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Rab1 GTPase regulates phenotypic modulation of pulmonary artery smooth muscle cells by mediating the transport of angiotensin II type 1 receptor under hypoxia

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Abstract

Previous studies have demonstrated that Rab1 is involved in the export of angiotensin II (Ang II) type 1 receptor (AT1R) to the cell surface in endothelial cells and cardiomyocytes. The aim of this study was to evaluate whether the modification of Rab1-mediated endoplasmic reticulum (ER) to the Golgi body transport alters the cell surface expression and function of endogenous AT1R and AT1R-mediated phenotypic modulation in primary cultures of pulmonary artery smooth muscle cells (PASMCs). Lentiviral expression of wild-type Rab1 (Rab1WT) significantly increased cell surface expression of endogenous AT1R. However, Rab1 siRNA had the opposite effect, and attenuated downregulation of the expression of PASMCs phenotype markers, α smooth muscle actin (α-SMA) and vimentin (VIM) in rat pulmonary artery smooth muscle cells (RPASMCs) during hypoxia. Analysis of the subcellular localization of AT1R revealed that Rab1 regulated AT1R transport from the ER to the Golgi in PASMCs. Consistent with their effects on AT1R export, Rab1 modified the AT1R-mediated cell growth and the phosphorylation of signal transducing activator of transcription 3 (STAT3) during hypoxia. We found that hypoxia promoted Rab1 expression and strongly correlated with the repressed expression of PASMC phenotype markers in RPASMCs. These data strongly indicate that Rab1 modulates PASMCs function by manipulating AT1R traffic from the ER to the Golgi and provide the first evidence implicating ER-to-Golgi transport as a regulatory step for the control of RPASMCs growth.

Keywords

Pulmonary artery smooth muscle cells; Phenotypic modulation; Rab1 GTPase; Angiotensin II type 1 receptor; Hypoxia

1. Introduction

Phenotypic modulation of the VSMCs plays a key role in a number of major diseases in humans. In systemic arteries, atherosclerosis, restenosis, pulmonary hypertension, and leiomyogenic tumorigenicity are the most widely acknowledged human diseases that are

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believed to involve VSMCs phenotypic modulation. At least some of the changes in the arterial wall structure result from medial VSMCs becoming highly proliferative and secretory. The transition of VSMCs from a contractile, differentiated phenotype to a synthetic, undifferentiated phenotype is an early event in systemic vascular changes in diseases (Owens et al., 2004; Faries et al., 2002; Hao et al., 2003; Lincoln et al., 2006). Phenotypic modulation of pulmonary artery smooth muscle cells can be triggered by hypoxia and involves many signaling pathways (Howell et al., 2003; Zhou et al., 2007). Recent research showed that angiotensin II (Ang II) is involved in hypoxia-induced proliferation of cultured pulmonary artery smooth muscle cells (Yu and Ma, 2000).

Ang II, an octapeptide hormone, elicits biological actions by binding to specific membrane-bound receptors on target cells to activate multiple intracellular signal transduction pathways. Using selective receptor ligands, two major Ang II receptor subtypes were identified and subsequently cloned: angiotensin II type 1 receptor (AT1R) and angiotensin II type 2 receptor (AT2R). Both receptor subtypes belong to the seven transmembrane spanning receptor superfamily that is coupled with heterotrimeric G proteins (Matsubara, 1998; Timmermans et al., 1993; Kambayashi et al., 1993). Stimulation of AT1 receptors by Ang II activates the mitogen-activated protein kinases, such as extracellular-regulated kinases, by way of Src and Ras (small G proteins belonging to the Ras family of G proteins) and the JAK/STAT pathway (Schmitz and Berk, 1997). AT1R may play an important role in vascular remodeling and phenotypic modulation of VSMCs (Masakazu et al., 2004). Hypoxia does not affect the total expression of AT1R in heart and blood vessels but contributes to the expression of AT1R on the surface of cardiac myocytes and VSMCs (Della Bruna et al., 1995; Fletcher et al., 2002).

Proper AT1R function is dependent on its correct intracellular transport. After being synthesized, folded, and assembled in the ER, AT1R is transported to the Golgi apparatus, where post-translational modificationsoccur (Lanctôt et al., 1999). Afterward, it is transported to the plasma membrane. The AT1R at the plasma membrane undergoes internalization in the continued presence of Ang II agonist. The internalized AT1R may then be transported to the lysosome for degradation or recycled back to the plasma membrane (Hunyady et al., 2000; Claing et al., 2002; Marchese et al., 2003). Therefore, the number of AT1Rs on the plasma membrane is determined by the overall balance between AT1R export to the cell surface, internalization, recycling, and degradation. Most studies on AT1R trafficking have focused on the events involved in its internalization, recycling, and degradation (Hunyady et al., 2000; Claing et al., 2002; Marchese et al., 2003), while work examining the export of AT1R from the ER through the Golgi apparatus to the cell surface and the regulation of receptor function by these processes has not been performed.

Rab proteins, which are members of the Ras superfamily of monomeric GTPases, mediate intracellular protein trafficking between organelles (Zerial and McBride, 2001). Rab GTPases are defined as small GTP-binding proteins of ~20–30 kDa and include 60 proteins that are defined by their similar structure, function, and guanine nucleotide binding properties (Pereira-Leal and Seabra, 2000). The Rabs play essential roles in various aspects of vesicular membrane transport in both the exo- and endocytic pathways, and function as molecular switches through changes in their guanine nucleotide binding status. In its active, GTP-bound form, Rabs mediate vesicular transport by allowing transport carriers or vesicles to engage specific effectors (Zhao et al., 2007). Rab protein function is tightly regulated at the level of protein expression, localization, membrane association, and activation. It has been known since the early 1990s that Rab1 can localize to the ER and Golgi and regulates antegrade protein transport, specifically from the ER to the Golgi and between the Golgi compartments (Béraud-Dufour and Balch, 2002; Short et al., 2005; Duvernay et al., 2005; Duvernay et al., 2004; Wittinghofer, 2006; Saraste and Goud, 2007). Rab1 regulates the

transport of AT1R from the ER to the cell surface of epithelial cell and cardiocytes (Filipeanu et al., 2004; Wu, 2008). These data suggest that PASMCs phenotypic modulation and other activities may be modulated by altering Rab1 function, which could subsequently affect the cell surface expression of AT1R. Owing to the cited observations, the aim of our study was to assess whether revised ER-to-Golgi transport could change the cell surface expression and function of AT1R in RPASMCs during hypoxia. The role of Rab1-mediated alterations of phenotypic modulation and other activities of RPASMCs was also investigated. We show here that Rab1 was indeed significantly increased in RPASMCs exposed to hypoxia. Furthermore, Rab1 affected AT1R transport, specifically from the ER to the Golgi, and silencing of Rab1 with siRNA attenuated hypoxia-mediated phenotypic modulation. In addition, Rab1 siRNA suppressed hypoxia-induced RPASMC proliferation and phosphorylation of STAT3. All these data raised the possibility that Rab1 was involved in the process of hypoxia-induced phenotypic modulation and proliferation of RPASMCs by regulating AT1R transport from the ER to the Golgi.

2. Materials and methods

2.1. Materials

Sprague–Dawley rats were obtained from Chongqing City Laboratory Animal Center, Chongqing, China. High glycose Dulbecco's modified Eagle's medium (DMEM), trypsin and fetal bovine serum (FBS) were purchased from GIBCO (Carlsbad, CA). [^{3}H]Ang II (specific activity = 50.5 Ci/mmol) were purchased from PerkinElmer Inc. (Waltham, MA). Anti-FLAG M2 monoclonal antibody, human Ang II and PD 123319 (the specific AT2R antagonist) were obtained from Sigma (St. Louis, MO). ZD 7155 (the specific AT1R antagonist) was purchased from Tocris Bioscience (Ellisville, MI). Antibodies against Rab1, antibody against hemagglutinin (HA), antibody against VIM were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Antibody against α-SMA was purchased from Abcam plc (Cambridge, UK). High affinity fluorescein-conjugated anti-HA antibody 3F10 was purchased from Roche Applied Science (Mannheim, Germany). Antibody against β-actin, antibody against phospho-STAT3 and antibody against STAT3 was purchased from Cell Signaling Technology Inc. (Beverly, MA). CellTiter 96® AQueous One Solution Cell Proliferation Assay was obtained from Promega (Madison, WI). Lipofectamine 2000 reagent, Penicillin and streptomycin were purchased from Invitrogen (Carlsbad, CA). Wild type Rab1-pcDNA3 (Rab1WT), pcDNA3 plasmid, pDsRed2-ER, an ER marker, AT1R-pCDM8 tagged with green fluorescent protein (GFP-AR1T) and AT1R tagged with hemagglutinin (HA-AT1R) were kind gifts of Dr. Wu (Department of Pharmacology and Experimental Therapeutics, Louisiana State University Health Sciences Center, New Orleans, Louisiana). All other materials were described elsewhere (Wang et al., 2008; Li et al., 2010).

2.2. Cell culture

RPASMCs were isolated from the pulmonary arteries of male Sprague–Dawley rats weighing 150–200 g with an explant technique previously described (Bai et al., 2006), and with the approval of the local Ethical Committee. In brief, the pulmonary arteries were dissected free of fat and excess adventitial tissue and then opened along its longitudinal axis, and the endothelial lining was removed by scraping of the luminal surface. Small fragments (~1 mm^3) were transferred to a flask and cultured in DMEM containing 10% FBS supplemented with 4 mM L-glutamine, penicillin 100 U/ml and streptomycin 100 μg/ml in a humidified atmosphere (95% air–5% CO_2) at 37.0 °C. The purity and identity of RPASMCs were verified by their typical morphological pattern and by immunofluorescence using specific antibody against α-SMA. Cells from passages 3–5 were used in experiment, RPASMCs were grown on 25-cm^2 plates to 90% confluence and then cultured in DMEM

containing 0.5% FBS for 24 h. RPASMCs were transferred into an air-tight hypoxic chamber incubator (Thermo electron, USA) containing 3% O_2–5% CO_2–N_2 for different periods of time. In order to study roles of AngII receptor in hypoxia-induced effect, we used AT1R and AT2R antagonists (ZD7155 and PD123319) in medium. The concentration of AngII, ZD7155 and PD123319 was 100 nM, 100 nM and 1 μM respectively.

2.3. Lentivirus particles for rab1 and infection of RPASMCs

Rab1WT lentivirus particles encoding the FLAG epitope were constructed by Genechem Co., Ltd. (Shanghai, China) using a transient transfection procedure as described previously (Li et al., 2010). The recombinant lentivirus of Rab1 and the control lentivirus (empty lentivirus) were prepared and titered to 10^9 TU/ml (transfection unit). The cells were transiently transfected with control parent lentivirus or lentivirus expressing Rab1 (multiplicity of infection of 5–100). After infection for 6 h, the medium was changed, and the RPASMCs were further cultured in DMEM with 10% FBS. The efficiency of lentivirus infection of RPASMCs was evaluated by fluorescent microscopy.

2.4. RNA Interfering

Short hairpin RNA (shRNA) lentiviral particles of mouse Rab1, Control shRNA Lentiviral Particles and copGFP Control Lentiviral Particles were purchased from Santa Cruz Biotechnology. Gene silence of Rab1 in RPASMCs was performed using Rabl shRNA lentiviral particles containing the resistance gene for puromycin (Santa Cruz Biotechnology Inc.) according to the manufacturer's instruction manual. Transfectants were selected by culturing DMEM medium containing 5 μg/ml of puromycin and 10% FBS. Cloning of a transfectant with the lower Rab1 expression by Rab1 shRNA was performed by limiting dilution. Expression of Rab1 protein was assessed by immunoblotting with anti-Rab1 antibody. After 1 week, the cells were processed as described.

2.5. Plasmid transfection

Transfection of the RPASMCs was carried out using Lipofectamine 2000 reagent (Filipeanu et al., 2004; Wu et al., 2003). RPASMCs were cultured on 6 well dishes, for each transfection, 0.8 μg of GPF-AT1R, or pDsRed2-ER were diluted into 125 μl of serum-free Opti-MEM in a tube; in another tube, 7 μl of Lipofectamine was diluted into 125 μl of serum-free Opti-MEM. The two solutions were combined and mixed gently. After incubation for 20 min at room temperature, the mixture was added to each well containing 800 μl of fresh DMEM. Based on the fluorescence, 80% of the cells were transfected.

2.6. Measurement of AT1R expression at the cell surface

Cell-surface expression of AT1Rs in RPASMCs was measured by intact cell ligand binding as described previously (Filipeanu et al., 2004; Wu et al., 2003). Briefly, RPASMCs were cultured at a density of 5×10^5 cells/well on 12-well plates. The cells were incubated with phosphate-buffered saline (PBS) containing [^{3}H]Ang II at 4 °C (to limit AT1R internalization induced by ligand Ang II during the binding) overnight with constant shaking. To exclude the contribution of AT2R to ligand binding, all solutions were supplemented with 1 μM PD 123319, a specific AT2R antagonist. The nonspecific binding was determined in the presence of nonradioactive Ang II (10 μM). The cells were washed twice with 1 ml of ice-cold PBS, and the cell surface-bound [^{3}H]Ang II was extracted by mild acid treatment (2–5 min with 0.5 ml of buffer containing 50 mM glycine, pH 3, and 125 mM NaCl). The radioactivity for each sample was counted by a liquid scintillation counter. We determined the Ang II dose-dependent binding curve (2.5–20 nM) in RPASMCs. Ang II binding to RPASMCs increased linearly at concentrations between 2.5 and 20 nM and reached maximal binding at the concentration of 17.5 nM. Therefore, the cell

surface expression of AT1R in RPASMCs infected with Rab1 lentivirus was measured at a saturating concentration of 20 nM.

Total AT1R expression at the cell surface in RPASMCs was measured by flow cytometry as described previously. Briefly, RPASMCs were transfected with HA-AT1R, collected and resuspended in PBS containing 1% fetal bovine serum at a density of 4×10^6 cells/ml, and incubated with high affinity anti-HA antibody 3F10 conjugated fluorescein at a final concentration of 2 μg/ml for 30 min at 4 °C. After washing twice with 0.5 ml of PBS and 1% fetal bovine serum, the cells were resuspended, and the fluorescence was measured in a flow cytometer (Filipeanu et al., 2004; Wu et al., 2003).

2.7. Western blotting

Western blotting was carried out as described previously (Wang et al., 2008). 40 μg of protein samples was separated by 12% SDS-PAGE and transferred onto polyvinylidene difluoride membranes. The signal was detected by enhanced chemiluminescence detectionsystem (ECL Plus, Amersham, Buckinghamshire, England). Densitometric analysis was performed using Quantity One software.

2.8. Immunofluorescence microscopy

After treatments, the RPASMCs were fixed with a mixture of 4% paraformaldehyde and 4% sucrose in PBS for 15 min. The cover-slips were mounted, and fluorescence was detected with a Leica DMRA2 epifluorescence microscope. Images were deconvoluted using Slide-Book software and the nearest-neighbors deconvolution algorithm (Intelligent Imaging Innovations) as described previously (Li et al., 2010).

2.9. MTS assay for cell viability

The cell viability of RPASMCs were determined by MTS assay [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] according to the manufacturer's instructions, the cells were plated in 100 μl of culture medium at 5×10^3 cells/well in a 96-well plate, pipet 20 μl of CellTiter 96® AQueous One Solution Cell Proliferation Assay into each well. Thereafter, cells were treated with MTS at 37 °C for 2 h, and absorbance at 490 nm was then determined with a microplate reader (Bio-TEK Inc., Winooski, VT). All the experiments were repeated 5 times.

2.10. Statistical analysis

Statistical comparisons were performed using the paired, two-tailed Student's *t*-test for experiments consisting of two groups only and with one-way ANOVA with a multiple comparison method for experiments consisting of more than two groups. Results were considered statistically significant when $P < 0.05$. Data are presented as mean ± standard error.

3. Results

3.1. RPASMC characteristics

Under phase-contrast microscopy, normal primary RPASMCs had a fusiform-like structure and were grouped together in peak-valley formations (magnification 40×) (Fig. 1A). The cells were characterized as smooth muscle cells by α-SMA antigen expression. Using immunofluorescence, positive expression of α-SMA antigen in RPASMCs was demonstrated by the presence of lines in the cytoplasm that bound IgG marked with fluorescein isothiocyanate (FITC). The nuclei are appear blue following 4′,6-diamidino-2-phenylindole (DAPI) staining (magnification 400×) (Fig. 1B).

3.2. Expression of Rab1 in RPASMCs

We determined the effect of lentivirus-mediated expression of Rab1 in RPASMCs. The expression of Rab1 in RPASMCs was determined by Western blotting using a Rab1 antibody (Fig. 2A). Localization of FLAG-Rab1WT in infected RPASMCs was revealed by fluorescence microscopy following immunostaining with anti-FLAG antibodies. Similar results were obtained in at least three separate experiments. Infected Rab1WT was localized to the per-inuclear region of RPASMCs, based on the fluorescence, 70% of the cells were transfected (Fig. 2B).

3.3. Effect of hypoxia on the expression of Rab1 and cell surface expression of AT1R in RPASMCs

RPASMCs were cultured and exposed to hypoxia for 48 h. The expression of Rab1 increased by about 40%, as determined by Western blotting using the Rab1 antibody (Fig. 3A). The cell surface expression of AT1R in RPASMCs was determined by flow cytometry following staining with anti-HA antibody. The data showed that the cell surface expression of AT1R increased in RPASMCs after 48 h of hypoxic challenge (Fig. 3B). These results indicate that hypoxia induces the amplified expression of Rab1 and the cell surface expression AT1R in RPASMCs.

3.4. Effect of Rab1 on the cell surface expression

In our studies, we found that infection with lentiviruses expressing Rab1WT did not significantly change AT1R distribution at the plasma membrane, but we also determined that the cell surface expression of AT1R, as measured by a saturating concentration of Ang II, was significantly augmented in RPASMCs infected with these lentiviruses compared with cells infected with control lentivirus. On the other hand, lentiviral expression of Rab1 siRNA markedly attenuated AT1R expression at the cell surface by 54.2% (Fig. 4A and B). These data indicate that the activation of Rab1 GTPase is required for normal export of AT1R from the ER to the Golgi in RPASMCs. It is noteworthy that the cell surface expression of AT1R in RPASMCs infected with the Rab1WT lentivirus was significantly increased compared with cells infected with the control lentivirus following 48 h of hypoxic challenge (Fig. 4C), indicating that Rab1 can modify the cell surface expression of endogenous AT1R in RPASMCs.

3.5. Effect of Rab1 on the subcellular localization of AT1R

To further determine the functional role of Rab1 in regulating AT1R expression at the cell surface, we sought to determine the effect of Rab1 expression on the subcellular distribution of AT1R. For these studies, GFP-AT1R was transiently transfected into RPASMCs after infection with the control lentivirus or virus encoding Rab1 siRNA. Fluorescent microscopic analyses determined that about 70% of the RPASMCs were infected. GFP-AT1R was mainly localized at the cell surface in cells infected with control lentivirus. In contrast, GFP-AT1R accumulated in the perinuclear regions, co-localized with the ER marker, pDsRed2, and could not be transported to the cell surface in cells infected virus encoding Rab1 siRNA (Fig. 5). These data strongly indicate that normal Rab1 is required for the transport of AT1R to the cell surface.

3.6. Effect of Rab1 on the phenotypic modulation of RPASMCs during hypoxia

To determine if Rab1 affects modulation of the RPASMC phenotype, we detected examined the expression of VSMC phenotype marker proteins over a hypoxic period of 48 h by Western blotting. The expressions of the SMC phenotype marker proteins, α-SMA and VIM, were significantly decreased ($P < 0.05$). To further understand the role of Rab1 in modulating cell phenotype after hypoxia, we infected RPASMCs with Rab1 siRNA, and the

cells were then exposed to hypoxia for 48 h, followed by the determination of the expression of VSMCs phenotype marker proteins. The data showed that VSMC marker proteins, including α-SMA and VIM, were down-regulated after hypoxic exposure. However, the expression of the SMC marker proteins in RPASMCs infected with lentiviruses encoding Rab1 siRNA was not downregulated so much ($P < 0.05$) (Fig. 6), indicating that Rab1 siRNA can reverse the downregulation of VSMC marker protein expression in RPASMCs following hypoxia.

3.7. Effect of Rab1 on RPASMC proliferation

To examine whether Rab1 plays a regulatory role in hypoxia-induced RPASMC proliferation, quiescent cultured cells were exposed to hypoxia or normoxia in medium containing 0.5% FBS for 24 h, and MTS assay was assessed. As shown in Fig. 7, exposure to hypoxia for 48 h plus Ang II resulted in a significant, 159% increase of the optical density (OD) in comparison with normoxic controls. In contrast, the OD of RPASMCs infected with lentivirus encoding Rab1-siRNA under AngII and ZD7155 conditions, only increased by 27% when compared with that of the normoxic controls, which was a statistically significant difference ($P < 0.05$).

3.8. Effect of Rab1 on the phosphorylation of STAT3 in hypoxic RPASMCs

To address whether Rab1 alters hypoxia-induced STAT3 phosphorylation, we infected RPASMCs with lentiviruses expressing Rab1 siRNA and examined changes in activated STAT3 after subjection to hypoxia for 8 h. The levels of STAT3 phosphorylated at the active site tyrosine residue increased by 5.37-fold in cells being exposed to hypoxia. In contrast, STAT3 phosphorylation in RPASMCs treated with Rab1 siRNA only increased by 1.14 (Fig. 8, $P < 0.05$). Therefore, hypoxia strongly induces STAT3 phosphorylation, and Rab1 regulates this tyrosine phosphorylation of STAT3. These findings suggest that Rab1 enhances the activity of STAT3 in RPASMCs and might regulate the growth of these cells via the STAT3 pathway.

4. Discussion

In our research, we found that Rab1 affects the cell surface expression of endogenous AT1R in RPASMCs. The microscopy analysis revealed that the expression of Rab1 siRNA induced an accumulation of AT1R in the ER, suggesting that AT1R trafficking from the ER to the cell surface is mediated through a Rab1-dependent pathway and that Rab1 is involved in the transport from the ER to the Golgi. This finding is consistent with previous data in cardiocytes (Filipeanu et al., 2004). In contrast, treatment with Rab1 siRNA attenuated the cell surface expression of AT1R in RPASMCs, further demonstrating that Rab1 is a necessary factor for the normal export of AT1R. The overexpression of Rab1 significantly increased endogenous AT1R levels at the cell surface, suggesting that Rab1 may function as a rate-limiting factor for the transport of endogenous AT1R to the cell surface. The influence of Rab1 on AT1R expression at the cell surface is due to its effect on the export of the receptor from the ER to the Golgi compartment.

In this report, we first demonstrated that 48 h of hypoxia irritation increased the expression of Rab1 and increased the numbers of bounding AT1R in RPASMCs. And Rab1 siRNA reduced the export of endogenous AT1R and significantly decreased the total cell surface number of AT1R. However, Rab1 had no significant effect on the overall cell surface expression of AT1R in infected RPASMCs. Probably, AT1R overexpression has saturated the export potential of the cell, such that further increase in Rab1 expression has no effect on AT1R transport. These data indicate that Rab1 is involved in the regulation of AT1R expression at the cell surface. The increased expression of Rab1 may contribute to increased

AT1R levels at the cell surface, which may be a possible mechanism. These data raise the possibility that Rab1 is involved in the pathogenic process of hypoxia-induced changes in RPASMC activation and proliferation.

STAT3, a member of the STAT is implicated in the regulation of several genes involved in cell proliferation, differentiation (Bromberg et al., 1999), and coupled to AT1R (McWhinney et al., 1997). We examined changes in STAT3 activity in the cells exposure to hypoxia for 8 h, level of phosphorylation of STAT3 was significantly increased in the cells, however treatment of RPASMCs with Rab1 siRNA significantly suppressed hypoxia-induced STAT3 activation, suggesting that downregulation of Rab1 may effectively suppress STAT3 activation. Therefore, hypoxia strongly induces STAT3 phosphorylation, and Rab1 regulates phosphorylation of STAT3.

In normoxia, PASMC exhibited relatively low activity, following exposure to hypoxia and AngII, OD of the cells increased by 159%. After the cells infection with Rab1 siRNA under AngII and ZD7155 conditions, it only increased by 27% compared with normoxia. Thus, Rab1 was involved in promoting hypoxia-induced RPASMC proliferation.

Many VSMC-specific gene products have been identified that serve as useful markers of the relative state of differentiation, α-SMA is an excellent VSMC differentiation marker because it is the first known protein to be expressed during differentiation of the SMC in development and is required for the high force development properties of fully differentiated VSMCs (Owens et al., 2004). VIM is a cytoskeletal protein and is expressed in VSMCs, and can be used as a marker for the contractile, differentiated RPASMC phenotype (Zhou et al., 2007; Bochaton-Piallat et al., 1993). In our study, down-regulation of α-SMA and VIM expression was appeared after cells being subjected to hypoxia for 48 h, and Rab1 overexpression in RPVSMCs could strengthen the downregulation, nevertheless Rab1 siRNA attenuated the downregulation.

In conclusion, our findings indicate that Rab1 regulates the ER-to-Golgi transport and function of AT1R. Export from the ER to the cell surface involves the selection of transport pathways for different GPCRs at distinct steps and affects the functions and makeup of cells. It would be very important to better understand the function of abnormal Rab1 activation and endogenous regulatory mechanisms of this protein as a potentially useful means of developing targeted therapeutic strategies for diseases associated with vascular remodeling.

Acknowledgments

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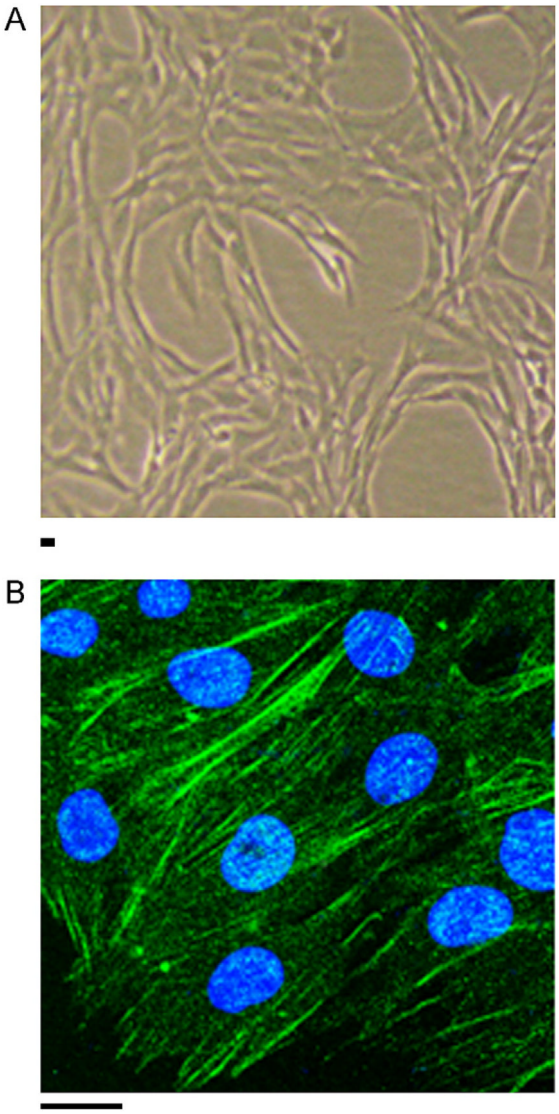

Fig. 1.

Rat pulmonary artery smooth muscle cell (RPASMC) morphology. (A) Under phase-contrast microscopy, normal primary RPASMC morphology appeared as “peak-valley” formations (magnification 40×). (B) RPASMCs were dually stained. Positive expression of α-SMA antigen in RPASMCs was visualized by staining with anti-rat α-SMA binding IgG marked with fluorescein isothiocyanate (FITC) in the cytoplasm by immunofluorescence (green). Nuclei were stained by 4′,6-diamidino-2-phenylindole (DAPI) stain (blue) (magnification 400×). Scale bar, 10 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

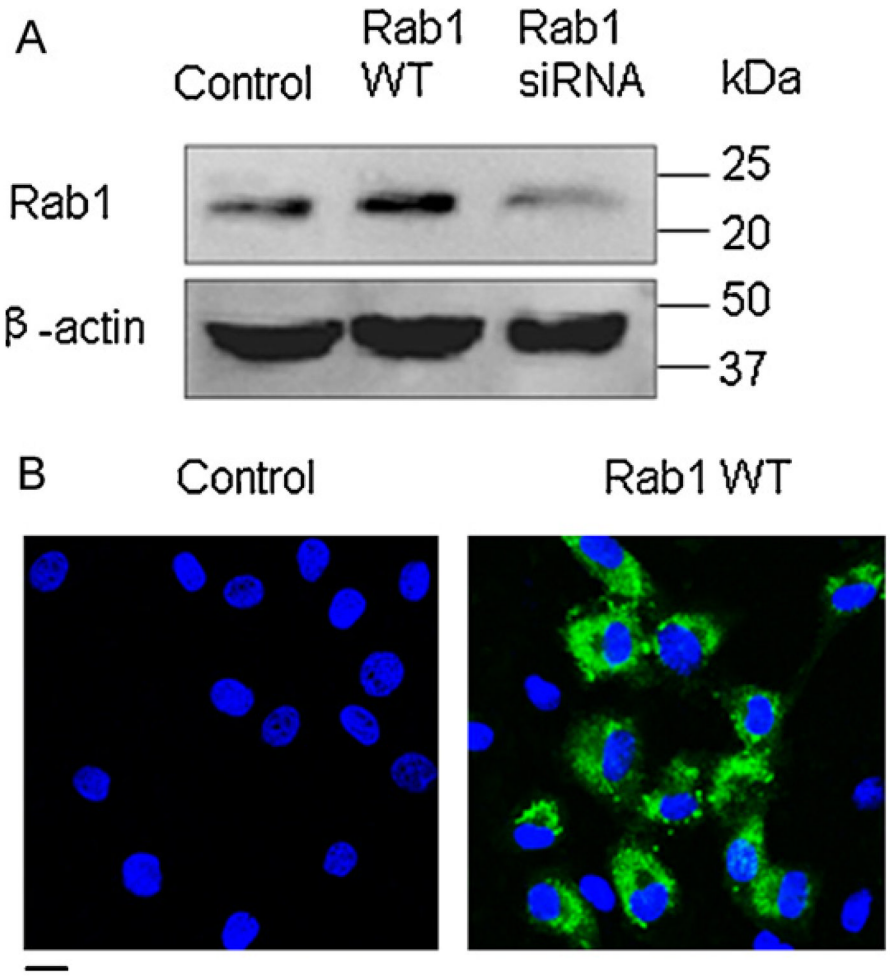

Fig. 2.

Lentivirus-mediated expression of Rab1 in RPASMCs. (A) Western blotting analysis of Rab1 following the expression of Rab1WT or Rab1 siRNA from lentiviruses. RPASMCs were infected with control lentiviral vector(Ctrl) or recombinant Rab1WT lentivirus or with Rab1 siRNA lentivirus. Whole RPASMCs lysate (40 μg) was separated by 12% SDS-PAGE, and Rab1 expression was detected by immunoblotting with anti-Rab1 antibody. The immunoblot shown is representative of results obtained in three different experiments. (B) Localization of Rab1WT and estimation of infection efficiency. RPASMCs were cultured on coverslips and infected with control lentiviral vector or FLAG-Rab1WT lentivirus. Localization of FLAG-Rab1WT in infected RPASMCs was determined by fluorescence microscopy following immunostaining with anti-FLAG antibodies as described in Section 2. Similar results were obtained in at least three separate experiments. Scale bar, 10 μm.

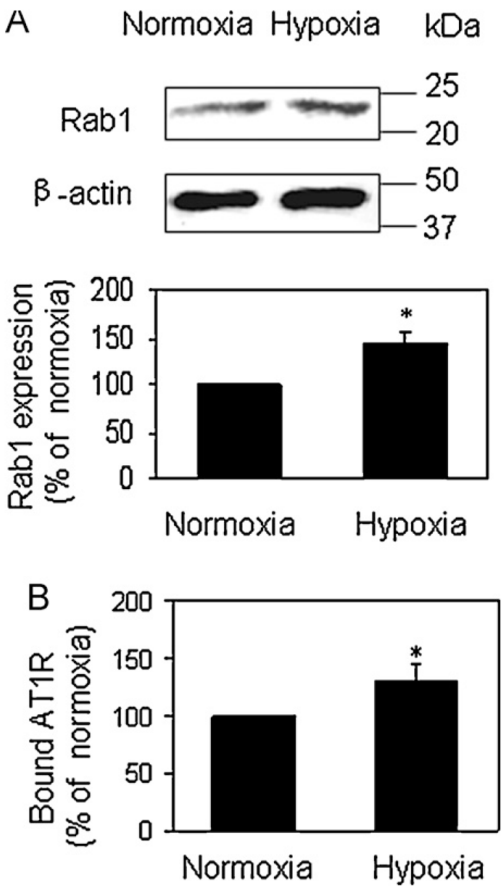

Fig. 3.

Effect of hypoxia on the expression of Rab1 and AT1R in RPASMCs. (A) RPASMCs were exposed to hypoxia for 48 h, and the expression of Rab1 was determined by Western blotting using antibody against Rab1. Representative blots showed Rab1 (upper panel) and β-actin expression (lower panel). Bargraph demonstrated quantitative data of Rab1 levels after hypoxic challenge. (B) RPASMCs were exposed to hypoxia for 48 h, and the cell surface expression of AT1R was determined by intact cell ligand binding as described in Section 2. The mean values of specific [^{3}H] Ang II binding were 556 ± 61 and 725 ± 83 cpm (n = 3 duplicate sets) for the RPASMCs in the normoxic group or those subjected to hypoxia for 48 h, respectively. The data are expressed as the percentage of the mean value obtained in the normoxic group and presented as the means ± S.E. of three separate experiments. * P < 0.05 versus normoxia (time 0).

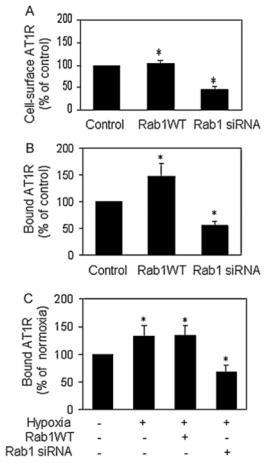

Fig. 4.

Effect of Rab1 on cell-surface expression of AT1R in RPASMCs. (A) The effect of Rab1WT and Rab1 siRNA lentiviruses on the cell surface expression of AT1R. RPASMCs were transfected with HA-AT1R together with control lentiviral, or those encoding Rab1WT or Rab1 siRNA. AT1R expression at the cell surface was quantitated by flow cytometry following incubation with anti-HA antibodies as described in "Materials and methods." The mean values of fluorescence obtained from the untransfected RPASMCs and from RPASMCs transfected with HA-AT1R and control lentivirus, Rab1WT, or Rab1 siRNA lentiviruses were 76 ± 7, 553 ± 35, 569 ± 38, and 259 ± 30, respectively. (B) Quantitation of the cell surface number of bound AT1R molecules in RPASMCs. RPASMCs were infected with control lentivirus, or with Rab1WT or Rab1 siRNA lentiviruses. The cell surface expression of AT1R was determined by binding to ligands. The mean values of specific [^{3}H] Ang II binding of AT1R were 550 ± 69, 804 ± 130, and 301 ± 46 cpm (n = 3 for duplicate experiments) from the cells infected with control lentivirus, Rab1WT lentivirus, or Rab1 siRNA lentivirus, respectively. (C) Modulation of the cell surface expression of AT1R by infection with Rab1WT or Rab1 siRNA lentivirus particles in RPASMCs exposed to hypoxia for 48 h. RPASMCs were infected with control lentivirus, Rab1WT lentivirus, or Rab1 siRNA lentivirus and were then exposed to hypoxia for 48 h. The cell surface expression of bound AT1R was determined by intact cell ligand binding. The mean values of specific [^{3}H] Ang II binding of AT1R were 728 ± 109, 738 ± 98 and 371 ± 68 cpm from the cells infected with control lentivirus, Rab1WT lentivirus, and Rab1 siRNA lentivirus (all cells were subjected to hypoxia) versus 547 ± 59 cpm for the cells infected with control lentivirus in normoxia. All the data shown are the percentage of the mean value obtained from the RPASMCs infected with control lentivirus and are presented as the means ± S.E. (n = 3 for duplicate experiments). *P < 0.05 versus RPASMCs infected with control lentivirus.

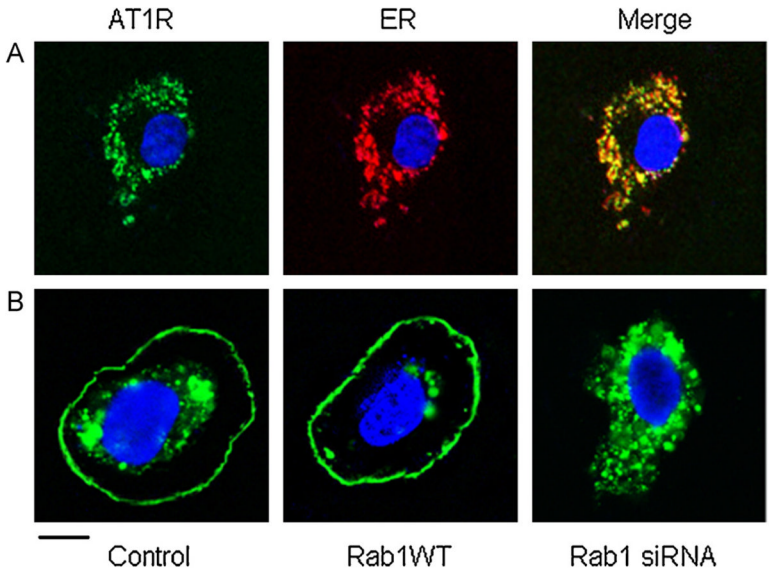

Fig. 5.

Effect of Rab1 on the subcellular localization of AT1R in RPASMCs. (A) Co-localization of receptors with the ER. RPASMCs were grown on coverslips and infected with Rab1 lentivirus. The cells were then transfected with GFP-tagged AT1R together with the ER marker, pDsRed2-ER. Blue, nuclei staining with DAPI; green, GFP-tagged receptor; red, ER marker, pDsRed2-ER; yellow, co-localization of GFP-tagged AT1R with pDsRed2-ER. (B) The effect of lentiviral expression of Rab1 on the subcellular distribution of AT1R. RPASMCs were infected as above. The subcellular distribution of the receptor was revealed by fluorescence microscopy. Scale bar, 10 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

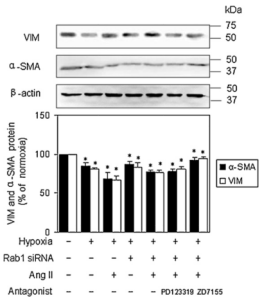

Fig. 6.

Effect of Rab1 on the expression of smooth muscle cell phenotypic markers of RPASMCs during hypoxia. RPASMCs were infected with Rab1 siRNA lentivirus, and then exposed to hypoxia for 48 h. Select cultures were then subjected to AngII (100 nM), ZD 7155 (100 nM) and PD 123319(1 μM). Whole RPASMCs lysate (40 μg) was separated by 12% SDS-PAGE, and α-SMA, vimentin, and β-actin expression was detected by immunoblotting. Bargragh meant quantitative data for changes in α-SMA and vimentin expression relative to β-actin. It showed that α-SMA and vimentin expression was downregulated in RPASMCs exposure to hypoxia for 48 h. These data are expressed as the percentage of the mean value obtained from the normoxia and presented as the means ± S.E. of three separate experiments. **P* < 0.05 versus the normoxic control (time 0).

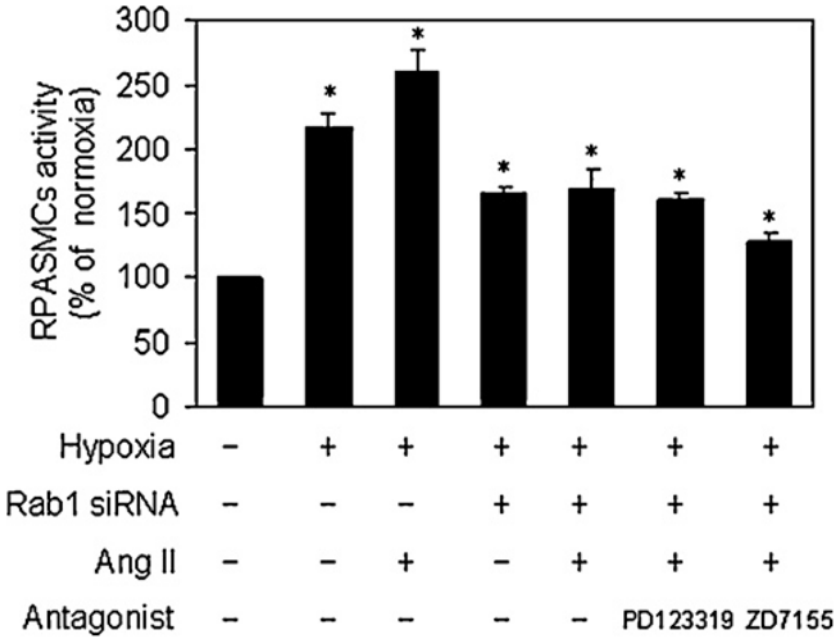

Fig. 7.

Effect of Rab1 on cells activity in RPASMCs exposed to hypoxia. RPASMCs were infected with Rab1 siRNA lentiviruses, the cells were then exposed to hypoxia in select culture medium as above for 48 h. OD was determined as described in Section 2. The mean values of OD were 0.538 ± 0.023, 1.170 ± 0.058, 1.396 ± 0.096, 0.891 ± 0.025, 0.910 ± 0.081, 0.862 ± 0.032, 0.684 ± 0.037 respectively. The data were shown at percentage of the mean value obtained from the normoxia as the mean ± S.E. in three separate experiments. *P < 0.05 versus the normoxic control (time 0).

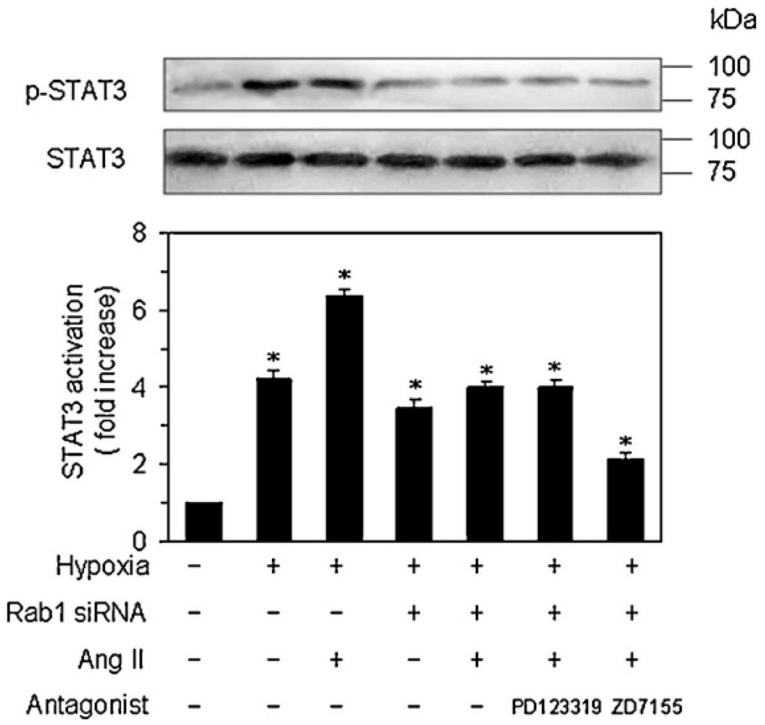

Fig. 8.

Effect of Rab1 on hypoxia-induced STAT3 phosphorylation. The cells were treated as above for 8 h. STAT3 was measured by Western blotting followed by densitometric analysis. Upper panel, representative bands showing phosphorylated STAT3, total STAT3 expression was shown in middle panel. Quantitative data of STAT3 phosphorylation was shown in lower panel. All data were shown as the mean ± S.E. of three separate experiments. *$P <$ 0.05 versus the normoxic control.