Silencing of p21-activated kinase attenuates vimentin phosphorylation on Ser-56 and reorientation of the vimentin network during stimulation of smooth muscle cells by 5-hydroxytryptamine

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Vimentin intermediate filaments undergo spatial reorganization in endothelial cells and fibroblasts in response to stimulation with platelet-derived growth factor and epidermal growth factor. In the present study, the vimentin network exhibited a curved filamentous structure in unstimulated smooth muscle cells. Vimentin filaments became straight and were arranged along the long axis of cells upon stimulation with 5-hydroxytryptamine (5-HT; serotonin). Stimulation of smooth muscle cells with 5-HT also induced phosphorylation of vimentin on Ser-56. Treatment of cells with small interfering RNA selectively down-regulated the expression of PAK1 (p21-activated kinase 1) without affecting the content of smooth muscle α -actin. The silencing of PAK1 inhibited the site-specific phosphorylation and spatial rearrangement of the vimentin network in response to stimulation with 5-HT. Neither the disruption of stress fibres by cytochalasin D

nor the inhibition of protein tyrosine phosphorylation affects the spatial reorganization of vimentin intermediate filaments in response to stimulation with 5-HT. In addition, stimulation of smooth muscle cells with 5-HT increased the ratio of soluble to insoluble vimentin. PAK1 silencing attenuated increases in the ratio of soluble to insoluble vimentin upon stimulation with 5-HT. These results suggest that the PAK-mediated site-specific phosphorylation of vimentin may play a role in regulating the reorganization of vimentin intermediate filaments during stimulation of smooth muscle cells with 5-HT.

Key words: cytoskeleton, contraction, 5-hydroxytryptamine (serotonin), intermediate filament, p21-activated kinase (PAK), vimentin.

INTRODUCTION

The intermediate filament network, myofilaments and microtubules comprise the cytoskeletal systems of smooth muscle cells. Intermediate filaments connect to dense bodies in the cytoplasm and to desmosomes at the membrane [1–3]. There are five types of structurally related intermediate filament proteins; their expression is cell-type specific [4]. In airway and vascular smooth muscle cells, vimentin is a major intermediate filament protein [5,6].

Until recently, the intermediate filament network was thought to be a fixed structure that maintains the integrity of cell architecture. Studies on endothelial cells and fibroblasts indicate that the spatial reorganization of vimentin intermediate filaments occurs in response to stimulation with PDGF (platelet-derived growth factor) and EGF (epidermal growth factor) [7,8]. The reorganization of vimentin filaments was also observed in fibroblasts overexpressing the small GTPases Cdc42 and Rac, which can be activated by PDGF and EGF [7,9]. Moreover, external stimulation alters the amount of soluble (depolymerized) vimentin in chromaffin cells, human fibroblasts, rat RVF-SM cells and BHK-21 fibroblasts [10–12].

The reorganization of the intermediate filament network may be regulated by phosphorylation of vimentin on serine/threonine residues [13–15]. Stimulation by angiotensin II induces vimentin phosphorylation in cultured rat aortic vascular smooth muscle cells [16]. *In vitro* studies have shown that a major phosphoryl-

ation site on mouse vimentin is Ser-55, which is similar to Ser-56 in human vimentin [14] (NCBI accession number XM 167414). Phosphorylation of vimentin on this site is associated with mitosis in mouse connective tissue cells, bovine kidney cells and human glioma cells [14,15].

PAK (p21-activated kinase) has been shown to phosphorylate vimentin in *in vitro* studies [17]. PAK is a serine/threonine kinase that was first identified as a Rac-interacting protein [18]. So far, six isoforms of the PAK family have been identified in mammalian cells; PAK1 is a major isoform in smooth muscle cells [19]. PAK may be involved in force generation in smooth muscle; introduction of a constitutively active PAK protein into skinned smooth muscle strips induces a slow tension development at low intracellular Ca^{2+} concentrations [20]. However, the role of PAK in regulating the phosphorylation and spatial organization of vimentin intermediate filaments in smooth muscle cells has not been investigated.

The present study was undertaken to assess the spatial rearrangement, site-specific phosphorylation and depolymerization of vimentin filaments in smooth muscle cells upon stimulation with 5-HT (5-hydroxytryptamine; serotonin), a well-known contractile agonist. We also evaluate the role of PAK in regulating the reorganization of vimentin intermediate filaments. Our results suggest that vimentin undergoes reorganization and phosphorylation on Ser-56 in response to stimulation with 5-HT. PAK plays an essential role in the regulation of vimentin spatial reorganization and phosphorylation in smooth muscle cells.

Abbreviations used: CAS, p130 Crk-associated substrate; DMEM, Dulbecco's modified Eagle's medium; ECL, enhanced chemiluminescence; EGF, epidermal growth factor; FAK, focal adhesion kinase; HBSS, Hanks balanced salt solution; 5-HT, 5-hydroxytryptamine (serotonin); PAK, p21-activated kinase; PDGF, platelet-derived growth factor; siRNA, small interfering RNA.

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EXPERIMENTAL

Cell culture

Six to eight canine tracheal muscle strips (0.5 mm × 0.2 mm × 8 mm) were incubated for 25 min with 5 ml of dissociation solution [130 mM NaCl, 5 mM KCl, 1.0 mM CaCl₂, 1.0 mM MgCl₂, 10 mM Hepes, 0.25 mM EDTA, 10 mM D-glucose, 10 mM taurine, pH 7, 400 units/ml collagenase (type I), 30 units/ml papain (type IV), 1 mg/ml BSA and 1 mM dithiothreitol]. All enzymes were obtained from Sigma. The strips were then washed three times with a Hepes-buffered saline solution (composition, in mM: 10 Hepes, 130 NaCl, 5 KCl, 10 glucose, 1 CaCl₂, 1 MgCl₂, 0.25 EDTA, 10 taurine, pH 7), and they were triturated with a pipette to liberate individual smooth muscle cells from the tissue. The cell suspension was mixed with DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% (v/v) fetal bovine serum and antibiotics (100 units/ml penicillin, 100 µg/ml streptomycin and 2.5 µg/ml amphotericin-B). Cells were cultured in 100-mm dishes at 37°C in the presence of 5% CO₂ in the same DMEM. The medium was changed every 3–4 days until the cells reached confluence, and confluent cells were passaged with trypsin/EDTA solution. Cells within passage 4 were then serum-deprived 24 h before biochemical and immunofluorescence/fluorescence experiments. These smooth muscle cells in culture express high levels of smooth-muscle-specific α -actin, as determined by immunoblot analysis.

Immunoprecipitation

Cells in dishes were stimulated with 5-HT or left unstimulated. These cells were then washed with ice-cold HBSS (Hanks balanced salt solution; Invitrogen, Carlsbad, CA, U.S.A.). They were mixed with extraction buffer containing 20 mM Tris/HCl, pH 7.4, 2% Triton X-100, 0.2% SDS, 2 mM EDTA, phosphatase inhibitors (2 mM sodium orthovanadate, 2 mM molybdate and 2 mM sodium pyrophosphate) and protease inhibitors (2 mM benzamidine, 0.5 mM aprotinin and 1 mM PMSF). Each sample was centrifuged at 15 800 g for 20 min at 4°C for the collection of supernatant. Cell extracts containing equal amounts of protein were precleared for 30 min with 50 µl of 10% (w/v) Protein A-Sepharose (Sigma) to remove cellular proteins that associate non-specifically with Protein A. The precleared extracts were centrifuged at 15 800 g for 2 min. The supernatant was then incubated with monoclonal antibody against vimentin (clone RV202; BD Biosciences, San Diego, CA, U.S.A.; 1:2000 dilution) or CAS (p130 Crk-associated substrate, clone 21; BD Biosciences; 1:1500 dilution) overnight, followed by the addition of 10% (w/v) Protein A-Sepharose conjugated to rabbit anti-(mouse Ig). Immunocomplexes were washed three times in Tris-buffered saline containing 0.1% Triton X-100. All immunoprecipitation procedures were performed at 4°C.

Analysis of protein phosphorylation

The immunoprecipitates or cell extracts were boiled in sample buffer [1.5% dithiothreitol, 2% SDS, 80 mM Tris/HCl, pH 6.8, 10% (v/v) glycerol and 0.01% Bromophenol Blue] for 4 min and separated by SDS/PAGE. Proteins were transferred to a nitrocellulose membrane, after which the membrane was blocked with 2% (w/v) gelatin for 1 h and probed with site-specific, state-dependent antibody for vimentin Ser-56 (custom-made by SynPep Inc., Dublin, CA, U.S.A.; against the synthetic phosphopeptide sequence Ser-Leu-Tyr-Ala-Ser-phosphoSer⁵⁶-Pro-Gly-Gly-Ala-Tyr-Cys; antibody dilution 1:1000) followed by horseradish peroxidase-conjugated anti-rabbit Ig (ICN Biomedicals, Inc.,

Irvine, CA, U.S.A.). Proteins were visualized by ECL (enhanced chemiluminescence). The membrane was stripped and reprobed with monoclonal anti-vimentin antibody (BD Bioscience; 1:10 000 dilution) followed by horseradish peroxidase-conjugated anti-(mouse Ig) (Amersham Life Sciences, Arlington Heights, IL, U.S.A.) to normalize for minor differences in protein loading. For protein tyrosine phosphorylation, blots of CAS immunoprecipitates or cell extracts were probed with anti-phosphotyrosine antibody (PY20; ICN Biomedicals, Inc.; 1:1500 dilution), stripped and reprobed with anti-CAS antibody (BD Biosciences; 1:1500 dilution) or anti-paxillin antibody (clone 349; BD Biosciences; 1:10 000 dilution). Phosphoprotein and total protein were quantified by scanning densitometry (Bio-Rad Molecular Analyst software) after visualization by ECL. Changes in protein phosphorylation were expressed as a magnitude increase over levels of phosphorylation in unstimulated cells.

Immunofluorescence and fluorescence analysis

Cells were plated in six-well cell culture plates containing coverslips and incubated in serum-free medium for 1 day. After different treatments, these cells were fixed for 15 min in 4% (v/v) paraformaldehyde, and were then washed three times in Tris-buffered saline containing 50 mM Tris, 150 mM NaCl and 0.1% NaN₃, followed by permeabilization with 0.2% Triton X-100 dissolved in Tris-buffered saline for 5 min. These cells were immunofluorescently stained for vimentin using monoclonal anti-vimentin antibody (1:100 dilution) followed by goat anti-(mouse IgG) conjugated to Alexa 488 (Molecular Probes, Eugene, OR, U.S.A.; 1:100 dilution). Rhodamine-labelled phalloidin (Molecular Probes; 1:50 dilution) was used to visualize F-actin (filamentous actin). The cellular localization of fluorescently labelled proteins was viewed by laser scanning confocal microscopy (Zeiss LSM 510 microscope) using an Apo × 100 oil immersion objective. Alexa 488-labelled proteins (green) were excited with a 488 nm argon laser light and fluorescent emissions were collected at 500–550 nm. The fluorescence of rhodamine-labelled phalloidin (red) was excited with a helium/neon laser at 543 nm and emissions were collected at 565–615 nm. For each independent experiment, 20 cells were selected randomly for observation. The length of straight vimentin filaments and total cell length on cell images were assessed by use of Photoshop Measure Tool. Vimentin filaments with a straight section longer than one-quarter of the cell length were considered as straight filaments (In these cells, the length of nucleus is approx. one-third of cell length). Cells exhibiting more than four straight filaments were counted as cells with rearranged filaments. The percentage of cells with rearranged vimentin filaments was calculated as follows: (number of cells with rearranged vimentin filaments)/(number of total cells observed) × 100.

siRNA (small interfering RNA), cell transfection and protein expression analysis

PAK1 siRNA duplexes with the sequence 5'-CAUCAAUA-UCACUAAGUCdTdT-3' were purchased from Dharmacon (Lafayette, CO, U.S.A.). The 21-base sequences of PAK1 siRNA target to human PAK1 (NCBI accession no. NM 002576). The siRNA duplexes possessed a 5'-phosphorylated antisense strand and they were deprotected. Non-specific control siRNA duplexes were also purchased from Dharmacon. Control RNA or siRNA (100 nM) was introduced into smooth muscle cells by using TransFast Transfection Reagent (Promega, Madison, WI, U.S.A.), according to the manufacturer's guidelines. Briefly, cells in 35-mm dishes were incubated with 1 ml of medium containing Transfast reagent and RNA for 1 h, followed by the addition of

2 ml of regular medium. These cells were incubated for 4 days to allow for protein down-regulation.

Protein expression was assessed by immunoblot analysis. Blots of protein extracts from cells were probed with anti-PAK1 antibody (Cell Signaling, Beverly, MA, U.S.A.; 1:1000), stripped and reprobed with antibody against smooth muscle α -actin (clone 1A4; Sigma; 1:10000 dilution). Densitometric values of PAK1 and actin from immunoblots were determined for cells treated with control RNA or siRNA and normalized to those of untreated cells. The ratios of PAK1 to actin were calculated to verify that protein silencing was selective for PAK1.

Analysis of the ratio of soluble to insoluble vimentin

The amounts of soluble and insoluble vimentin were evaluated by modification of the method described previously [7,8]. Smooth muscle cells were washed with ice-cold HBSS, and then scraped, collected and centrifuged at 8000 *g* for 5 min. The resulting pellets were incubated at 37°C for 30 min with a buffer containing 1 % Nonidet P-40, 10 % (v/v) glycerol, 20 mM Hepes, pH 7.6, 150 mM NaCl, 2 mM sodium orthovanadate, 2 mM molybdate, 2 mM sodium pyrophosphate and protease inhibitors (2 mM benzamide, 0.5 mM aprotinin and 1 mM PMSF). The soluble (depolymerized) and insoluble (polymerized) fractions were collected after centrifugation at 2100 *g* at 4°C for 30 min, and analysed by SDS/PAGE and transferred to nitrocellulose membranes. The membranes were probed with anti-vimentin antibody followed by ECL visualization. The ratio of soluble/insoluble vimentin was determined after scanning densitometry of the immunoblots.

Site-directed mutagenesis and preparation of recombinant proteins

PCR-mediated mutagenesis was carried out on pEGFP-vimentin (kindly provided by Dr Robert D. Goldman, Northwestern University, Chicago, IL, U.S.A.) to generate the vimentin mutant S56A (alanine substitution at serine-56). The resulting PCR products were digested with BamHI and EcoRI, and subcloned into pGEX-2T followed by transformation of *Escherichia coli* (BL21). DNA sequencing was used to confirm the mutation of vimentin. Wild-type vimentin was also subcloned into the same vector. Bacterial cells carrying the cDNA constructs were grown overnight in Luria broth medium, after which they were collected by centrifugation (2700 *g* for 15 min at 4°C). Cells were then sonicated on ice and centrifuged at 20000 *g* for 30 min at 4°C. Recombinant proteins were purified as fusion proteins by conjugating the proteins in the supernatant to glutathione–Sephadex beads. After the beads were washed, recombinant proteins were released from the beads by thrombin treatment. Thrombin was removed from recombinant proteins by incubation with *p*-aminobenzamide–agarose beads (Sigma) [21,22].

Analysis of vimentin phosphorylation *in vitro*

Purified wild-type vimentin or the S56A vimentin mutant (0.1 mg/ml) was incubated with 2 μ g/ml active PAK1 (Upstate, Chicago, IL, U.S.A.) for 15 min in a buffer containing 25 mM Tris/HCl (pH 7.6), 0.5 mM MgCl₂ and 100 μ M ATP. The reaction mixture was boiled in the SDS sample buffer, followed by immunoblot analysis using the phospho-vimentin (Ser-56) antibody.

Statistical analysis

All statistical analysis was performed using Prism 4 software (GraphPad Software, San Diego, CA, U.S.A.). Comparison among multiple groups was performed by one-way ANOVA

followed by a *post hoc* test (Tukey's multiple comparison test). Differences between pairs of groups were analysed by the Student–Newman–Keuls test or Dunn's method. Values of *n* refer to the numbers of experiments used to obtain each value. *P* < 0.05 was considered to be significant.

RESULTS

Stimulation of smooth muscle cells with 5-HT leads to spatial rearrangement of the vimentin network

To determine whether activation by 5-HT induces reorganization of the vimentin network, cultured tracheal smooth muscle cells were stimulated with 10 μ M 5-HT for 5 or 15 min, and these cells were then fixed and immunofluorescently stained by using anti-vimentin antibody. These cells were also stained with rhodamine-conjugated phalloidin for the observation of F-actin under agonist stimulation [23,24].

Exposure of smooth muscle cells to 5-HT induced the spatial reorientation of vimentin filaments (Figure 1). In most unstimulated cells, vimentin exhibited a curved filamentous structure with a random arrangement (Figure 1A, panel a). Vimentin filaments became straight, and orientated along the long axis of cells 5 or 15 min after stimulation with 5-HT (Figure 1A, panels b and c). The numbers of cells displaying the reorganized vimentin filaments in response to stimulation with 5-HT were significantly increased compared with those not treated with 5-HT (Figure 1B; *n* = 5, *P* < 0.05). In addition, the intensity of F-actin staining was relatively lower in unstimulated cells (Figure 1A, panel a). Stimulation of cells with 5-HT led to increases in F-actin staining (Figure 1A, panels b and c), indicating an increase in the polymerization of F-actin in stimulated smooth muscle cells [23,24].

Vimentin undergoes phosphorylation on Ser-56 in response to stimulation with 5-HT

To investigate the role of vimentin phosphorylation at Ser-56 in smooth muscle cells, a polyclonal antibody against the synthetic phosphopeptide was produced (see the Experimental section). The phospho-vimentin (Ser-56) antibody reacted with the wild-type vimentin phosphorylated by PAK1, but not with the unphosphorylated wild-type protein (Figure 2A). Absorption of phosphopeptide (pS56), but not non-phosphopeptide (S56), inhibited the immunoreactivity of the phospho-vimentin antibody against phosphorylated wild-type vimentin. The pS56 antibody did not recognize the unphosphorylated S56A vimentin mutant or the mutant treated with PAK.

We sought to evaluate whether agonist activation stimulates phosphorylation of vimentin on Ser-56 in smooth muscle cells. Tracheal smooth muscle cells were stimulated with 10 μ M 5-HT for 1, 5, 15 or 30 min, or were left unstimulated. Vimentin phosphorylation on Ser-56 was determined by immunoblot analysis of vimentin immunoprecipitates obtained from these cells.

The stimulation of smooth muscle cells with 5-HT resulted in increases in vimentin phosphorylation at Ser-56. As shown in Figure 2(B), vimentin phosphorylation at Ser-56 was increased 1 min after stimulation with 5-HT, reached a maximal level 15 min after stimulation, and was maintained for the 30-min period. The phosphorylation levels in smooth muscle cells were increased by 2.5-fold 15 min after stimulation with 5-HT (Figure 2C; *n* = 4).

To determine the extent of vimentin phosphorylation at Ser-56, half portions of protein extracts of unstimulated or stimulated cells were precleared with the antibody against phospho-vimentin (Ser-56), and then they were immunoprecipitated with anti-vimentin antibody (P fraction). The other half of the same protein

Figure 1 Stimulation with 5-HT induces the reorientation of vimentin filaments in smooth muscle cells

(A) Cultured tracheal smooth muscle cells were stimulated with $10 \mu\text{M}$ 5-HT for 5 or 15 min, or they were not stimulated. These cells were stained with anti-vimentin antibody (green) and rhodamine/phalloidin for F-actin (red). In unstimulated cells, vimentin exhibited curved filamentous structures (a). During stimulation with 5-HT for 5 min (b) or 15 min (c), the vimentin filaments became straight and were arranged along the long axis of cells. In addition, more F-actin was found in stimulated cells (b and c) than in unstimulated cells (a). The bar ($10 \mu\text{m}$) indicates the magnification of the main figures; the insets are $1.5\times$ the magnification of the main figures. (B) Vimentin filaments displaying straightness longer than one-quarter of cell length are considered as straight filaments. Cells with more than four straight vimentin filaments are counted as cells with rearranged filaments. Quantitative analysis is expressed as (numbers of cells with rearranged vimentin filaments)/(numbers of total cells observed) $\times 100$. The asterisk (*) denotes a significantly higher percentage of cells with rearranged vimentin filaments in stimulated cells relative to unstimulated cells ($P < 0.05$). The values represent means $\pm$ S.E.M. ($n = 5$). (C) An immunoblot of whole-cell extracts was obtained with monoclonal anti-vimentin antibody.

extracts was immunoprecipitated with anti-vimentin antibody (N fraction). Both fractions were subjected to immunoblot analysis using anti-vimentin antibody. In unstimulated cells, the density of the P fraction (normalized to the N fraction) was 0.87 ± 0.07 ($n = 3$), indicating that 13 % of total vimentin was phosphorylated at Ser-56 site in unstimulated cells. In response to 5-HT stimulation for 15 min, the normalized density of the P fraction decreased to 0.35 ± 0.09 ($n = 3$), suggesting that the extent of vimentin phosphorylation at Ser-56 was 65 % in 5-HT-stimulated cells (Figure 2D).

Treatment of smooth muscle cells with siRNA depresses the expression of PAK1

We evaluated the effects of siRNA for PAK1 (a major PAK isoform in smooth muscle) on protein expression in smooth muscle cells. Tracheal smooth muscle cells were transfected with 100 nM control RNA or PAK1 siRNA, or they were subjected to the transfection procedure in the absence of RNA duplexes. These cells were incubated for 4 days to allow for protein down-regulation. The expression of PAK1 was assessed by immunoblot analysis. We also determined the level of smooth muscle α -actin protein to evaluate the selectivity of siRNA for gene silencing.

Transfection of smooth muscle cells with control RNA did not affect the expression of PAK1 or actin. In contrast, treatment of cells with siRNA suppressed the expression of PAK1 without affecting actin (Figure 3A). The ratio of PAK1 to actin in siRNA-treated cells was significantly lower than the ratios in cells not treated with RNA or in cells treated with control RNA (Figure 3B; $n = 4$, $P < 0.05$). These results demonstrated that siRNA selectively inhibits the expression of PAK1 in smooth muscle cells.

Down-regulation of PAK1 by siRNA inhibits vimentin phosphorylation at Ser-56 elicited by 5-HT

To assess the role of PAK1 in regulating vimentin phosphorylation at Ser-56, we determined the effects of PAK1 down-regulation on the site-specific phosphorylation of vimentin in smooth muscle cells during receptor activation. Smooth muscle cells that had been treated with no RNA, control RNA or siRNA for PAK1 were stimulated with $10 \mu\text{M}$ 5-HT for 15 min, or they were left unstimulated. Phosphorylation of vimentin at Ser-56 in these cells was then determined.

In cells not treated with RNA duplexes, stimulation with 5-HT increased phosphorylation of vimentin on Ser-56. The treatment of cells with control RNA did not inhibit vimentin phosphorylation in response to stimulation with 5-HT. The down-regulation of PAK1 by siRNA did not inhibit the level of vimentin site-specific phosphorylation in unstimulated smooth muscle cells (Figure 4A). In contrast, the silencing of PAK1 suppressed vimentin phosphorylation elicited by 5-HT. Vimentin phosphorylation at Ser-56 upon stimulation with 5-HT was significantly lower in cells that had been treated with PAK1 siRNA than in cells treated with control RNA or with no RNA (Figure 4B; $n = 4$, $P < 0.05$).

Silencing of PAK1 by siRNA attenuates the spatial rearrangement of vimentin filaments stimulated by 5-HT

We evaluated the effects of PAK silencing on the spatial reorganization of the vimentin network. Smooth muscle cells that had been treated with control RNA or PAK1 siRNA were stimulated with $10 \mu\text{M}$ 5-HT for 15 min, and then stained for vimentin and F-actin.

Figure 2 Vimentin undergoes phosphorylation on Ser-56 in smooth muscle cells stimulated with 5-HT

(A) Characterization of the antibody (Ab) against phospho-vimentin (Ser-56). Wild-type vimentin or the S56A vimentin mutant was phosphorylated by PAK1 *in vitro*. Blots of the proteins (200 ng) were probed using the anti-phospho-vimentin (Ser-56) antibody (pS56 antibody), stripped and reprobed by use of anti-vimentin antibody in the presence or absence of synthetic peptides. The phospho-vimentin antibody recognizes the wild-type vimentin phosphorylated by PAK, but not unphosphorylated wild-type protein. Preincubation with phosphopeptide (pS56), but not non-phosphopeptide (S56), inhibits the immunoreactivity of pS56 antibody towards phosphorylated wild-type vimentin. The pS56 antibody does not recognize the S56A vimentin mutant in the absence or presence of PAK. (B) Cultured smooth muscle cells were stimulated with 10 μ M 5-HT for 1, 5, 15 or 30 min, or they were unstimulated. Blots of vimentin immunoprecipitates obtained from these cells were probed using the pS56 antibody, stripped and reprobed by use of the vimentin antibody. Representative immunoblots show that stimulation of cells with 5-HT induces vimentin phosphorylation at Ser-56 at different time points. C, unstimulated cells; IB, immunoblot; IP, immunoprecipitation. (C) Vimentin phosphorylation is quantified as multiples of phosphorylation levels obtained in unstimulated cells. The values represent means $\pm$ S.E.M. ($n = 4$); *significantly higher phosphorylation levels in the stimulated cells compared with the level in unstimulated cells ($P < 0.05$). (D) To determine the extent of vimentin phosphorylation at Ser-56, half-portion of protein extracts of unstimulated or stimulated cells was precleared with pS56 antibody, and then immunoprecipitated with vimentin antibody (P fraction). The other halves of the same protein extracts were immunoprecipitated with vimentin antibody (N fraction). Both fractions were subjected to immunoblot analysis using vimentin antibody. In response to stimulation with 5-HT, the level of the P fraction (normalized to the N fraction) was decreased to 0.35 ± 0.07 ($n = 3$), suggesting that 65% of total vimentin was phosphorylated in 5-HT-stimulated cells.

Figure 3 Treatment with siRNA depresses the expression of PAK1 in cultured smooth muscle cells

(A) Representative immunoblots illustrating PAK1 silencing by siRNA in smooth muscle cells. Blots of protein extracts from tracheal smooth muscle cells that had been treated with siRNA or control RNA (C RNA), or with no RNA, were detected with antibodies against PAK1 and smooth muscle α -actin. The amount of PAK1 was lower in siRNA-treated cells than in cells treated with no RNA or control RNA. Similar amounts of α -actin were detected in all three groups of cells. Molecular mass markers are indicated on the left. (B) Ratios in siRNA-treated and control-RNA-treated cells are normalized to the ratio obtained from cells not treated with RNA. The values represent means $\pm$ S.E.M. ($n = 4$); *significantly lower ratio in siRNA-treated cells relative to cells treated with no RNA or control RNA ($P < 0.05$).

In cells treated with control RNA, vimentin filaments underwent spatial reorganization upon stimulation with 5-HT (Figure 5A, panels a and b). However, the vimentin network remained a curved and random structure in siRNA-treated cells after 5-HT stimulation (Figure 5A, panels c and d). Upon stimulation with 5-HT, the numbers of cells with reorganized vimentin filaments after treatment with control RNA was significantly greater than that of those that had been treated with siRNA (Figure 5B; $n = 4$, $P < 0.05$). Additionally, the silencing of PAK1 did not depress the increase in F-actin staining in response to stimulation with 5-HT (Figure 5A, panels a–d), indicating that PAK1 is not involved in the regulation of F-actin polymerization in these cells.

The effects of siRNA on PAK1 expression and vimentin reorganization are reversible

Smooth muscle cells that had been treated with control RNA, siRNA or no RNA were incubated in the absence of control RNA or siRNA for an additional 4 days. Protein expression and the spatial reorganization of vimentin filaments from these cells were then determined. After additional incubation without the nucleotides, PAK1 protein expression in siRNA-treated cells was similar to that in cells treated with control RNA or cells not treated with RNA (Figures 6A and 6B). Vimentin filament reorganization in response to stimulation with 5-HT was also recovered in siRNA-treated cells after an additional 4-day incubation without siRNA (Figure 6C).

Figure 4 PAK1 down-regulation inhibits vimentin phosphorylation at Ser-56 in response to stimulation with 5-HT

Smooth muscle cells that had been treated with no RNA, control RNA (C RNA) or PAK1 siRNA were stimulated with $10 \mu\text{M}$ 5-HT for 15 min, or left unstimulated. The site-specific phosphorylation of vimentin was determined by immunoblot analysis of vimentin immunoprecipitates obtained from these cells. **(A)** Representative immunoblots illustrating the effects of PAK1 silencing on Ser-56 phosphorylation on vimentin in cells stimulated with 5-HT. Treatment with siRNA, but not with control RNA, inhibited vimentin phosphorylation on Ser-56 after stimulation with 5-HT. pS56 antibody, antibody against phospho-vimentin (Ser-56); IB, immunoblot; Ab, antibody. **(B)** Vimentin phosphorylation is quantified as multiples of phosphorylation levels obtained in unstimulated cells not treated with RNA. *Significantly higher phosphorylation levels in stimulated cells than in corresponding unstimulated cells ($n = 4$, $P < 0.05$); **significantly lower vimentin phosphorylation upon stimulation with 5-HT in cells treated with siRNA than in cells not treated with RNA or cells treated with control RNA ($n = 4$, $P < 0.05$).

Down-regulation of PAK1 inhibits the increase in the soluble vimentin/insoluble vimentin ratio in response to stimulation with 5-HT

We assessed the effects of stimulation with 5-HT on vimentin filament depolymerization/polymerization in smooth muscle cells. Smooth muscle cells were stimulated with $10 \mu\text{M}$ 5-HT for 5 min or 15 min, and then the ratio of soluble vimentin/insoluble vimentin was evaluated using a method of fractionation. The soluble/insoluble vimentin ratios in cells in response to stimulation with 5-HT for 5 min or 15 min were significantly higher than that in unstimulated cells (Figures 7A and 7B; $n = 5-7$, $P < 0.05$). The results suggest that stimulation with 5-HT induces the depolymerization of vimentin intermediate filaments in smooth muscle cells. The ratios of soluble to insoluble vimentin in cells stimulated with 5-HT for 5 min were not significantly different from those in cells elicited by 5-HT for 15 min (Figure 7B; $n = 5-7$, $P > 0.05$).

To evaluate whether PAK affects vimentin depolymerization/polymerization, smooth muscle cells that had been treated with control RNA, PAK1 siRNA or no RNA were stimulated with $10 \mu\text{M}$ 5-HT for 5 min, and then assayed for the soluble/insoluble

Figure 5 PAK1 silencing attenuates the spatial rearrangement of vimentin filaments in response to stimulation with 5-HT

(A) Smooth muscle cells that had been treated with control RNA or PAK1 siRNA were stimulated with $10 \mu\text{M}$ 5-HT for 15 min, or unstimulated. These cells were stained for vimentin (green) and F-actin (red). In the unstimulated cells treated with control RNA **(a)** or siRNA **(c)**, the vimentin network displayed a curved filamentous structure. Stimulation with 5-HT induced the spatial reorganization of the vimentin network in cells treated with control RNA **(b)**. However, vimentin filaments remained curved structures after stimulation with 5-HT of cells that had been treated with PAK1 siRNA **(d)**. The increase in F-actin formation upon agonist stimulation was not affected in cells treated with control RNA or siRNA **(a-d)**. The bar ($10 \mu\text{m}$) indicates the magnification of the main figures; the insets are $1.5\times$ the magnification of the main figures. **(B)** Quantitative analysis of the effects of PAK1 silencing on the spatial reorganization of vimentin filaments. *Significantly higher percentage of cells with rearranged vimentin filaments in stimulated cells relative to unstimulated cells treated with control RNA ($P < 0.05$); **significantly lower percentage of cells with rearranged vimentin in stimulated cells treated with siRNA compared with stimulated cells treated with control RNA. The values represent means $\pm$ S.E.M. ($n = 4$).

vimentin ratio. Treatment with siRNA or control RNA did not affect the ratio of soluble vimentin/insoluble vimentin in unstimulated cells; however, the soluble/insoluble vimentin ratio upon stimulation with 5-HT was significantly lower in cells treated with siRNA than in cells treated with no RNA or control RNA (Figure 7C; $n = 4$, $P < 0.05$).

Disruption of the actin cytoskeleton does not affect the reorganization of the vimentin network upon stimulation with 5-HT

Agonist stimulation also induces actin polymerization and re-modelling of the actin cytoskeleton in smooth muscle cells

Figure 6 The effects of siRNA on PAK1 expression and vimentin reorganization are reversible

(A) Smooth muscle cells that had been treated with control RNA, siRNA or no RNA were incubated in the absence of control RNA or siRNA for an additional 4 days. The representative immunoblot shows that PAK1 protein expression in siRNA-removed cells was similar to that in control RNA-removed cells or in cells not treated with RNA. (B) PAK1/actin ratios in cells after removal of siRNA or control RNA are normalized to the ratio obtained from cells not treated with RNA. The values represent means $\pm$ S.E.M. ($n = 4$). (C) After an additional 4-day incubation without siRNA or control RNA, vimentin filament reorientation in response to stimulation with 5-HT was also recovered. The values represent mean $\pm$ S.E.M. ($n = 4$).

(Figure 1) [23,24]. Thus the reorientation of vimentin filaments in response to stimulation with 5-HT could be secondary to the remodelling of the actin cytoskeleton. To evaluate whether actin microfilaments affect the vimentin intermediate filament network, smooth muscle cells were treated with cytochalasin D, an actin-disrupting agent, and then stimulated with $10 \mu\text{M}$ 5-HT for 15 min. These cells were double stained for F-actin and vimentin.

In cells treated with $5 \mu\text{M}$ cytochalasin D for 30 min, the actin cytoskeleton was clearly disrupted compared with control cells (Figure 8A, panels a–d). However, treatment with cytochalasin D did not affect the reorientation of the vimentin intermediate filament network in response to stimulation with 5-HT (Figure 8A, panels c and d). The percentage of cells with rearranged vimentin filaments after treatment with cytochalasin D was not significantly different from that of those without cytochalasin D treatment upon agonist activation (Figure 8B; $n = 4$, $P > 0.05$).

Figure 7 PAK1 down-regulation inhibits the increase in the ratio of soluble vimentin to insoluble vimentin in smooth muscle cells stimulated with 5-HT

(A) Immunoblot illustrating the effects of 5-HT on the amount of soluble (supernatant) and insoluble (pellet) vimentin in cells. Smooth muscle cells were stimulated with $10 \mu\text{M}$ 5-HT for 5 min or 15 min, or unstimulated. Supernatant (S) and pellet (P) fractions were assessed by fractionation. The stimulation of cells with 5-HT increased soluble vimentin and decreased insoluble vimentin. (B) The ratios of soluble to insoluble vimentin were determined by scanning densitometry of immunoblots for the fractions; *significantly greater ratios of soluble vimentin/insoluble vimentin in 5-HT-stimulated cells than in unstimulated cells ($P < 0.05$, $n = 5-7$). (C) Smooth muscle cells that had been treated with no RNA, control RNA (C RNA) or PAK1 siRNA were activated with $10 \mu\text{M}$ 5-HT for 5 min. The ratios of soluble vimentin/insoluble vimentin were then determined; *significantly greater ratios of soluble vimentin/insoluble vimentin in stimulated cells than in corresponding unstimulated cells ($P < 0.05$, $n = 4$); **significantly lower ratios of soluble vimentin/insoluble vimentin upon stimulation with 5-HT in cells treated with siRNA than in cells not treated with RNA or treated with control RNA ($n = 4$, $P < 0.05$).

Inhibition of protein tyrosine phosphorylation does not affect the spatial reorganization of vimentin filaments in response to stimulation with 5-HT

Protein tyrosine phosphorylation has been implicated in the regulation of spatial changes in vimentin filaments [7]. Stimulation with contractile agonists induces the tyrosine phosphorylation of the integrin-associated proteins FAK (focal adhesion kinase), CAS and paxillin in smooth muscle tissues and cells [25–27]. To determine whether the spatial reorganization of vimentin filaments is regulated by protein tyrosine phosphorylation, smooth muscle cells were stimulated with $10 \mu\text{M}$ 5-HT for 15 min in the absence or presence of the protein tyrosine kinase inhibitor genistein ($100 \mu\text{M}$, 30 min). These cells were then double-labelled for F-actin and vimentin filaments.

Figure 8 Disruption of the actin cytoskeleton and inhibition of protein tyrosine phosphorylation do not inhibit the spatial reorganization of vimentin filaments in response to stimulation with 5-HT

(A) Smooth muscle cells were pretreated with or without 5 μ M cytochalasin D or 100 μ M genistein for 30 min, and then activated with 10 μ M 5-HT for 15 min, or unstimulated. These cells were stained for vimentin (green) and F-actin (red). Treatment with cytochalasin D decreased F-actin and disrupted actin stress fibres (c and d). Genistein inhibited the increase in F-actin formation in response to 5-HT stimulation (e and f). Curved vimentin filaments were observed in unstimulated control cells (a) and in cells treated with cytochalasin D (c) or genistein (e). Stimulation with 5-HT induced the reorganization of vimentin filaments in control cells (b) and cells pretreated with cytochalasin D (d) or genistein (f). The bar (10 μ m) indicates the magnification of the main figures; the insets are 1.5 $\times$ the magnification of the main figures. (B) Quantitative analysis of the effects of cytochalasin D and genistein on the spatial reorganization of vimentin filaments. The values represent means $\pm$ S.E.M. ($n = 4$).

F-actin formation in response to stimulation with 5-HT in cells treated with genistein was reduced compared with that in cells not treated with genistein. In contrast, treatment with genistein did not affect vimentin filament rearrangement in response to stimulation with 5-HT (Figure 8A, panels e and f). During stimulation with 5-HT, numbers of cells with reorganized vimentin filaments were not significantly different between con-

Figure 9 Treatment with genistein inhibits protein tyrosine phosphorylation stimulated by 5-HT

Smooth muscle cells were stimulated with 10 μ M 5-HT for 15 min or unstimulated in the absence or presence of treatment with 100 μ M genistein for 30 min. Blots of protein extracts (A) or CAS immunoprecipitates (B) obtained from these cells were probed with anti-phosphotyrosine antibody (PY20 Ab), stripped and reprobed with antibodies against paxillin (A) or CAS (B) to ensure even protein loading. Molecular mass markers are indicated on the left. CAS and paxillin are indicated on the right. The blots are representative of three identical experiments. IB, immunoblot; IP, immunoprecipitate.

trol cells and those treated with genistein (Figure 8B; $n = 4$, $P > 0.05$).

To ensure that genistein inhibits protein tyrosine phosphorylation, extracts of smooth muscle cells that had been treated with genistein and 5-HT were analysed for protein tyrosine phosphorylation. Stimulation of cells with 5-HT led to the tyrosine phosphorylation of several proteins. Exposure of cells to genistein inhibited protein tyrosine phosphorylation (Figure 9A). In addition, the level of tyrosine phosphorylation in immunoprecipitates of CAS (a major focal adhesion-associated protein) in response to stimulation with 5-HT was reduced in cells treated with genistein (Figure 9B).

DISCUSSION

Our present results demonstrate that spatial reorientation, phosphorylation and depolymerization of the vimentin network occur in smooth muscle cells in response to stimulation with 5-HT. Moreover, PAK is required for the site-specific phosphorylation and reorganization of vimentin filaments during stimulation with 5-HT. We propose that the PAK-mediated site-specific phosphorylation of vimentin may play a role in regulating the reorganization of vimentin intermediate filaments in smooth muscle cells in response to stimulation with 5-HT.

Previous studies on fibroblasts and endothelial cells have found that vimentin filaments undergo spatial reorganization in response

to stimulation with PDGF and EGF, or in response to over-expression of Cdc42Hs and Rac1 [7,8]. The reorganization of vimentin filaments in these non-muscle cells during activation via growth factor-mediated pathways may promote cell division and crawling [7]. In the present paper, we observed that stimulation with 5-HT induces the spatial reorientation of vimentin intermediate filaments in smooth muscle cells. The physiological relevance of the spatial reorientation of vimentin in smooth muscle cells is currently unknown. Studies with vimentin knockout mice suggest that vimentin is necessary for shear stress (flow)-induced mechanical response in mesenteric resistance arteries [28]. Stiffness and contractile capacity are also impaired in vimentin-deficient fibroblasts [29,30]. Thus we speculate that the spatial rearrangement of vimentin filaments may form part of the cytoskeletal remodelling that affects force development during activation with 5-HT. As stimulation with 5-HT also exerts mitogenic effects on smooth muscle cells [31], the reorganization of vimentin filaments upon 5-HT stimulation might also be associated with mitogenic processes in smooth muscle cells.

The occurrence of straight vimentin filaments in the stimulated cells indicates that vimentin may undergo polymerization during stimulation. However, we found that stimulation with 5-HT increases the ratio of soluble vimentin/insoluble vimentin, suggesting overall depolymerization of vimentin in these cells. In unstimulated cells, the ratio of soluble vimentin/insoluble vimentin is approx. 0.1, indicating that 10% of total vimentin is in the depolymerized form. Stimulation with 5-HT increases the ratio of soluble vimentin/insoluble vimentin to approx. 0.3, implying that 20% of total vimentin is depolymerized. These results lead us to hypothesize that activation with 5-HT may trigger simultaneous polymerization and depolymerization of vimentin to facilitate the transition from curvature to straightness. However, the overall extent of vimentin depolymerization is slightly higher than its polymerization in these cells.

We considered the possibility that stimulation with 5-HT may also induce vimentin phosphorylation in these cells. Phosphorylation of vimentin has been shown to be associated with its spatial reorganization in a number of non-muscle cells, including mouse connective tissue cells, bovine kidney cells, human glioma cells, fibroblasts and COS-7 cells [13–15]. Stimulation with angiotensin II induces vimentin phosphorylation in cultured rat aortic vascular smooth muscle cells [16]. Biochemical studies have revealed that Ser-55/56 is a major phosphorylation residue [14]. We have shown previously that stimulation of tracheal smooth muscle tissues with acetylcholine triggers phosphorylation of vimentin on Ser-56 [32]. In the present paper, we demonstrate that activation with 5-HT induces phosphorylation of vimentin on Ser-56 in canine tracheal smooth muscle cells. These results suggest that vimentin undergoes phosphorylation on Ser-56 in smooth muscle cells in response to agonist stimulation.

In the present study, PAK1 was able to phosphorylate vimentin on Ser-56 *in vitro*. To determine the role of PAK in regulating vimentin in cells, smooth muscle cells were treated with siRNA duplex. Treatment of smooth muscle cells with siRNA for PAK1 selectively decreased the protein level of PAK1 without affecting the expression of smooth muscle α -actin. The siRNA duplex may induce the assembly of the RNA-induced silencing complex and trigger post-transcriptional gene silencing [33,34]. The down-regulation of PAK1 inhibited phosphorylation of vimentin on Ser-56 during stimulation with 5-HT, suggesting that PAK1 is an essential molecule for vimentin phosphorylation on this residue.

We hypothesized that the site-specific phosphorylation of vimentin may be a cellular mechanism that regulates vimentin filament reorganization in smooth muscle cells under 5-HT stimulation. In the present study, PAK1 down-regulation suppressed

the rearrangement of the vimentin network in response to 5-HT stimulation. The effects of siRNA on protein expression and vimentin filament reorganization were reversible. Since phosphorylation of vimentin on Ser-56 occurs concurrently with vimentin filament reorientation, and the inhibition of PAK1 expression depresses both vimentin phosphorylation on this residue and vimentin reorganization upon 5-HT stimulation, we propose that the PAK-mediated site-specific phosphorylation of vimentin may play a role in regulating the reorganization of vimentin intermediate filaments during agonist stimulation.

The mechanisms by which vimentin phosphorylation might regulate its reorganization are currently unclear. In the present study, stimulation with 5-HT also induced vimentin depolymerization, as indicated by the increase in the ratio of soluble vimentin/insoluble vimentin in stimulated cells (see above). Furthermore, the down-regulation of PAK1 inhibited vimentin phosphorylation, depolymerization and spatial rearrangement in response to stimulation with 5-HT. These results suggest that vimentin phosphorylation may lead to its partial disassembly and thus the spatial reorganization. This assumption is supported by previous observations whereby vimentin phosphorylation induced its distribution to soluble fractions or its reorganization [10–13,15,17].

Stimulation of smooth muscle cells with 5-HT induces the formation of F-actin, indicating that actin polymerization occurs in response to agonist activation. The results are consistent with previous studies in smooth muscle tissues and cells [23,24,35–39]. The down-regulation of PAK1 does not suppress the formation of filamentous actin, indicating that PAK is not involved in the regulation of stress fibre formation. PAK is a major downstream effector of Cdc42 and Rac [19]; these GTPases and PAK are involved in the regulation of cortical actin that is associated with membrane ruffles and lamellipodium formation [7,40–42]. The formation of cortical actin also requires the activation and co-ordination of several signalling proteins, such as Paxillin, N-WASP and the Arp2/3 complex [38,43–45]. Thus it is not surprising that PAK has not been implicated in regulating the formation of actin stress filaments in cultured smooth muscle cells. Rho-mediated pathways have been shown to mediate stress fibre formation in cultured smooth muscle cells [23].

Stimulation of smooth muscle cells with 5-HT triggers both actin polymerization and vimentin filament reorientation. It has been thought that the actin cytoskeleton is associated with the vimentin filament system [46]. Therefore the remodelling of the actin cytoskeleton could influence the rearrangement of vimentin intermediate filaments during agonist stimulation. However, in the present study, disruption of the actin cytoskeleton by the actin-disrupting agent cytochalasin D did not affect the spatial reorganization of vimentin filaments in response to agonist stimulation. These results suggest that the rearrangement of vimentin filaments is independent of actin cytoskeleton remodelling. Since the inhibition of actin polymerization depresses force generation and stiffness in smooth muscle tissues and cells [24,35,36,47], these results also suggest that spatial reorganization of the vimentin network upon stimulation with 5-HT does not stem from changes in force development and stiffness.

In the present study, the inhibition of PAK protein expression prevented the spatial reorganization of vimentin intermediate filaments without influencing F-actin polymerization. This observation suggests to us that the PAK-mediated vimentin network reorganization does not affect actin polymerization and remodelling in smooth muscle cells in response to stimulation with 5-HT.

Inhibition of protein tyrosine phosphorylation impairs spatial reorganization of the vimentin network in fibroblasts overexpressing Cdc42Hs and Rac1 [7]. Stimulation with contractile

agonists initiates the tyrosine phosphorylation of FAK, paxillin and CAS in smooth muscle tissues and cells [25–27], and these proteins have been shown to play a role in regulating smooth muscle contraction [26,36,38,48,49]. In the present study, several focal adhesion proteins, including CAS and paxillin, underwent tyrosine phosphorylation in response to stimulation with 5-HT, which could be suppressed by the protein tyrosine phosphorylation inhibitor genistein. Nevertheless, the inhibition of protein tyrosine phosphorylation did not affect the rearrangement of vimentin intermediate filaments. This observation indicates that the tyrosine phosphorylation of focal adhesion proteins does not modulate the vimentin network during stimulation of smooth muscle cells. The inhibition of protein tyrosine phosphorylation by the inhibitor impairs F-actin polymerization, consistent with a role of focal adhesion proteins in actin dynamics in smooth muscle cells [26,36,38,48,50].

In summary, stimulation of cultured smooth muscle cells with 5-HT induces the spatial reorientation, phosphorylation on Ser-56 and partial depolymerization of the vimentin network. The silencing of PAK1 inhibits the site-specific phosphorylation, depolymerization and spatial rearrangement of the vimentin filaments during 5-HT stimulation. Neither the disruption of stress fibres by cytochalasin D nor the inhibition of protein tyrosine phosphorylation affects the spatial reorganization of vimentin intermediate filaments in response to stimulation with 5-HT. We conclude that PAK1 plays an essential role in the regulation of the phosphorylation of vimentin on Ser-56 and the reorganization of the intermediate filament network in smooth muscle cells during stimulation with 5-HT.

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