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Role of the G-protein and tyrosine kinase—Rho/ROK pathways in 15-hydroxyeicosatetraenoic acid induced pulmonary vasoconstriction in hypoxic rats

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It has been previously reported by us that hypoxia activates lung 15-lipoxygenase (15-LO), which catalyzes arachidonic acid to 15-hydroxyeicosatetraenoic acid (15-HETE), leading to the constriction of pulmonary artery (PA). Rho-associated serine/threonine kinase (ROK), a downstream effector of small GTPase RhoA that may be modulated by G-protein and tyrosine kinase, plays an important role in smooth muscle contraction. However, whether the 15-HETE induced PA vasoconstriction involves the Rho/ROK pathway remains to be demonstrated. Therefore, we studied the contribution of ROK as well as G-protein and tyrosine kinase to the 15-HETE induced pulmonary vasoconstriction using PA ring technique, RNA interference technology, RP-HPLC, western blot and RT-PCR combined with the blockers. The hypoxia-induced expression of ROK is regulated by 15-HETE in rat PA smooth muscle cells (PASMCs), leading to vasoconstriction. The up-regulation of ROK expression caused by 15-HETE appears to be mediated by the G-protein and tyrosine kinase pathways. The translocation of ROK2 from the nucleus to the cytoplasm during hypoxia exposure relies on the mechanism for 15-HETE production. These results suggest that 15-HETE may mediate the up-regulation of ROK expression through G-protein and tyrosine kinase pathways under hypoxic condition, leading to PA vasoconstriction.

Keywords: 15-hydroxyeicosatetraenoic acid/hypoxia/pulmonary artery smooth muscle cells/pulmonary vasoconstriction/ROK.

Abbreviations: AA, arachidonic acid; CDC, cinnamyl 3,4-dihydroxy-[alpha]-cyanocinnamate; 15-HETE, 15-hydroxyeicosatetraenoic acid; 15-LO, 15-lipoxygenase; NDGA, nordihydroguaiaretic acid; ROK, Rho-associated serine/threonine kinase; RP-HPLC, reverse-phase high-pressure liquid chromatography; RT–PCR, reverse transcription polymerase chain reaction.

It has long been recognized that hypoxic pulmonary vasoconstriction (HPV) is an important adaptive process by which the ventilation-perfusion inequality in lungs is corrected. Many factors, including nitric oxide, prostanoids and endothelin-1 (ET-1), have been suggested in the pulmonary response to hypoxia although the underlying mechanisms are still unclear.

Rho/ROK is involved in diverse physiological functions associated with cytoskeletal rearrangements, such as cell morphology, cell motility, cytokinesis and smooth muscle contraction (*1*). Experimental evidence has shown that hypoxia induces activation of Rho-kinase (ROK) in pulmonary artery smooth muscle cells (PASMCs), leading to the better maintenance of the phosphorylation of myosin light chain (MLC) and the constriction of pulmonary artery (PA) (*2*). The ROK activated by binding to GTP-bound RhoA has also been demonstrated to increase Ca^{2+} sensitivity and plays an important role in smooth muscle contraction (*3*, *4*). Like ROK, tyrosine kinase is also involved in excitation–contraction coupling in PASMCs (*5*). It has been reported that heterotrimeric G-protein coupled receptors involving members in the Gα12/13 family are linked to agonist-induced RhoA activation in aortic smooth muscle cells (*6*), and tyrosine kinase is required as the upstream of RhoA in acute CNS trauma (*7*). Since how the RhoA/ROK pathway is activated in PASMCs is unclear, it is necessary to identify potential cell-endogenous mediators and demonstrate the relationship of RhoA/ROK with G-protein and tyrosine kinase under hypoxic condition.

15-Hydroxyeicosatetraenoic acid (15-HETE), a metabolite of arachidonic acid (AA) catalyzed by 15-lipoxygenase (15-LO), plays an important role in several pulmonary diseases. Our previous studies have shown that hypoxia activates 15-LO in PA increasing 15-HETE production, leading to an inhibition of voltage-gated K^+ channels, PASMCs depolarization and vasoconstriction (*8*–*10*). We have also found that 15-HETE constricts the PA of hypoxic rat through the ERK1/2 signalling pathway and Ca^{2+}-dependent signalling pathway (*11*, *12*). Consistently, previous studies have shown that AA induces Ca^{2+} sensitization of smooth muscle contraction through

the up-regulation of ROK (*13*). Thus, it is possible that 15-HETE induces pulmonary vasoconstriction during hypoxia through a ROK-dependent mechanism. To address this issue, we performed studies on how 15-HETE modulated ROK expression, affecting pulmonary vasoconstriction under hypoxic condition, and whether this involved G-protein and tyrosine kinase pathways, using PA ring technique, western blot, RT-PCR, RNA interference technology, RP-HPLC and immunocytochemistry.

Materials and Methods

Reagents

All reagents were of the highest available grade from common commercial sources. 15-HETE, 12-HETE, 5-HETE, cinnamyl 3,4-dihydroxy-[alpha]-cyanocinnamate (CDC) and NDGA (nordihydroguaiaretic acid) were purchased from Cayman Chemical Co., Inc (Ann Arbor, USA); suramin, SCH-202676 [*N*-(2,3-diphenyl-1,2,4-thiadiazol-5-(2H)-ylidene) methanamine hydrobromide] and Y-27632 [(+)-(R)-*trans*-4-(1-aminoethyl)-*N*-(4-pyridyl) cyclohexanecarboxamide dihydrochloride] were purchased from Sigma-Aldrich Co. (St. Louis, USA); tyrphostin A25 was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); genistein was produced by Beyotime Institute of Biotechnology (Haimen, China); anti-ROK2 and anti-ROK1 were purchased from Boster Biological Technology Co. Ltd. (Wuhan, China); RT-PCR kit was from Tiangen Biotech Co. Ltd (Beijing, China). GTPase assay kit and P_iBind^{TM} resin were purchased from Innova Biosciences (Cambridge, UK). X-tremeGene siRNA Transfection Reagent was purchased from Roche Applied Science (Mannheim, Germany). Anti-MYPT1 and phosphor-specific antibodies against MYPT1 were purchased from Cell Signaling Technology (Danvers, MA).

Animals

Male Wistar rats (200–250 g) were used in present study. They were housed in the Animal Research Center of Harbin Medical University, which is fully accredited by the Institutional Animal Care and Use Committee (IACUC), at a controlled ambient temperature of 20–25°C with 50 ± 10% relative humidity and at a 12-h light dark cycle. Food and water were given unrestrictedly.

Hypoxic rat model

Hypoxic exposures were carried out according to the method published by Zhu *et al.* (*8*). Briefly, adult male Wistar rats were raised in an environmental chamber, where the fractional inspired O_2 (FIO_2) was reduced to 12% (FIO_2 0.12) for 9 days to make hypoxic model, the rats raised in normoxic condition (FIO_2 0.21) were served as control. To test the effects of NDGA on hypoxia, one group of rats had been given NDGA (650 mg/kg b.w. orally, once daily) since 2 days before hypoxia till they were anaesthetized (*14*). There were several exit holes on the hypoxic box for CO_2 discharge. O_2 concentration was measured in the chamber and controlled by PROOX-100 (BiosPherix, Ltd., USA) with supplemental N_2. Appropriate amount $CaCl_2$ and boric acid were placed in the hypoxic chamber to absorb CO_2, H_2O and NH_3. There were sufficient food and water in the hypoxic box. After 9 days the rats were anesthetized with pentobarbital sodium (50 mg/kg, I.P.), and then the thorax was opened. The heart and lungs were taken immediately and placed in ice-cold Krebs solution containing NaCl 116.0, KCl 4.2, $CaCl_2$ 2.5, NaH_2PO_4 1.6, $MgSO_4$ 1.2, $NaHCO_3$ 22.0 and D-glucose 11.0 mM, followed by micro-dissection of PA rings. In some experiments, the lungs were quickly removed and further processed for RP-HPLC.

Tension studies of PA rings

The arterial ring preparation was performed similarly as our previous reports (*8*). Briefly, PA (1–1.5 mm in diameter) was isolated and cut into small segments (3 mm in length). Eight rings were mounted on tungsten wire, and recoded simultaneously with 3–4 rings as controls. The rings were bathed in pH-adjusted Krebs solution gassed with 95% O_2/5% CO_2 at 37°C. The apparatus was connected with a force transducer. Tension data were relayed from the pressure transducers to a signal amplifier (600 series eight-channel amplifier, Gould Electronics). Data were acquired and analyzed with CODAS software (Data Q Instruments, Inc). The following protocols were implemented after 1-h equilibration. Protocol 1 examined the responsiveness of PA rings of normoxic rat to phenylephrine (PE) in the presence of NDGA and Y-27632. Concentration–response curves were constructed by cumulatively increasing the concentration of PE (10^{-7}–10^{-5} M) every 5 min and recording the tension changes at the end of every 5-min period. Protocol 2 examined the effects of NDGA and Y-27632 on responses to PE (10^{-7}–10^{-5} M) in hypoxic PA rings. Protocol 3 examined the effects of NDGA and suramin on responses to PE (10^{-7}–10^{-5} M) in PA rings of hypoxic rat. Protocol 4 examined the effects of NDGA and SCH-202676 on responses to PE (10^{-7}–10^{-5} M) in PA rings of hypoxic rat. Protocol 5 examined the responses to PE (10^{-7}–10^{-5} M) in PA rings of hypoxic rat pretreated with NDGA and genistein. Protocol 6 examined the effects of NDGA and tyrphostin A25 on responses to PE (10^{-7}–10^{-5} M) in PA rings of hypoxic rat.

Dissociation and culture of PASMCs

Primary cultured PASMCs were prepared using tissue from Wistar rats. The isolated PA rings were denuded by rubbing the luminal surface with a cotton swab and then digested in PBS containing 1.5 mg/ml of collagenase (typeII, Worthington, Shanghai, and P.R. China) and bovine serum albumin (1.5 mg/ml) for 2 h at 37°C. The dispersed cells were centrifuged and resuspended in DMEM containing 20% fetal bovine serum (FBS) and 1% penicillin/streptomycin and then plated in T75 culture flask in a humidified atmosphere of 5% CO_2 at 37°C. The purity of PASMCs in the primary cultures was confirmed by the specific monoclonal antibodies raised against smooth muscle α-actin. Cell viability (usually >98%) was determined by Trypan Blue exclusion. Before each experiment, the cells were incubated in serum-free-DMEM for 24 h to stop cell growth, and then exposed to normoxia (21% O_2/5% CO_2/balance N_2) or hypoxia (2.5% O_2/5% CO_2/balance N_2).

siRNA design and transfection

To silence the expression of 15-LO1 protein, transfect the PASMCs with small interfering RNA, which was designed and synthesized by GenePharma. Non-targeted control siRNA (siNC) was used as negative control. The sense sequence of siRNA against 15-LO1 and non-targeted control sequence were listed below: accession No. ds-siRNA sequence corresponding nucleotides, 15-LO1: (NM_031010) 5′-GGUUCUACUGGGUUCCUAATT-3′, 1650–1670, NC control: 5′-UUCUCCGAACGUGUCACGUTT-3′. Briefly, the PASMCs were cultured till 30– 50% confluence and incubated in serum-free-DMEM to starve. Then 2 μg siRNA and 10 μl X-tremeGene siRNA Transfection Reagent were, respectively, diluted in serum-free Opti-MEM-1 medium and mixed them together. Incubated the mixture (siRNA/Transfection Reagent) at room temperature for 20 min and added directly onto cells. After 6–8 h exposure to siRNA, removed the transfection reagents and continued to culture the PASMCs in DMEM containing 5% FBS for another 24 h under normoxic or hypoxic condition. The efficiency of protein silencing was analyzed by western blot.

Western blot analysis

Pulmonary arteries from rats (normoxia, hypoxia and hypoxia with NDGA) were homogenized in a hand-held micro-tissue grinder in ice-cold lysis buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 100 μg/ml of phenylmethylsulfonyl fluoride and 30 μl/ml of aprotinin). The homogenates were sonicated on ice and then centrifuged at 16,099*g* for 10 min at 4°C. The supernatants were collected and stored at −80°C until use in western blot analysis.

PASMCs were gently washed twice with ice-cold PBS, scraped into 0.3 ml of lysis buffer and incubated for 30 min on ice. The lysates were then sonicated and centrifuged at 16,099*g* for 10 min, and the insoluble fraction was discarded. The protein concentration of each supernatant was determined with Bradford method. Equal amount of protein (30 μg) from each sample was resolved in an 8% sodium dodecyl sulphate–polacrylamide gel electrophoresis (SDS–PAGE) and then transferred to nitrocellulose membranes. After blocked with Tris-buffered saline containing 0.1% Tween-20 (TBS-T) and 5% nonfat milk, the blot was probed with monoclonal

antibody specific for 160 kDa ROK2 (1 : 500), 160 kDa ROK1 (1 : 500), 140 kDa MYPT1 (1 : 500), 140 kDa phospho-MYPT1 (Thr853) (1 : 500), 75 kDa 15-LO1 (1 : 500) and 43 kDa β-actin (1 : 5,000), respectively, overnight at 4°C and washed before incubating with anti-rabbit horseradish peroxidase-conjugated IgG (1 : 5,000) for 1 h at room temperature. The bound antibody was detected with an enhanced chemiluminescence detection system (Amersham).

RT–PCR

The primers were designed according to a rat ROK2 genomic sequence (GenBank # NM-013022.1), as follow: ROK2 sense primer: 5′-TTACTATGGACGAGAATGT-3′, anti-sense primer: 5′-AGTTGCTGCTGTCTATGT-3′, fragment size: 344 bp. β-Actin (GenBank # NM_031144.2), as follow: sense primer: 5′-ACTATCG GCAATGAGCG-3′, anti-sense primer: 5′-GAGCCAGGGCAGTA ATCT-3′, fragment size: 230 bp. Total RNAs were extracted from cultured PASMCs by the acid guanidinium thiocyanate–phenol–chloroform extraction method and reversely transcribed with the superscript first-strand cDNA synthesis kit. The cDNA samples were amplified in a DNA thermal cycler (PerkinElmer) and the PCR products were visualized by ethidium bromide-stained agarose gel electrophoresis. To quantify the PCR products, an invariant mRNA of β-actin was used as an internal control. The OD values in the channel signals, measured by a Kodak electrophoresis documentation system, were normalized to those in the β-actin signals. The ratios were expressed as arbitrary units for quantitative comparison.

Immunocytochemistry

PASMCs were fixed with 4% paraformaldehyde fixative in PBS at room temperature for 15 min, and then treated with 0.5% Triton X-100 for 10 min. After washout with PBS, the cells were blocked with 3% normal bovine serum in PBS at 37°C for 30 min, then incubated with anti-ROK1 and anti-ROK2 primary antibody (1 : 50), respectively, in PBS at 4°C overnight. After washed, the cells were incubated with FITC-conjugated secondary antibody (1 : 100) diluted by PBS at 37°C for 2 h away from light. Slides were examined with a microscope (Nikon, Japan), and images were recorded by digital photomicrography (Olympus, Japan). The experiment was repeated three times from the cell culture step.

GTPase activity assay

GTPase activities in PASMCs were assayed by the GTPase colorimetric assay kit. The protein was abstracted by the method mentioned-above. Eliminated free P_i in samples with P_iBind™ resin and set up GTPase assays mixture with 100 µl sample plus 100 µl substrate/buffer mix (0.5 M Tris pH 7.5, 0.1 M $MgCl_2$, 10 mM specially purified GTP and water). After reactions at 37°C for 30 min, added 50 µl of Gold mix (by adding Accelerator to P_iColorLock™ Gold) to stop reactions, and added 20 µl of stabilizers 2 min later. After 30 min, read the plate at a wavelength in the range 590–660 nm.

RP-HPLC

The contents of 15- and 12-HETE in rat lung tissues which contain pulmonary arteries were analyzed by RP-HPLC according to the published method (*15*). Briefly, the tissues were homogenized within ethyl acetate, acidified to pH 3.0 with formic acid and centrifuged at 16,099*g* for 10 min. The supernatants were collected, dried down under argon, reconstituted in 0.5 ml of 20% acetonitrile: water (pH 3.0), and applied to a Sep-Pak Vac that was pre-washed with water followed by acetonitrile and water. The column was washed with different proportional acetonitrile: water to remove polar lipids and then was eluted with 500 µl of ethyl acetate to capture the free fatty acids. The samples were labelled with 2-(2,3-naphthalimino)-ethyl trifluoromethanesulfonate (36.4 mmol/l). *N*,*N*-diisopropylethylamine was added to catalyze the reaction. Endogenous 15- and 12-HETE were separated on an ODS column (4.6 mm × 250 mm, 5 µm) at 1.3 ml/min isocratically with methanol/water/glacial acetic acid (80 : 20 : 0.01) and detected with fluorometer. WIT-002 (10 ng/µl) was used as an internal standard. Retention times of 15-, 12-HETE and WIT-002 were 45, 49 and 72 min, respectively. Recovery of samples averaged 93.6 ± 2.3%. The precision relative SD was 12.7% ($n = 6$). Linearity of the samples in the range 1–200 ng/ml was good, $r^2 = 0.992$.

Statistical analysis

All values were expressed as mean ± SEM. Potential differences among the experimental groups were evaluated by using two-tailed analyses of variance (ANOVA) followed by Dunnett test to test the means that were identified as significantly different between groups. Values were considered significantly different when $P \leqslant 0.05$.

Results

Effects of Y-27632 and NDGA on 15-HETE-induced PA constriction

To determine whether the ROK and 15-HETE pathways were involved in the constriction of hypoxic PA rings, the ROK inhibitor Y-27632 and the 15-LOX inhibitor NDGA were used in rat PA tension studies. PA rings isolated from normoxic and hypoxic rats were incubated with 30 µM NDGA, 1 µM Y-27632 and NDGA plus Y-27632 for 30 min, respectively. The PA rings without any treatment were used as control. A series of concentration–response curves were produced when the PA rings were treated with increment concentrations of PE in the range from 10^{-7} to 10^{-5} M. The PE concentration response shifted to the right significantly in the groups of PA rings treated with NDGA, Y-27632 or NDGA plus Y-27632 compared with the control group under both normoxic and hypoxic conditions, with the group of NDGA plus Y-27632 more remarkable than that of NDGA alone (Fig. 1A and B), suggesting that 15-HETE-induced PA constriction in hypoxia may be mediated by the activation of the ROK pathway.

Rho/ROK activity assay in PAs

We evaluated the activity level of RhoA/ROK from PA by measuring the amount of phospho-Thr853 in total MYPT1, the ratio of phosphorylated MYPT1 to total MYPT1 was used as an index of the Rho/ROK activity (*16*). The results indicated that hypoxia increased the amount of phospho-Thr853 in total MYPT1 compared with normoxia, but administration of NDGA to hypoxic rats reversed this effect (Fig. 2), suggesting that Rho/ROK pathway was involved in the HPV mediated by 15-HETE.

Time-dependent changes in ROK expression under hypoxic condition

One way to up-regulate the ROK pathway is to enhance ROK expression, which we performed experiments to address. To optimize the experimental conditions, we examined time-dependent changes in ROK2 expression under hypoxic condition in cultured rat PASMCs. After serum-deprivation for 24 h, the cells cultured in 10% FBS were exposed to hypoxia at 0, 12, 24 and 48 h, respectively. We found that the ROK2 expression at both protein and mRNA levels started increasing in 12 h-exposure to hypoxia, and reached the plateau level in 24 h (Fig. 3A and B).

Effects of 15-HETE on the expression of ROK in cultured rat PASMCs

To investigate whether 15-HETE plays a role on the up-regulation of ROK expression, exogenous 15-HETE, the endogenous 15-LOX inhibitors

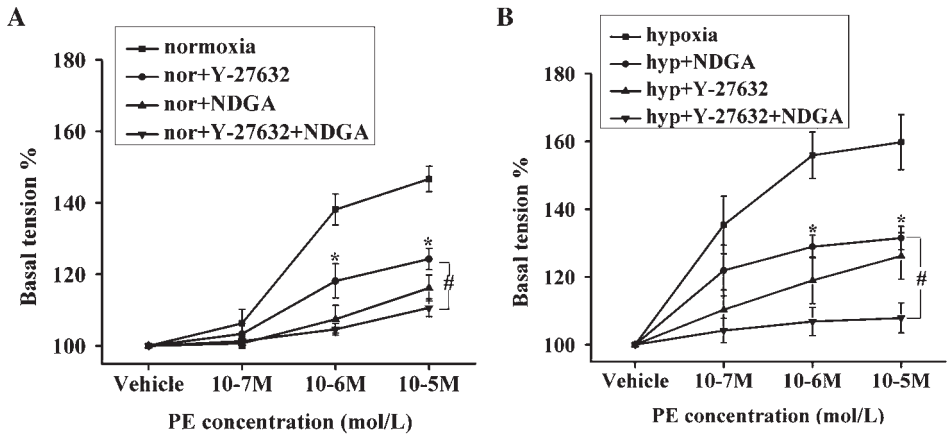

Fig. 1 Effects of Y-27632 on 15-HETE-induced pulmonary artery constriction. PA rings isolated, respectively, from normoxic and hypoxic rats were incubated with 30 μM NDGA, 1 μM Y-27632 and NDGA plus Y-27632 for 30 min, respectively. The PA rings without any treatment were taken as control. Then the PA rings were treated with increasing concentration of PE in the range from 10^{-7} to 10^{-5} M. (A) The vascular tension of the normoxic rats PA rings, $^{*}P<0.05$ versus normoxic control, $^{\#}P<0.05$ versus NDGA, $n=6$ from two rats. (B) The vascular tension of the hypoxic rats, $^{*}P<0.05$ versus hypoxic control, $^{\#}P<0.05$ versus Hyp+NDGA, $n=6$ from two rats.

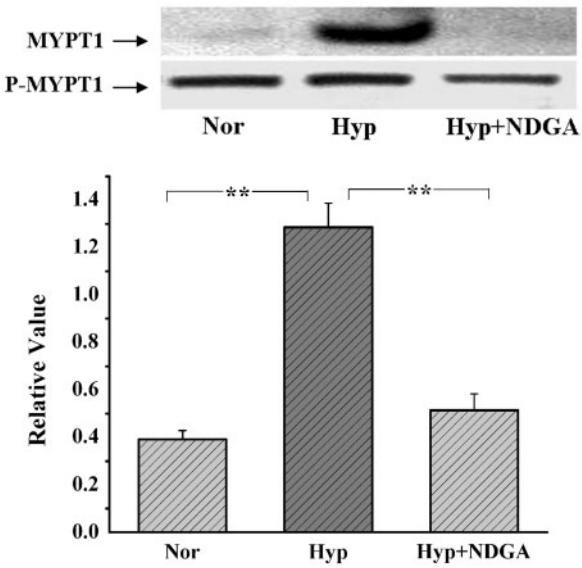

Fig. 2 The measurement of Rho-activity in PAs. MYPT1 phosphatase, represent Rho activity, was examined with western blot using PA rings from rats. 'Nor' means normoxic rat, 'Hyp' means hypoxic rat, 'Hyp+NDGA' means the rat treated with NDGA under hypoxic condition. All values are denoted as mean ± SEM ($n=3$, $^{**}P<0.01$).

NDGA and CDC were used in cultured rat PASMCs. Our results showed that both exogenous 15-HETE and hypoxia up-regulated the expression of ROK2 significantly in comparison with normoxic control, whereas either NDGA or CDC significantly reversed the up-regulation of ROK2 expression induced by hypoxia (Fig. 4A–D). In the presence of NDGA or CDC, exogenous 15-HETE still increased the ROK2 expression under hypoxic condition (Fig. 4A–D). We also studied the effects of 15-HETE on the expression of ROK1. The results showed that 15-HETE up-regulated the expression of ROK1, NDGA and CDC could reverse the effects of hypoxia on ROK1 expression (Fig. 4E). These data implicated that 15-HETE had a noticeable effects on both ROK1 and ROK2, suggesting that the up-regulation of ROK expression induced by hypoxia appears to be mediated by endogenous 15-HETE.

Effects of siRNA against 15-LO1 on the expression of ROK

As NDGA and CDC are chemical blockage of 15-lipoxygenase, we performed RNA interference technology to make the expression of 15-LO1 specifically partial deficiency. To assess the efficiency and specificity of RNA interference, we measured intracellular expression of 15-LO1 relative to β-actin by western blot assays. Our results showed that expression of 15-LO1 treated with siNC was not different from expression in untreated control cells or cells treated with the Transfection Reagent alone (Fig. 5A). Then the expression of ROK was tested by western blot assays. The results showed that the expression of both ROK1 and ROK2 decreased after the 15-LO was silenced under both normoxic and hypoxic conditions (Fig. 5B–E). These data suggested that the role of Rho/ROK pathway in PASMCs was mediated by 15-HETE under both normoxic and hypoxic conditions.

15-HETE-mediated translocation of ROK2 protein in cultured rat PASMCs

Previous studies have suggested that ROK2 protein in PASMCs were translocated from nucleus to cytoplasm during exposure to hypoxia (*17*), a process that is required for its activation and therefore precedes phosphorylation of MYPT-1 (*3*). To determine whether 15-HETE was involved in this process, the cultured PASMCs were divided into four groups: normoxic control, cells treated with 15-HETE during normoxia for 24 h, hypoxic control, and cells treated with NDGA during hypoxia for 24 h. We found that hypoxia and 15-HETE induced the ROK2 protein translocation from the nucleus to the cytoplasm, and NDGA could reverse the effect of hypoxia, while there was no notable translocation of ROK1 in cultured

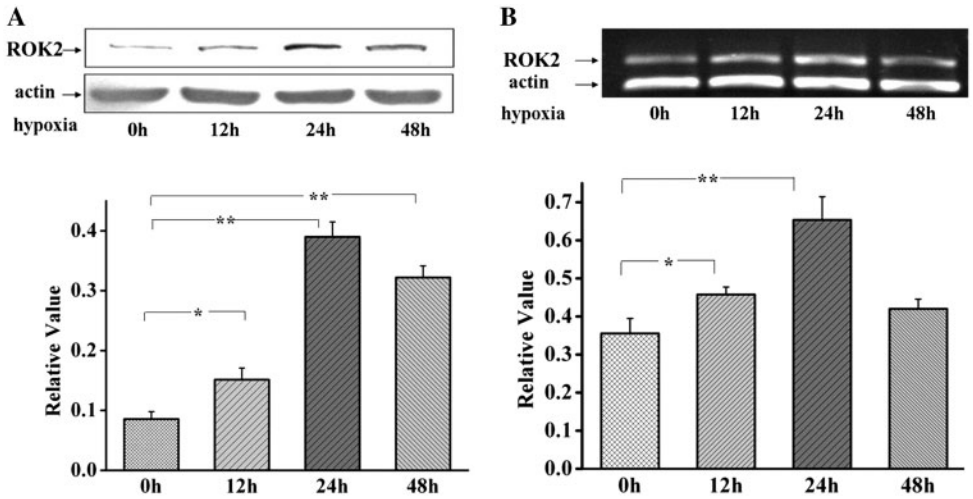

Fig. 3 Time-dependent changes in ROK expression under hypoxic condition. Cultured PASMCs were exposed to hypoxia for 0, 12, 24 and 48 h, respectively; hypoxia increased the expression of ROK in a time-dependent manner. (A) The expression of ROK protein in PASMCs. (B) The expression of ROK mRNA in PASMCs. All values are denoted as mean ± SEM ($n = 3$, $^*P < 0.05$, $^{**}P < 0.01$).

PASMCs (Fig. 6), indicating that 15-HETE was involved in ROK2 protein translocation during hypoxia.

Effects of G-protein and tyrosine kinase on 15-HETE-induced constriction of hypoxic PA rings

To determine whether G-protein and tyrosine kinase were involved in 15-HETE-induced contraction in hypoxic PA rings, G-protein inhibitors suramin and SCH-202676, tyrosine kinase blockers genistein and tyrphostin A25 and 15-LOX blocker NDGA were employed in the PA ring technique. Our results showed that the PE-induced contraction curve shifted to the right significantly in the groups of PA rings treated with NDGA, suramin, SCH-202676, NDGA plus suramin, NDGA plus SCH-202676, genistein, tyrphostin A25, NDGA plus genistein or NDGA plus tyrphostin A25 when compared with the control group (Fig. 7). In addition, the inhibitions of PA rings constriction with NDGA in the presence of suramin, SCH-202676, genistein and tyrphostin A25 were more obvious than that of NDGA alone (Fig. 7). These data indicated that NDGA, suramin, genistein, SCH-202676 and tyrphostin A25 decreased the PA rings contraction while being induced by hypoxia, in which G protein and tyrosine kinase pathways seem to play an important role.

Effects of G-protein on the up-regulation of ROK2 expression induced by 15-HETE in cultured rat PASMCs and the measurement of GTPase activity

To elucidate whether 15-HETE up-regulates the ROK expression through G-protein pathway, we used two G-protein inhibitors suramin and SCH-202676, and exogenous 15-HETE. Exogenous 15-HETE increased ROK2 protein expression compared with the control, an effect that was reversed by suramin or SCH-202676 under normoxic condition (Fig. 8A and B). Hypoxia raised the expression of ROK2 at both protein and mRNA levels in comparison with the normoxic control, while suramin and SCH-202676 could significantly reverse the effect of hypoxia on the expression of ROK2. Exogenous 15-HETE also increased the expression of ROK2 under hypoxic condition when compared with hypoxia alone. Such an effect was inhibited remarkably by suramin or SCH-202676 (Fig. 8C–F). We also measured the GTPase activity to examine the effect of 15-HETE on the G-proteins. The results showed that both 15-HETE and hypoxia increased the GTPase activity, but administration of NDGA or CDC to PASMCs decreased the GTPase activity (Fig. 9). These results therefore suggested that the up-regulation of the ROK2 expression by 15-HETE seemed to require G-protein-dependent processes.

Effects of tyrosine kinase on the up-regulation of ROK2 expression induced by 15-HETE in cultured rat PASMCs

To examine whether 15-HETE up-regulates the ROK expression through tyrosine kinase pathway, we tested two tyrosine kinase blockers: genistein and tyrphostin A25 and exogenous 5-HETE. Our results showed that exogenous 15-HETE increased ROK2 expression and this effect was reversed by genistein or tyrphostin A25 under normoxic condition (Fig. 10A and B). Hypoxia increased the expression of ROK2, while both genistein and tyrphostin A25 could significantly reverse this process. Furthermore, exogenous 15-HETE increased the expression of ROK2 under hypoxic condition compared with the hypoxic control which was inhibited remarkably by genistein or tyrphostin A25 (Fig. 10C–F). These results suggested that the up-regulation of the ROK2 expression induced by 15-HETE may also involve tyrosine kinase.

Measurements of endogenous 15- and 12-HETE levels by RP-HPLC

To measure the endogenous 15- and 12-HETE levels, RP-HPLC was employed. The results showed that the

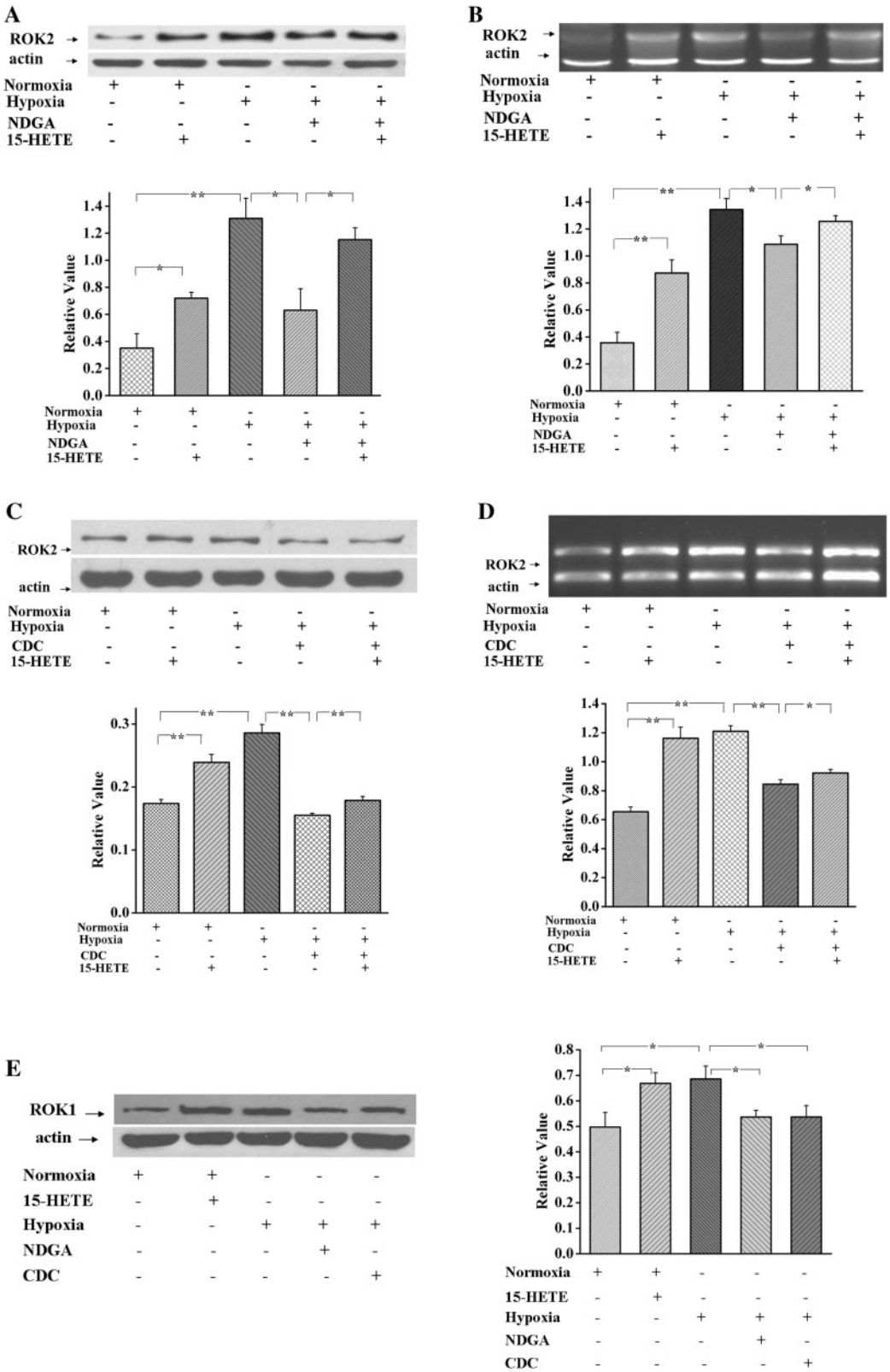

Fig. 4 Effects of 15-HETE on the expression of ROK in cultured PASMCs. The experimental groups were as follows: group 1 was normoxic control; group 2 was 15-HETE group, in which the cells were treated with 1 μM 15-HETE for 24 h under normoxic condition; group 3 was hypoxic group in which the cells were treated under hypoxic condition for 24 h; group 4 was subjected to inhibition of endogenous 15-HETE, in which the cells were pretreated with 30 μM NDGA or 5 μM CDC for 24 h under hypoxic condition; group 5 was exposed to exogenous 15-HETE and hypoxia, in which the cells were treated with 1 μM 15-HETE under hypoxic condition after blocks of endogenous 15-HETE with NDGA or CDC for 24 h. ROK2 protein expression in PASMCs treated with NDGA (A) and CDC (C) under hypoxic condition. ROK2 mRNA expression in PASMCs treated with NDGA (B) and CDC (D) under hypoxic condition. E: represented the effect of 15-HETE on the expression of ROK1 protein. All values are denoted as mean ± SEM ($n = 3$, $^{*}P < 0.05$, $^{**}P < 0.01$).

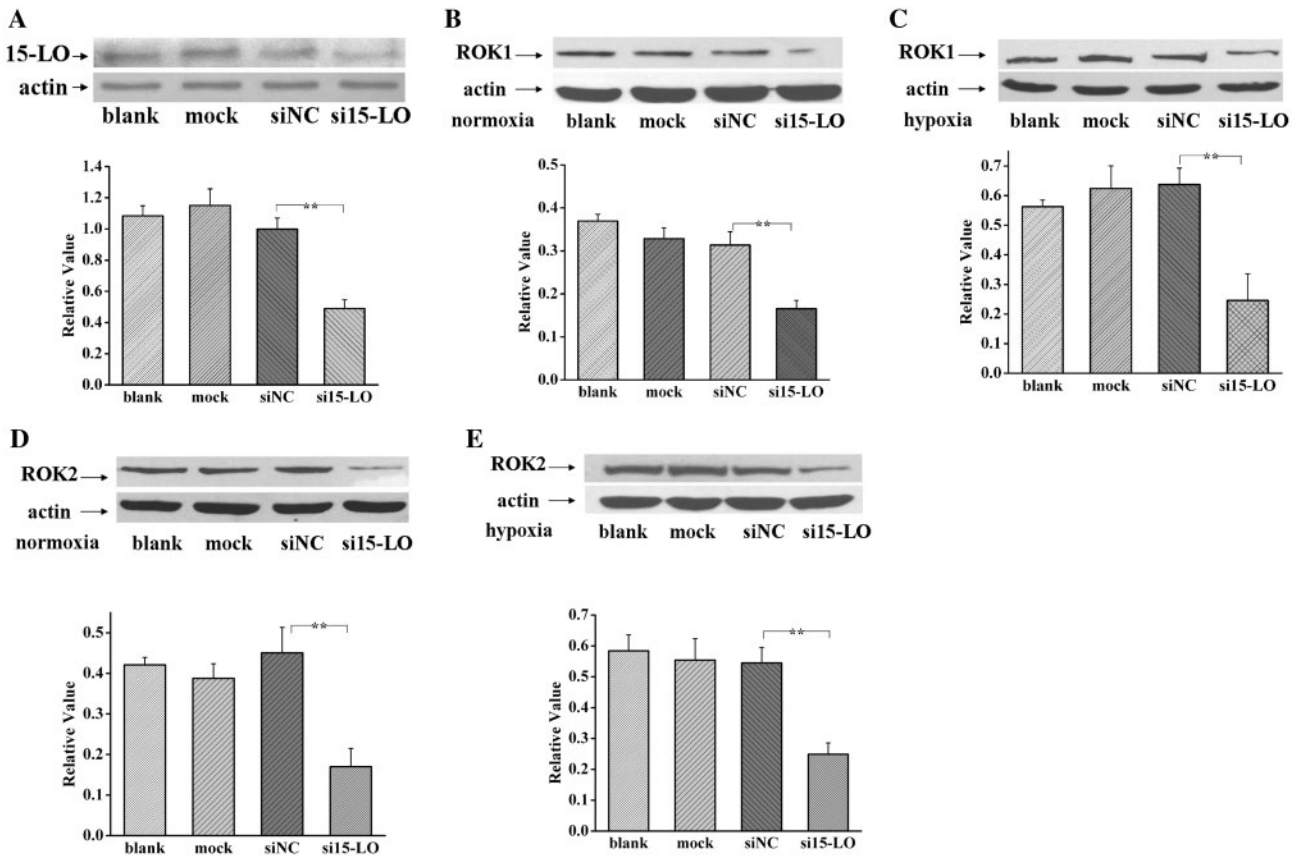

Fig. 5 The efficiency and specificity of siRNA directed against 15-LO1 and the role of 15-LO1 on the expression of ROK. Cells treated with Transfection Reagent alone (mock) and untreated PASMCs (blank) from the same isolation served as additional negative controls, siNC transfected cells were used as negative control. (A) Represented the amount of 15-LO1 protein expression after the cells were treated with siRNA against 15-LO1. (B and C) Represented the expression of ROK1 protein after the treatment of siRNA against 15-LO1 under normoxic and hypoxic conditions, respectively. (D and E) Represented the expression of ROK2 protein after the treatment of siRNA against 15-LO1 under normoxic and hypoxic conditions, respectively. All values are denoted as mean ± SEM ($n = 3$, $^{**}P < 0.01$).

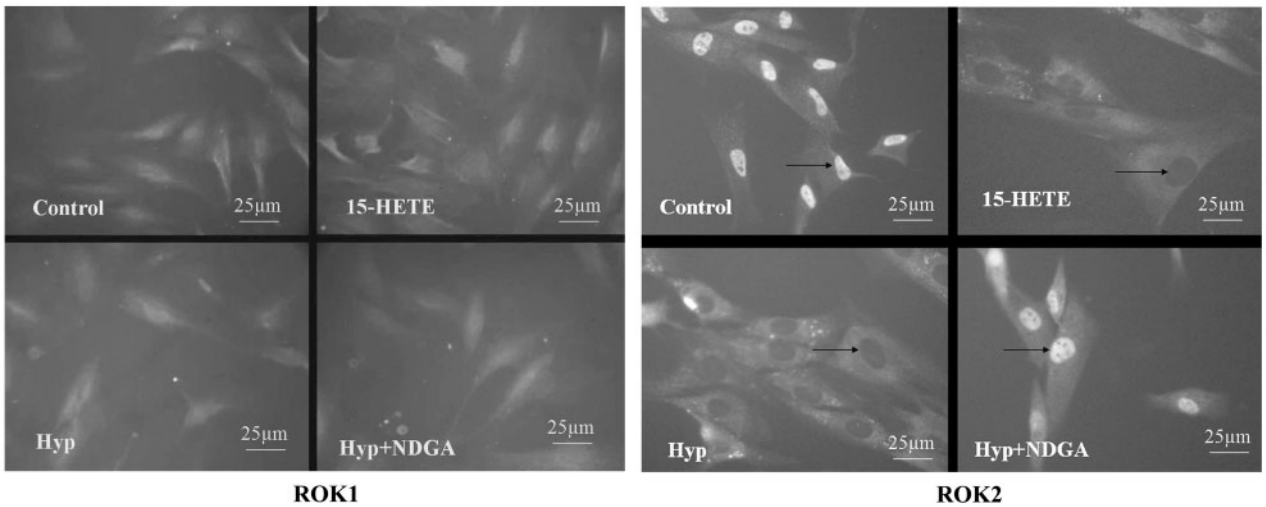

Fig. 6 15-HETE-mediated translocation of ROK2 protein in cultured PASMCs. The translocation of ROK2 protein in cultured PASMCs was induced by hypoxia and exogenous 15-HETE. There was no notable evidence of transloaction of ROK1 in cultured PASMCs. Control, the cells without any treatment exposed to normoxia; 15-HETE, the cells treated with 15-HETE (1 μM) under normoxic condition; Hyp, the cells without any treatment exposed to hypoxia; Hyp+NDGA, the cells treated with NDGA (30 μM) under hypoxic condition. Scale bars = 25 μm.

endogenous 15- and 12-HETE levels were both increased in rat lung tissues under hypoxic condition compared with under normoxic condition, while after treated with NDGA, the productions of 15- and 12-HETE decreased (Fig. 11).

Effects of other isoforms of HETEs

The specificity of 15-HETE in the up-regulation of ROK expression in rat PASMCs was examined by using exogenous 5-, 12- and 15-HETE. The results showed that 15-HETE increased the protein expression

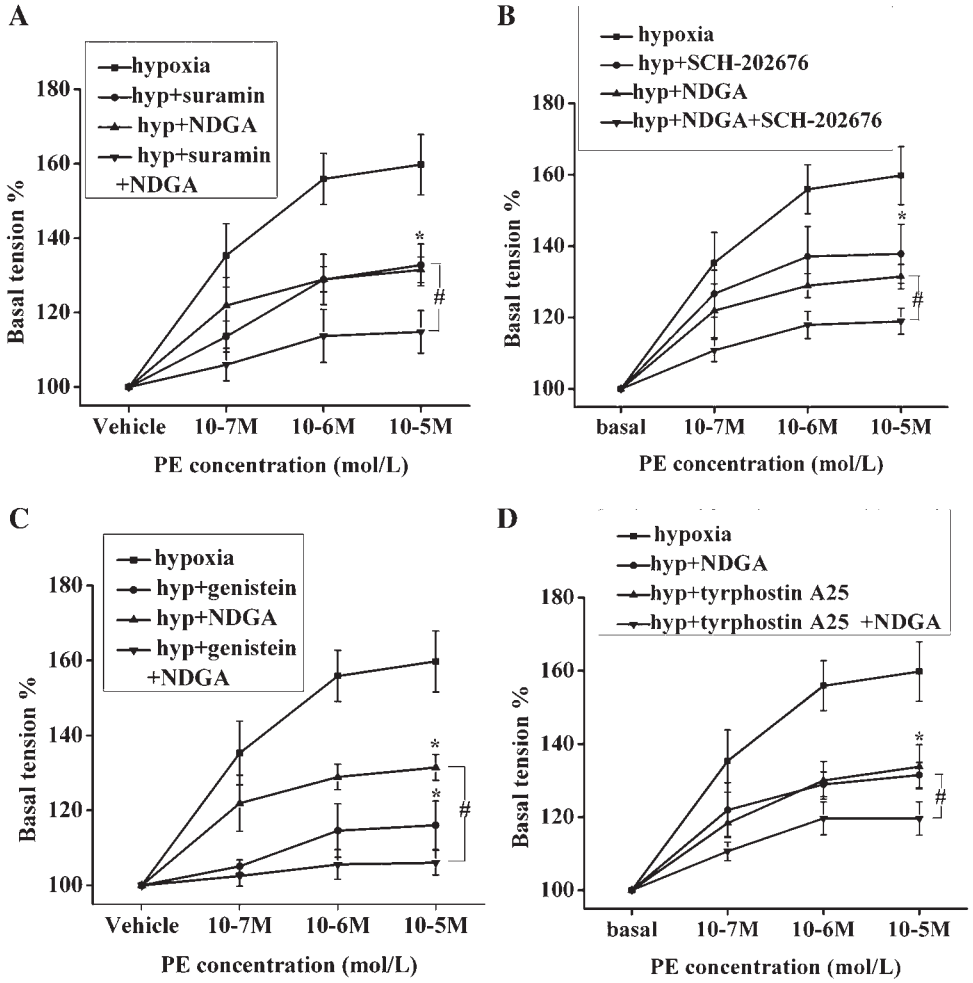

Fig. 7 Effects of G-protein and tyrosine kinase on 15-HETE-induced hypoxic rat PA rings constrictions. PA rings isolated from the rats exposed to hypoxia for 9 days were incubated with G-protein inhibitors, tyrosine kinase blockers and 15-LOX blocker NDGA, respectively, for 30 min. The PA rings without any treatment were served as control. Then the PA rings were treated with increasing concentration of PE in the range from 10^{-7} to 10^{-5} M. The vascular tension of the hypoxic rat PA rings treated with NDGA in the presence and absence of G-protein inhibitors suramin (100 μM) (A) and SCH-202676 (10 μM) (B). The vascular tension of the hypoxic rat PA rings treated with NDGA in the presence of tyrosine kinase blockers genistein (30 μM) (C) and tyrphostin A25 (150 μM) (D). All values are denoted as mean ± SEM, $^{*}P<0.05$ versus hypoxic control, $^{\#}P<0.05$ versus Hyp+NDGA, $n=12$ from four rats.

of both ROK1 and ROK2, while 5- and 12-HETE had no noticeable effects on it (Fig. 12). These data implicated that 15-HETE was more potent than 5- and 12-HETE in up-regulation of ROK expression.

Discussion

The present study provided novel evidence that Rho/ROK signalling pathway is involved in 15-HETE-induced PA constriction. Firstly, hypoxia increases the expression of ROK, which is regulated by 15-HETE, leading to pulmonary vasoconstriction. Secondly, 15-HETE induced up-regulation of ROK expression is likely to be mediated by G-protein and tyrosine kinase pathways, both of which are considered as the upstream signalling molecules of ROK. Thirdly, 15-HETE seems accountable for the ROK2 translocation from the nucleus to the cytoplasm during hypoxia.

A finding from the present study is that 15-HETE causes pulmonary vasoconstriction under hypoxic condition through Rho/ROK pathway. In our previous studies, we have found that hypoxic exposure induces the expression of 15-LO in the PAs (*8*). The 15-LO catalyzes the production of 15-HETE, which is an important mediator in regulating the HPV, while NDGA and CDC have been demonstrated to reduce the production of 15-HETE by blocking 15-LO (*18*, *19*).

It is reported that increased expression of ROK is an important signalling event in hypoxia-induced vascular adaptations in the lung. The activation and expression of ROK can lead to chronic hypoxic pulmonary hypertension by inducing sustained vasoconstriction (*20*), we suspected that whether 15-HETE-induced vasoconstriction is vial ROK signalling pathway. In the present studies, we have found that NDGA and Y-27632 remarkably attenuate the tension of hypoxic PA rings when compared with that of hypoxic PA rings without any treatment. These findings are strengthened by the data that 15-HETE increases the expression of ROK during hypoxia. We have shown that both

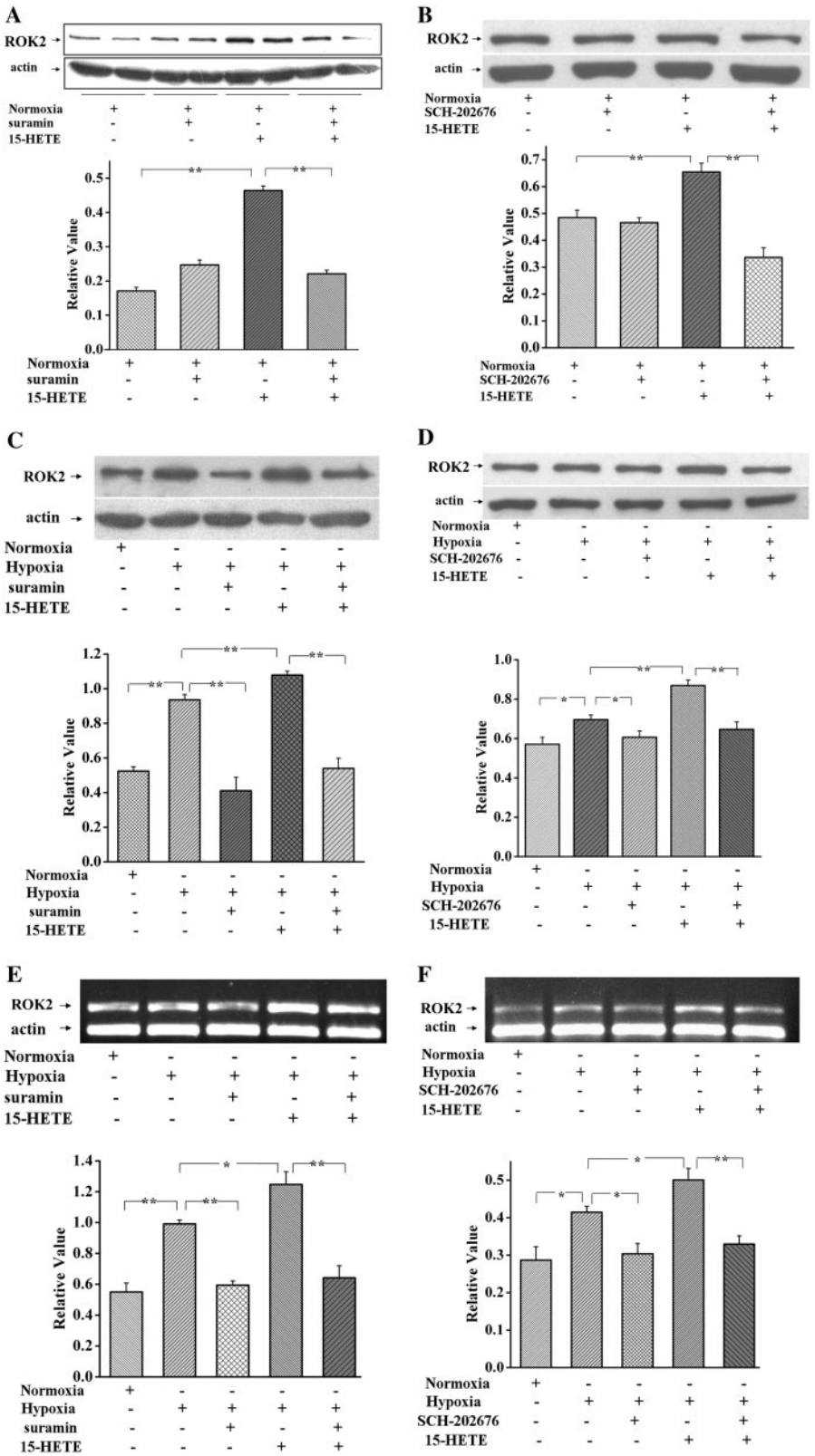

Fig. 8 Effects of G-protein on the up-regulation of ROK expression induced by 15-HETE in cultured PASMCs. The experiments of each G-protein inhibitors were divided into three parts: the first part was normoxic part for protein which was divided into four groups: control, inhibitor, 15-HETE, 15-HETE plus inhibitor; the other two parts were hypoxic parts, one is to determine protein, the other is to detect mRNA, each of parts included five groups: normoxic control, hypoxic control, hypoxia plus inhibitor, hypoxia plus 15-HETE and hypoxia plus 15-HETE plus inhibitor. ROK protein expression in PASMCs treated with suramin (A) and SCH-202676 (B) under normoxic condition. ROK protein expression in PASMCs treated with suramin (C) and SCH-202676 (D) under hypoxic condition. ROK mRNA expression in PASMCs under hypoxic condition with the presence of suramin (E) and SCH-202676 (F). All values are denoted as mean ± SEM ($n = 3$, $^*P < 0.05$, $^{**}P < 0.01$).

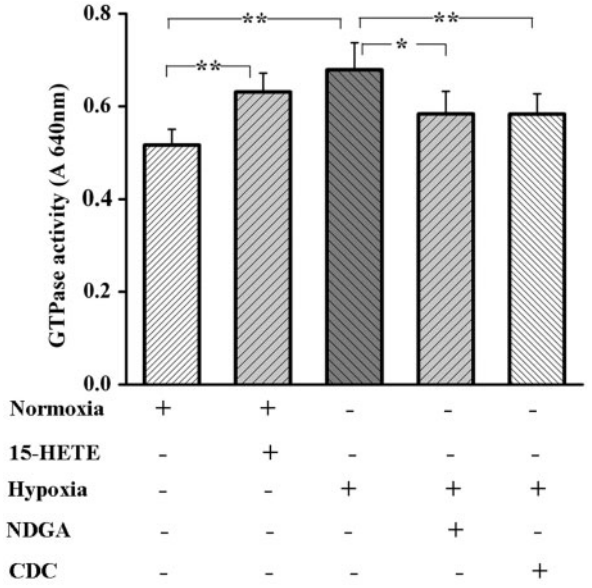

Fig. 9 GTPase activity assay in PASMCs. The experiments were divided into five groups: the cells treated with 15-HETE under normoxic condition; the cells treated with NDGA or CDC under hypoxic condition; the cells without adding any reagents grown during normoxic and hypoxic conditions were taken as control. All values are denoted as mean ± SEM ($n=6$, $^{*}P<0.05$, $^{**}P<0.01$).

hypoxic exposure and exogenous 15-HETE increase the expression of ROK in PASMCs significantly in both protein and mRNA levels. Inhibiting the formation of endogenous 15-HETE with NDGA or CDC decreased the expression of ROK in PASMCs under hypoxic condition. Moreover, in the presence of NDGA or CDC during hypoxia, exogenous 15-HETE still promotes the expression of ROK, identifying that up-regulation of ROK in PASMCs under hypoxic condition resulted from endogenous 15-HETE. Besides using the chemical blockage of 15-lipoxygenase, we also transfect PASMCs with siRNA directed against 15-LO1 to confirm the effect of 15-HETE on the expression of ROK. Moreover, MYPT1 phosphatase, represented Rho activity, was increased in PA rings from hypoxic rats, which was reversed by inhibiting the formation of endogenous 15-HETE. These data suggest that 15-HETE causes the hypoxic vasoconstriction through Rho/ROK pathway.

The present study also extends our understanding of signalling pathway network involved in 15-HETE-induced PA vasoconstriction. It is well known that G-protein coupled with G-protein coupled receptors (GPCRs) regulate Rho/ROK-kinase-dependent signalling processes (*6*, *21–23*). The heterotrimeric G-proteins consist of an α subunit (Gα) with GTPase activity and a βγ dimmer (Gβγ), signal-activated G protein-coupled receptors (GPCRs) induce GDP release from a Gα subunit, which is followed by the binding of GTP and turns on the system and causes conformational changes, then the signal transduction is turned off by the intrinsic GTPase activity of the Gα protein (*24*, *25*). To identify whether G-protein is involved in 15-HETE-induced PA vasoconstriction, we employed suramin and SCH-202676 to block the upstream of Rho/ROK-kinase-dependent signalling pathway and measured the GTPase activity. Some researchers have reported that suramin can not only disrupt receptor-G protein coupling but also inhibit guanine nucleotide release, as well as promoting the development of selective inhibitors of G protein signalling and G protein selective drugs (*26*, *27*). Furthermore, suramin could significantly reduce the contractile response of some agonists such as uridine nucleotides in PA smooth muscle (*28*), while SCH-202676 has been reported to regulate GPCR function as an allosteric modulator of both agonist and antagonist binding to G-protein-coupled receptors (*29*). Our data have shown that both suramin and SCH-202676 decrease the expression of ROK which is up-regulated by hypoxia in PASMCs. Moreover, they reduce the up-regulation of ROK expression induced by 15-HETE under both hypoxic and normoxic conditions in PASMCs, suggesting that the expression of ROK up-regulated by hypoxia and 15-HETE is fulfilled through G-protein pathway. G-protein blockers suramin and SCH-202676 also decreased the tension of PA rings increased by hypoxia, which is similar with the effect of inhibition of endogenous 15-HETE on PA rings. Furthermore, the GTPase activity increased after treated with 15-HETE. Our studies present the new evidence that hypoxia up-regulated the expression of ROK is mediated by 15-HETE via G-protein pathway.

In addition to the G-protein pathway, we also examined the contribution of tyrosine kinase pathways to 15-HETE-regulated Rho/ROK pathway. It is previously reported that tyrosine kinase regulates the activity and the expression of Rho through the tyrosine phosphorylation of RGL-containing RhoGEFs which promotes the exchange of GDP for GTP bound to Rho and activate its function (*30*). In our study, the tyrosine kinase inhibitors genistein and tyrphostin A25 decreased the expression of ROK up-regulated by 15-HETE, moreover, they attenuated the tension of PA rings induced by hypoxia, which coordinates well with the demonstration that tyrosine kinase is involved in excitation–contraction coupling in pulmonary vascular smooth muscle.

The question raised here is that whether 15-HETE up-regulated ROK expression is specific compared to other monoHETEs. Other HETEs were employed to test the possibility. The results showed that 12- and 5-HETE had no noticeable effects on the expression of ROK. The data were consistent with our HPLC results, in which endogenous 15-HETE levels was largely increased in rat lung tissues under hypoxic condition compared with that of 12-HETE under the same condition, implying that the up-regulation of ROK expression is mediated by 15-HETE under hypoxic condition.

The cellular location and subcellular distribution of ROK are another important factors relating to the function of ROK. It is reported that the translocation of ROK2 from nucleus to cytoplasm is a key process for the activation of the kinase (*3*). Therefore, the question arises as to whether hypoxia induces translocation

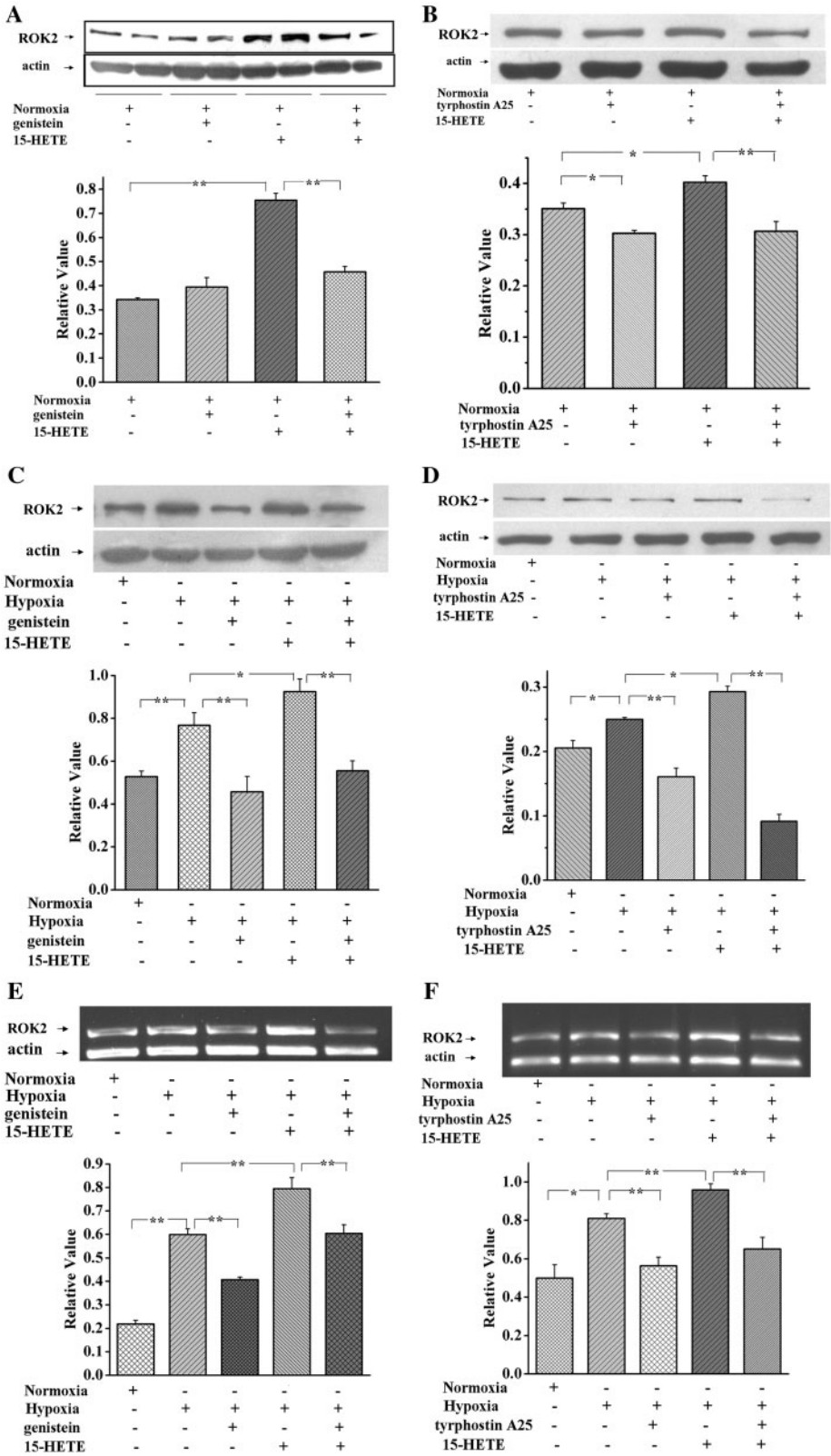

Fig. 10 Effects of tyrosine kinase on the up-regulation of ROK expression induced by 15-HETE in cultured PASMCs. The experimental groups with tyrosine kinase blockers were similar with Fig. 5. ROK protein expression in PASMCs treated with genistein (A) and tyrphostin A25 (B) under normoxic condition. ROK protein expression in PASMCs treated with genistein (C) and tyrphostin A25 (D) under hypoxic condition ROK mRNA expression was determined by RT-PCR in PASMCs under hypoxic condition in the presence of genstein (E) and tyrphostin A25 (F). All values are denoted as mean ± SEM ($n = 3$, $^{*}P < 0.05$, $^{**}P < 0.01$).

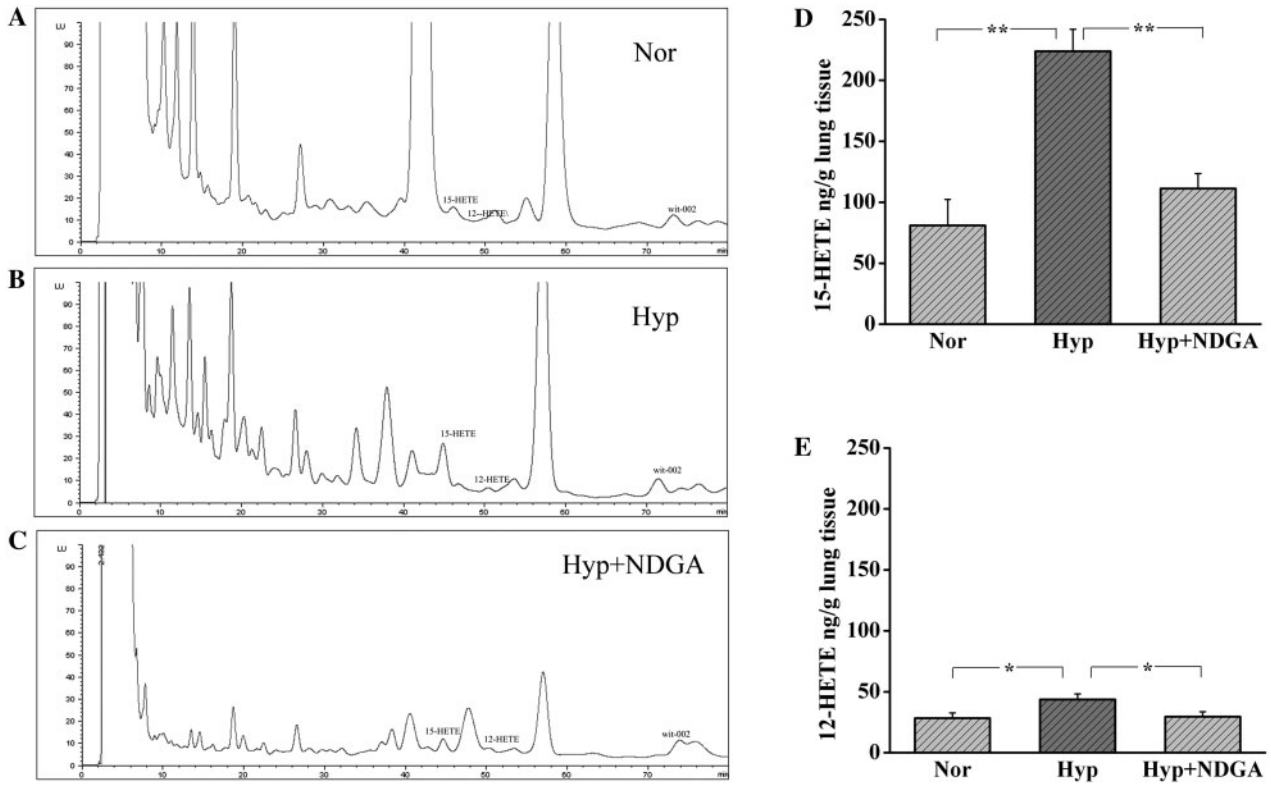

Fig. 11 Measurement of endogenous 15-HETE and 12-HETE levels by RP-HPLC. The endogenous 15- and 12-HETE levels were assayed with rat lung tissues under normoxic condition (A) and hypoxic condition in absence (B) or presence (C) of NDGA. (D and E), respectively, represented the statistical analysis of the productions of 15- and 12-HETE. All values are denoted as mean ± SEM ($n = 3$, $^*P < 0.05$).

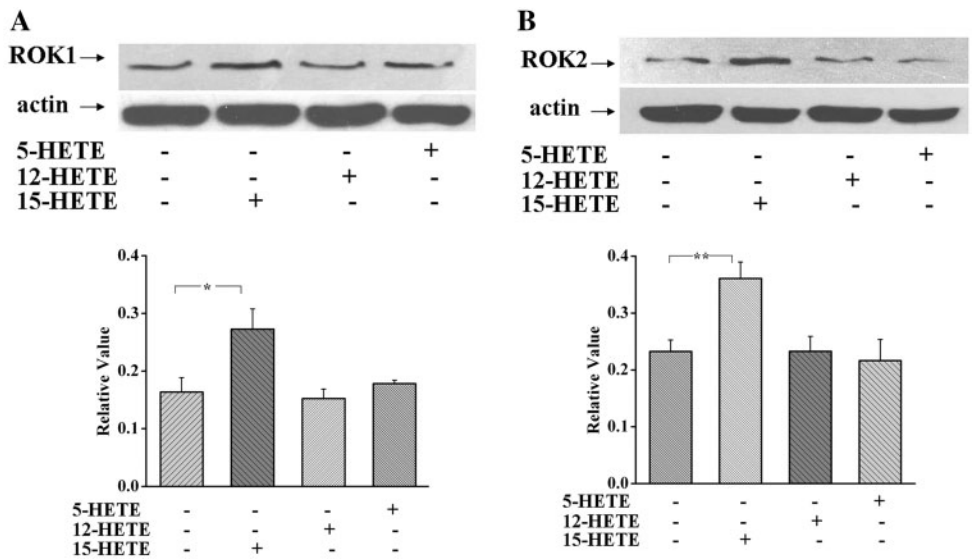

Fig. 12 Effects of other isoforms of HETEs. Exogenous 5-, 12-, 15-HETE were added to compare the influence of them on ROK protein expression in cultured rat PASMCs, the cells without any treatment were taken as control. (A) ROK1 protein expression in PASMCs. (B) ROK2 protein expression in PASMCs. All values are denoted as mean ± SEM ($n = 3$, $^*P < 0.05$, $^{**}P < 0.01$).

of ROK2 protein and whether the translocation is mediated by 15-HETE. In controlled cells, ROK2 staining is concentrated in the nucleus, as has been shown in the report by Knock *et al.* (*31*), but with the experience of hypoxia and 15-HETE, it is translocation to the cytoplasm. Importantly, this movement is reversed by pre-treatment with NDGA, indicating that the movement of ROK2 in response to stimulation by hypoxia is likely to be mediated by 15-HETE.

It is well-known that the carboxy-terminal regions of ROK, which contain the PH domain and the RBD, serve as an auto-regulatory inhibitor of the amino-terminal kinase domain (*32*). ROK could be activated not only by RhoA through amino-terminal transphosphorylation (*33*), but also by AA through binding of AA to the PH domain which is essential for targeting of the kinase to actomyosin compartment and subsequent assembling myosin (*33*, *34*). 15-HETE, as a

capital metabolite of AA, is likely to translocate from nucleus to cytoplasm by binding to the PH domain of the ROK2. The recent studies also show that there are several nuclear transport signals within the ROK2 amino acid sequences and some of the nuclear export signals are functionally active in exporting ROK2 from the nucleus, this regulation might involve post-translational modifications of ROK2 (*35*). The mechanism of the translocation mediated by 15-HETE remained unclear and need to be proved in our future study.

In conclusion, we have found that the increased expression of ROK up-regulated by 15-HETE is a key signalling event in the hypoxia-induced vascular contraction in the lung, G-protein and tyrosine kinase appear to raise the expression of ROK as the upstream of RhoA. These findings together with previous reports indicate that 15-HETE is an important contributor to pulmonary vascular resistance acting seemingly via the G-protein and tyrosine kinase-Rho/ROK pathways.

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Conflict of interest

None declared.

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