hypoxia-induced PAH (Eddahibi *et al.*, 2000; Keegan *et al.*,

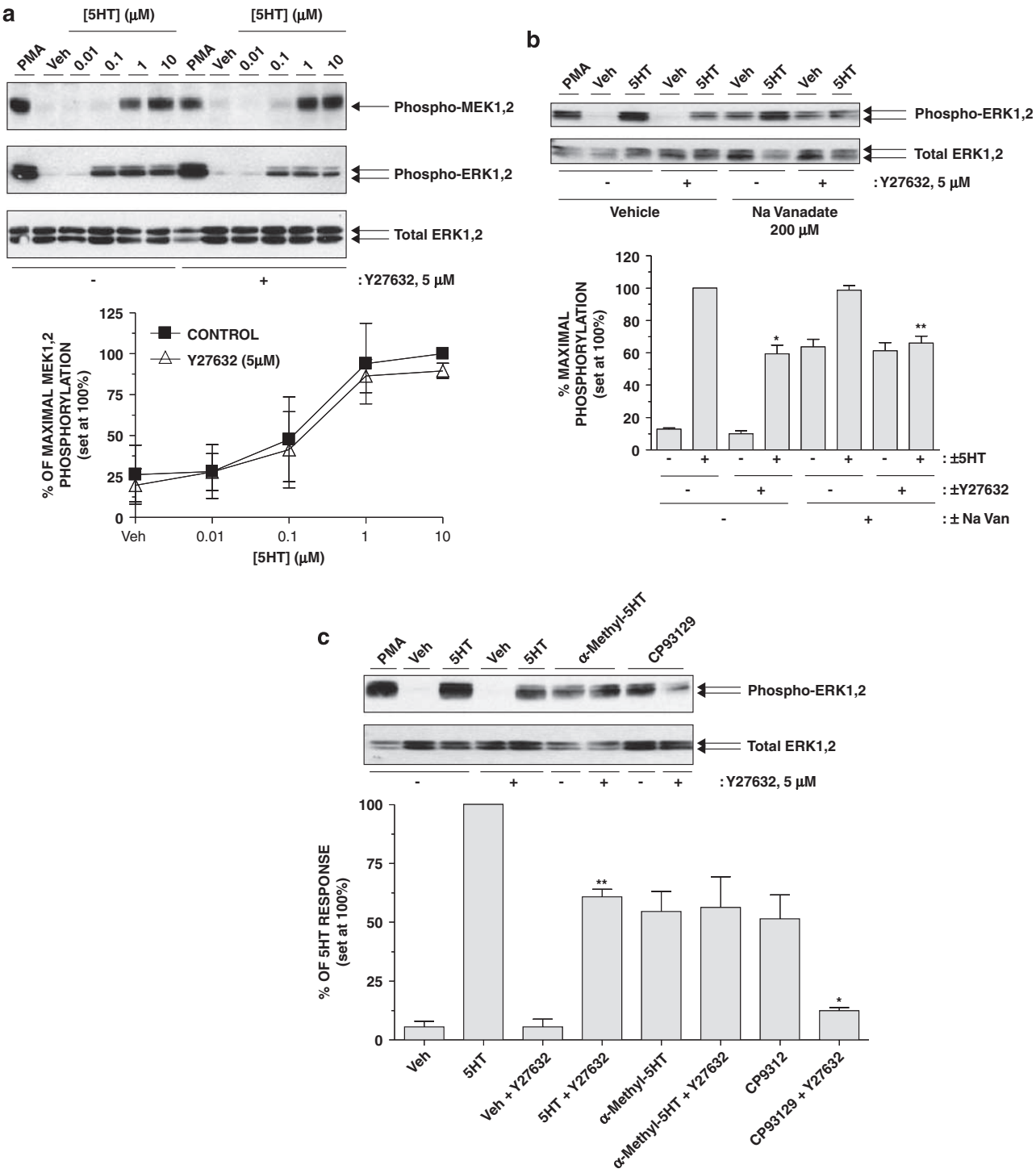

Figure 5 ROCK selectively regulates MEK–ERK interaction in response to 5-HT_{1B} receptor stimulation. (**a**) Upper—effect of 5 μM Y27632 on MEK and ERK phosphorylation before or after stimulation with either 1 μmol L^{-1} PMA or increasing concentrations of 5-HT. A representative blot from four experiments is shown. Lower—quantitative analysis of MEK phosphorylation; combined data from four experiments. (**b**) Upper—effect of 5 μM Y27632 or 200 μM sodium vanadate (Na Van) on ERK phosphorylation after stimulation with either 1 μM PMA or 10 μM 5-HT. A representative blot from three experiments is shown. Lower—quantitation of combined densitometric data from three experiments (*$P<0.05$ versus 5-HT-stimulated ERK phosphorylation, **$P<0.05$ versus vanadate-pretreated 5-HT-stimulated ERK phosphorylation). (**c**) Upper —effect of 5 μM Y27632 on ERK phosphorylation after stimulation with either vehicle (Veh), 1 μM PMA, 10 μM 5-HT, 1 μM α-methyl-5-HT (5-HT_{2A} agonist) or 1 μM CP93129 (5-HT_{1B} agonist). A representative blot from three experiments is shown. Lower—quantitation of combined densitometric data from three experiments (**$P<0.05$ versus 5-HT-stimulated ERK phosphorylation, *$P<0.05$ versus CP93129-stimulated ERK phosphorylation). ERK, extracellular signal-regulated kinase; MEK, mitogen-activated protein kinase/ERK kinase; PMA, phorbol 12-myristate 13-acetate; ROCK, Rho kinase.

2001). It is also well established that ROCK inhibition decreases hypoxia-induced PAH (Fagan *et al*., 2004; Abe *et al*., 2006). We wished to examine the effect of SERT overexpression on this phenomenon and further investigate our hypothesis that overexpression of SERT, and thus increased intracellular 5-HT levels, would result in a potentiation of ROCK-dependent pulmonary vascular remodelling. As predicted by previous studies, in WT mice, Y27632 inhibited hypoxia-induced pulmonary remodelling and also hypoxia-induced increases in sRVP. Confirming our previous studies (MacLean *et al*., 2004), hypoxia-induced remodelling and hypoxia-induced RVP elevation were greater in SERT+ mice than in WT mice. The effect of ROCK inhibition was twofold greater on these indices than in WT mice, suggesting that the component of hypoxia-induced pulmonary vascular remodelling and hypertension that is dependent on ROCK activity is increased when SERT is overexpressed and intracellular 5-HT levels are elevated. The development of right ventricular hypertrophy in response to chronic hypoxia was similar in WT and SERT+ animals as we have previously described (MacLean *et al*., 2004). However, the inhibition of pulmonary vascular remodelling and pulmonary pressures by Y27632 was reflected in a decrease in right ventricular hypertrophy in SERT+ hypoxic mice. These changes were restricted to the pulmonary system as neither hypoxia, SERT overexpression nor treatment with Y27632 had any significant effects on mean systemic arterial pressure or heart rate. Y27632 inhibited both pulmonary vascular remodelling and sRVP and hence ROCK may influence both vascular tone and remodelling. Nevertheless, our results would suggest that inhibition of 5-HT-mediated ERK-dependent proliferation by Y27632 may have contributed to the observed reduction in pulmonary vascular remodelling. Hypoxia has previously been shown to increase ROCK expression *in vivo* (Guilluy *et al*., 2005; Jin *et al*., 2006; McNamara *et al*., 2008). Given that *in vivo*, SERT overexpression increased the component of hypoxia-induced pulmonary vascular remodelling and hypertension that is dependent on ROCK activity, we wished to examine the effect of SERT overexpression on ROCK1 and ROCK2 proteins at a cellular level. The results show that, as predicted by previous studies but not previously demonstrated in isolated PFs, hypoxia induced an increase in ROCK1 and ROCK2 levels in PFs derived from WT mice. Curiously, although ROCK2 expression remained elevated in PFs from SERT+ animals following the induction of hypoxia, ROCK1 levels declined markedly. However, the mechanisms behind this phenomenon are unclear and are beyond the scope of the current study.

In conclusion, we have, for the first time, demonstrated that *in vivo* overexpression of SERT leads to an increase in ROCK-sensitive PAH both in normoxia and in hypoxia. Furthermore, our results demonstrate a novel interaction between the SERT, 5-HT_{1B} receptors, ROCK and ERK in pulmonary fibroblasts. Specifically, they suggest that 5-HT internalized through SERT activates the Rho/ROCK pathway, which plays a permissive role in 5-HT_{1B} receptor-mediated activation of ERK downstream from Raf by allowing efficient phosphorylation of ERK by its upstream kinase MEK. Defining the mechanisms by which ROCK specifically regulates 5-HT_{1B}-mediated activation of ERK will undoubtedly shed new light on how these critical pathways interact to trigger fibroblast proliferation in PAH.

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Conflict of interest

The authors state no conflict of interest.

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