

The P2Y₁₂ receptor antagonist Ticagrelor ameliorates pulmonary hypertension

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Abstract

Purpose

Pulmonary arterial hypertension (PAH) is a disease that the pulmonary artery is abnormally elevated. P2Y₁₂ is an adenosine diphosphate (ADP) receptor and it act as the target of thienopyridine antiplatelet drugs by controlling vascular remodeling. Inhibition of P2Y₁₂ receptor in the process of PAH was explored in this study.

Methods

The PAH model was established in Sprague-Dawley rats by single subcutaneous injection of 60 mg/kg monocrotaline (MCT). The ticagrelor solution (a selective P2Y₁₂R inhibitor) was intraperitoneally injected into rats at a dose of 14 mg/kg from the time of MCT injection to day 28.

Results

In the lung tissues of PAH rats, the marked P2Y₁₂R was detected. Treatment with ticagrelor greatly decreased P2Y₁₂R level and efficiently abolished the upregulation of α -SMA as demonstrated by Western blot and RT-PCR. The wall thickness and occlusion score of the pulmonary arterioles showed that blockade of P2Y₁₂R could relieve lung remodeling caused by PAH. The haemodynamic changes at 4 weeks determined that P2Y₁₂R inhibition affected RV pressure and right heart hypertrophy.

Conclusions

P2Y₁₂R might be involved in the pathogenesis of PAH. Blockade of P2Y₁₂R has potential in treating PAH.

Introduction

Pulmonary arterial hypertension (PAH) is a disease that the pulmonary artery is abnormally elevated and ultimately leads to pulmonary vascular remodeling. The proliferation of pulmonary arterial smooth muscle cell (PASMC) and the dysfunction of pulmonary arterial endothelial cell are determining factors involved in PAH pathogenesis. And it has been confirmed that, the two processes have significant roles in pulmonary vascular resistance, right heart failure, and death [1–3]. Besides that, various pathologic conditions have been revealed to be risk factors of PAH, such as hypoxia, oxidative, and infections.

P2Y₁₂ receptor is one of the members of P2 receptor family. The P2Y₁₂ receptor consist of ion-channel P2X and G-protein–coupled P2Y receptors. P2Y₁₂ receptor was originally found to be expressed in platelet. Recent studies demonstrated that, P2Y₁₂ receptor also expressed in vascular smooth muscle

cells (VSMCs)[4]. In platelets, endothelial cells, or immune cells, the adenosine triphosphate (ATP) and adenosine diphosphate (ADP) generated from cell are able to active P2 receptors, including P2Y12 [5]. The elevated P2Y12 suppresses adenylyl cyclase level and then participates in regulating the activation of platelet and thrombosis. Thus, the P2Y12 receptor has been clinically used as a target for thromboembolism treatment.

Antiplatelet drugs are widely used clinically, especially for cardiovascular events with thrombotic involvement. But recent clinical studies suggest that antiplatelet drugs may also be useful as agents for primary cardiovascular prevention [2, 6]. VSMCs are one of the main cell types involved in most stages of PAH. Inhibition the migration and proliferation of VSMCs are critical in the treatment of PAH. Ticagrelor is a relatively novel antiplatelet agent that has been shown to reversibly inhibit P2Y12 receptors on platelets and smooth muscle cells (SMCs). Here, the role of ticagrelor on the pathogenesis of PAH was tested for the first time, as well as the therapeutic role of ticagrelor on the treatment of PAH.

Materials And Methods

PAH model

SPF grade of Sprague-Dawley rats (all male, weighed 280-330g) were purchased from the Laboratory Animal Center, Chinese Academy of Science (Beijing, China). The rats were housed in a standard animal room at $21\pm 1^{\circ}\text{C}$ temperature and $55\pm 5\%$ humidity. The animal room were under a 12-h light/dark cycle and the rats were free access to water and food. After feeding in the animal room for 7 days, he experiments were began. The animal studies performed were all approved by the Shandong University Institutional Animal Care and Use Committee and were conducted according to the standard protocols and guidelines. The rats were randomly divided into 4 groups: Sham group in which rats received water alone (n = 15); Sham + T group in which the rats were intraperitoneally injected with 14 mg/kg ticagrelor solution (AstraZeneca) every day (n = 15); PAH group in which PAH was induced by left pneumonectomy plus MCT injection [7] (n = 30); and PH + T group in which PAH rats were injected with ticagrelor solution (n = 20). Ticagrelor solution (a selective P2Y12R inhibitor) was made using a 360 mg tablet diluted with 25.5 ml saline water and injected from the time MCT injection to day 28 [8].

The animals were anaesthetized using 2% xylazine (4 mg/kg)/ketamine (100 mg/kg). The rats received an adjusted rate of 60 breaths/min. Respiratory support was given to the rats using a small animal ventilator (HX-300S; Chengdu TME Technology Co., Ltd.) at a tidal volume of 1.1-1.3 ml/100 g, followed by a left unilateral pneumonectomy [9]. One week following surgery, the rats were subcutaneously injected with 60 mg/kg MCT. All rats were under monitored every day until the PAH symptoms were developed, such as body weight loss and tachypnea.

Echocardiography and haemodynamic measurements

Cardiac function was evaluated using a 14 MHz linear transducer equipped with an echocardiographic machine (Visual Sonics, Toronto, Canada). According to Simpson's method, cardiac output (CO) and B-

mode long axis was used to detect stroke volume, and pulmonary artery diameter and M-mode were used to measure RV wall thickness. The acceleration time of the pulmonary artery was obtained by applying ultrasonic Doppler to the pulmonary artery [10]. According to the tail-cuff method, a blood pressure recorder (BP-98A; Softron, Tokyo, Japan), was used to measure the blood pressure of the rats [11]. Pulmonary artery pressure transduction was conducted with correct jugular vein by a 1.4F Millar Mikro-Tip catheter transducer (Millar Instruments Inc., Houston, TX) directed to the main pulmonary artery after insertion into the right ventricular outflow duct, although RV systolic pressure (RVSP) was detected with a power laboratory monitoring device (Miller Instruments). Hemodynamic values were accurately computed by LabChart 7.0 physiological data acquisition system (AD Instruments, Sydney, Australia). The rats were anaesthetised during this process.

Tissue processing and histology

Following the test of echocardiography and haemodynamic measurements, the animals were sacrificed by inducing cardiac arrest by injection of 2 mmol KCl through the catheter. The lungs were isolated. The left one was weighed and the right one was inflated with 0.5% low melting agarose at a constant pressure of 25 cm H₂O, and fixed in 10% formalin for 24 h. Subsequently, the heart was excised.

Western blot

The lysis buffer used for the extraction of proteins from tissues was a mixture of RIPA (Beyotime Institute of Biotechnology) and PMSF at a ratio of 100:1[11]. The extracted proteins were detected using a BCA protein assay reagent kit (Pierce). The proteins were then subjected to a 5–12% SDS-PAGE gel and transferred onto polyvinylidene difluoride (PVDF) membrane. After blocking in the TBST for 1 h at 4°C, the target proteins were probed by incubation with following antibodies: 1:2000 for P2Y₁₂R (Abcam, USA) and 1:1500 for α -SMA (Abcam, USA). Primary antibodies were detected using horseradish peroxidase-conjugated antibodies: 1:5000 for anti-mouse (ZSJO-BIO, Beijing, China) and 1:5000 for anti-rabbit (ZSJO-BIO, Beijing, China), at room temperature for 2 h. The enhanced chemiluminescence (ECL) detection kit (Millipore) was used for blot development. The blots were visualized by the FluroChem E Imager (Protein-Simple, Santa Clara, CA, USA) and semi-quantified using ImageJ software (National Institutes of Health).

qRT-PCR

The RNAs in lung tissues were extracted using RNA-easy™ Isolation Reagent (cat. no. R701; Vazyme Biotech Co., Ltd.). The RNA was dissolved in DEPC-treated water and the concentration of the extracted RNA was determined using a superdifferential spectrophotometer (Thermo Fisher Scientific, Inc.). Subsequently, Mir-X™ miRNA First Strand Synthesis and TB Green™ (Cat. No. 638313; Takara Bio, Inc.) was used for reverse transcription and HiScript III RT SuperMix (cat. no. 323-01; Vazyme Biotech Co., Ltd.). The PCR procedure was conducted using an ABI 7500 fast Real Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) using ChamQ Universal SYBR qPCR Master Mix (cat. no. Q711-02; Vazyme Biotech Co., Ltd.). The thermocycling conditions were shown as following: 1 cycle of

pre-denaturation at 95°C for 30 sec, followed by 40 cycles of denaturation at 95°C for 10 sec, annealing and extension at 60°C for 30 sec, and step 3, 95°C for 15 sec, 60°C for 1 min and 95°C for 15 sec is an additional cycle for melting curve. Target gene expression was calculated using the $2^{-\Delta\Delta CT}$ method [12] by normalizing to GAPDH. The primers used in this study were as following. GAPDH: forward, 5'-AGATCCACAACGGATACATT-3', reverse, 5'-TCCCTCAAGATTGTCAGCAA-3'; α -SMA: forward, 5'-CCGACCGAATGCAGAAGGA-3', reverse 5'-ACAGAGTATTTGCGCTCCGGA-3'; P2Y12R: forward 5'-CTTCGTTCCCTTCCACTTTG-3', reverse 5'-AGGGTGCTCTCCTTCACGTA-3'.

Immunohistochemistry

The right lung tissues were formalin-fixed, paraffin-embedded and used for HE or regular immunohistochemistry staining [2]. The OCT-embedded tissue was placed into a freezing microtome (CM3050; Leica Microsystems GbmH) and tissue samples were cut into 5 μ m sections[9]. In each lung section, 30 small PAs (50–100 μ m in diameter) were analyzed at \times 40 magnification in a blinded manner. The medial wall thickness was expressed as the summation of two points of medial thickness/ external diameter \times 100 (%). Intraacinar (precapillary) PAs (20–30 μ m in diameter, 25 vessels each) were assessed for occlusive lesions, defined as Grade 0 when there was no evidence of neointimal lesion, Grade 1 when there was less than 50% luminal occlusion, and Grade 2 when there was more than 50% luminal occlusion [13]. There was no evidence of neointimal lesion formation in any PAs from normal rats (all PAs were graded as 0). Anti- α -SMA (1:200; Abcam) antibodies were used as primary antibodies. After fixing the frozen sections with cold acetone at 25°C for 5 min and blocking with QuickBlock™ Blocking Buffer for Immunol Staining (cat. no. P0260; Beyotime Institute of Biotechnology) for 10 min at 4°C, they were treated overnight at 4°C with anti-P2Y12R antibody (1:200; Novus) and α -SMA (1:200; Abcam). Following incubation with primary antibody, Alexa 546-conjugated donkey anti-rabbit (1:200; Invitrogen) and FITC-conjugated rabbit anti-mouse (1:200; Abcam) secondary antibodies were added, respectively, and the sections were incubated for 2 h at room temperature. The sections were counterstained with DAPI (Life Technologies) to identify nuclei. The sections were then washed and placed under a fluorescence microscope for observation and image capture. Nerve density was measured and evaluated using ImageJ software.

Statistics

Data are expressed as the mean \pm SEM. The significant difference between two groups were analyzed by unpaired t-test. For three or more groups, analysis of variance (ANOVA) followed by a Newman-Keuls test was utilized. Statistical analyses were performed using SPSS 20.0 software (SPSS Inc. Chicago, IL, USA), and p-value < 0.05 was considered statistically significant.

Results

1. PAH rats show significant high P2Y12R level in lungs

Co-staining of P2Y₁₂R with α -SMA shown that P2Y₁₂R was largely distributed in PASMCs from the hypertrophied media of pulmonary vessels in PAH lung tissue, indicating P2Y₁₂R as a central risk factor of PAH. To further investigate the role of P2Y₁₂R in PAH, a specific P2Y₁₂R inhibitor, ticagrelor, was applied.

2. Effects of ticagrelor on P2Y₁₂R and α -SMA expression in lung tissues

The effects of ticagrelor on P2Y₁₂R expression were assessed. The expression level of P2Y₁₂R (Fig. 2B, D, F) was upregulated in PAH rats. Treatment with ticagrelor greatly decreased P2Y₁₂R level and efficiently abolished the upregulation of α -SMA as demonstrated by Western blot and RT-PCR (Fig. 2A, C, E). Results showed that there was little difference between the two sham groups, which confirmed that interference by ticagrelor to PAH may be related to inhibition of P2Y₁₂R to expression of α -SMA.

3. P2Y₁₂R inhibition inhibits pulmonary vascular remodeling

PAH leads to pulmonary vascular remodeling [14], thus we further studied the effects of ticagrelor on remodeling. By measuring the wall thickness and occlusion score of the pulmonary arterioles, we found that wall thickness was remarkably increased from $60.8\% \pm 4.7\%$ – $81.2\% \pm 4.4\%$ ($p < 0.05$) in vessels with diameters ranging from 50 to 100 μm (Fig. 3). Treatment with ticagrelor suppressed the wall thickness to $67.6\% \pm 3.5\%$ ($p < 0.05$; Fig. 3C). Decreases in Grade I and II occlusion were also demonstrated (15 and 73% in PAH vehicle group vs. 25 and 29% in the ticagrelor administrated PAH group respectively; Fig. 3D). Therefore, blockade of P2Y₁₂R could relieve lung remodeling caused by PAH.

4. P2Y₁₂R inhibition ameliorates pulmonary hypertension

As shown in Fig. 4, RVSP was significantly inhibited by ticagrelor treatment in rats (39.3 ± 4.5 mm Hg, vs. 53.9 ± 4.8 mm Hg the P/MCT group, $p < 0.05$). Also, ticagrelor treatment prior to or after MCT administration significantly reduced the thickness of RV wall, RV area, and pulmonary artery diameter. It was also observed that, ticagrelor treatment increased the mean acceleration time of the pulmonary artery as compared with PAH group.

Discussion

Since platelet P2Y₁₂ ADP receptor has been considered as one important target of thienopyridine-type antiplatelet drugs, herein we investigated the impacts of ticagrelor (a selective P2Y₁₂R inhibitor) in the pathogenesis of PAH. We for the first time described a functionally active P2Y₁₂ in SMC proliferation post pulmonary hypertension. Firstly, it was demonstrated that P2Y₁₂R expression was updated in SMC

in PH rats. Secondly, this upregulation positively enhanced vascular proliferation. Therefore, the application of antiplatelet drugs could be important for the treatment of PH.

VSMCs are the major cell type in vessel walls and they play central roles in the most stages of pulmonary hypertension. Initially, P2Y12 receptors were found to be expressed in platelets and microglia in the brain sub region. Recently, studies shown that, it was also expressed in a variety of cells, such as VSMCs [15]. This is consistent with the results presented here which show significant P2Y12 upregulation on VSMC in pulmonary hypertension rats. In the current study, MCT-challenged left pneumonectomised rats showed a marked increase in the P2Y12R expression into peri-vascular and peri-alveolar areas of pulmonary tissues and bronchoalveolar lavage samples. It seems that inhibition of P2Y12 may have additional therapeutic benefits on pulmonary hypertension beyond anti-thrombotic effect, like anti-PAH.

Under the stimulations such as hypoxia or shear stress, and mediate vasodilatatory, inflammatory, and thrombotic responses, extracellular nucleotides including the purines ATP, ADP, and adenosine monophosphate (AMP) as well as pyrimidines uridine-5'-triphosphate (UTP) and uridine-diphosphate (UDP) are released within the pulmonary vascular bed and then involved in the pathogenesis of PH[16]. It has been reported that ADP induces VSMC contraction *via* P2Y12, and promotes proliferation. ADP elicits pulmonary vasoconstriction through P2Y1 and P2Y12 receptor activation [17]. It was shown here that P2Y12R was upregulated and co-stained with α -SMA in PAH rats. Furthermore, the P2Y12R level was positively related with α -SMA expression. The P2Y12 inhibitor ticagrelor reversed pulmonary hypertension, as well as α -SMA downregulation, indicating that activation of P2Y12 is required for proliferation of PSMCs.

The mechanism underlying P2Y12R mediated pulmonary remodeling may include cAMP/PKA signaling, which has been shown to be the key link in PSMCs proliferation [18], and is the downstream pathway under the stimulation of ADP [19]. Besides, the P2 receptor mediated Ca^{2+} signalosome of the human pulmonary endothelium may be implicated in pulmonary arterial hypertension [20]. The exact mechanism requires for further investigation.

The vessel wall P2Y12 receptor promotes vascular remodeling in the PAH pathological process. Therefore, antiplatelet agents such as ticagrelor may be used as a therapeutic target for pulmonary hypertension.

It remains to determine whether P2Y12 receptor has potentials in regulating other cell types of PAH pathogenesis, like pulmonary arterial endothelial cell. Besides, a mass of clinical trials are required before the use of ticagrelor as an anti-PAH agent.

Declarations

Acknowledgments

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Statements & Declarations

All procedures were conducted according to approved protocols and guidelines established by the Shandong University Institutional Animal Care and Use Committee. The datasets supporting the conclusions of this article are included within the article.

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Author Contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Xiaolu Li, Shuling You, Yugen Shi, Li Sun, Qingshan Zhang, Shuai Bao, Juan Zhang, Youlei Li, Miaomiao Wang, Yanwei Zhang, Weidong Cai, Hongxing Zhang, Yan Li and Suhua Yan. The first draft of the manuscript was written by Nannan Li and Jie Yin. And all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Ethics approval

All procedures were conducted according to approved protocols and guidelines established by the Shandong University Institutional Animal Care and Use Committee. (SYDWLS[2022]006)

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Table

Table 1 hemodynamics and echocardiography data at 28 day after MCT injection

Parameters	sham	Sham+T	MCT	MCT+T
No. of surviving rats	15	16	18	19
Body weight, g	436 \pm 6	426 \pm 10*	422 \pm 8*	418 \pm 11*
Heart Rate,bpm	431 \pm 7	440 \pm 10	448 \pm 9*	443 \pm 16
Pulmonary arterial acceleration time, ms	33.5 \pm 1.2	32.9 \pm 0.9	36.7 \pm 1.5*	34.4 \pm 1.7 [†]
LV area, mm ²	23.4 \pm 0.7	23.6 \pm 0.5	23.9 \pm 0.9	24.5 \pm 0.5
RV area, mm ²	13.5 \pm 0.5	13.6 \pm 0.4	14.9 \pm 1*	13.4 \pm 0.8 [†]

All values are mean \pm SD

Abbreviations: RV right ventricle, LV left ventricle

* p < 0.05 compared with sham group

[†] p < 0.05 compared with MCT group

Figures

Figure 1

Double-immunostained images for P2Y12R co-stained with α -SMA (A) sham group (B) sham+ T group, (C) PAH group, and (D) PAH+ T group. (Green, red and blue were α -SMA, P2Y12R and DAPI, respectively.) T= ticagrelor.

Figure 2

Activation of the P2Y12R and α -SMA in PH rats. α -SMA (42 KDa) and P2Y12R (41 KDa) protein expression was normalized against GAPDH (36 KDa), as measured by Western blot (A, B). Quantification of protein level is shown (C, D). Relative α -SMA and P2Y12R mRNA expression analyzed by qRT-PCR (E, F) in the sham, sham + T, PAH, and PAH+ T groups. * $p < 0.05$ and ** $p < 0.01$ mean that results had significant difference countered to sham and PAH group, respectively.

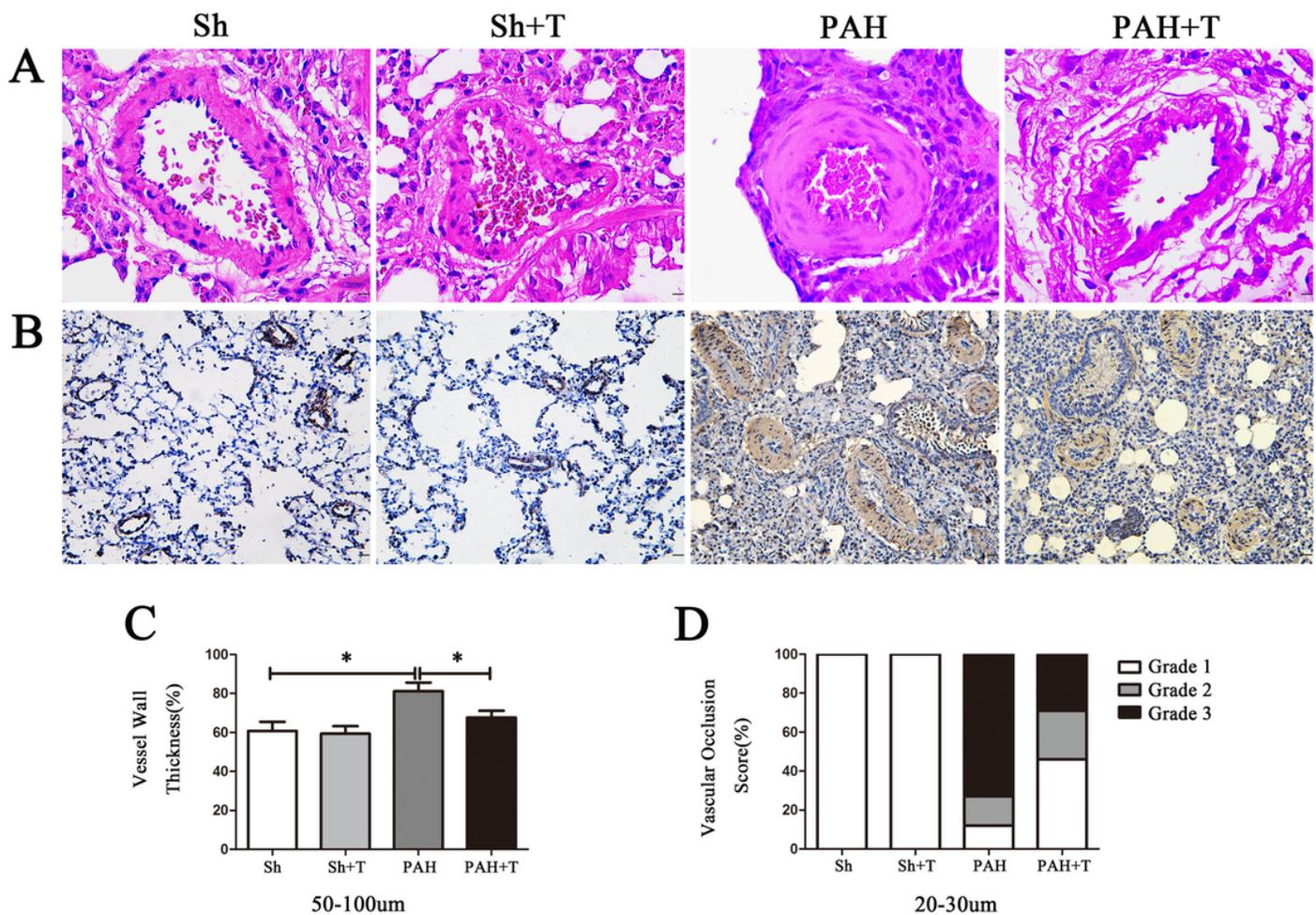


Figure 3

Effect of ticagrelor on pulmonary vascular remodeling. (A) Haematoxylin-eosin, and (B) IHC staining of α -actin with an α -SMA antibody in the sham, sham + T, PAH, and PAH+ T groups, respectively. (C) Ratios of rats' small pulmonary arteries thickness (diameter 50-100 μ m). (D) Vascular occlusion score of the rats' small pulmonary arteries (diameter 20-30 μ m). * p < 0.05 means that results had significant difference countered to sham and PAH group.

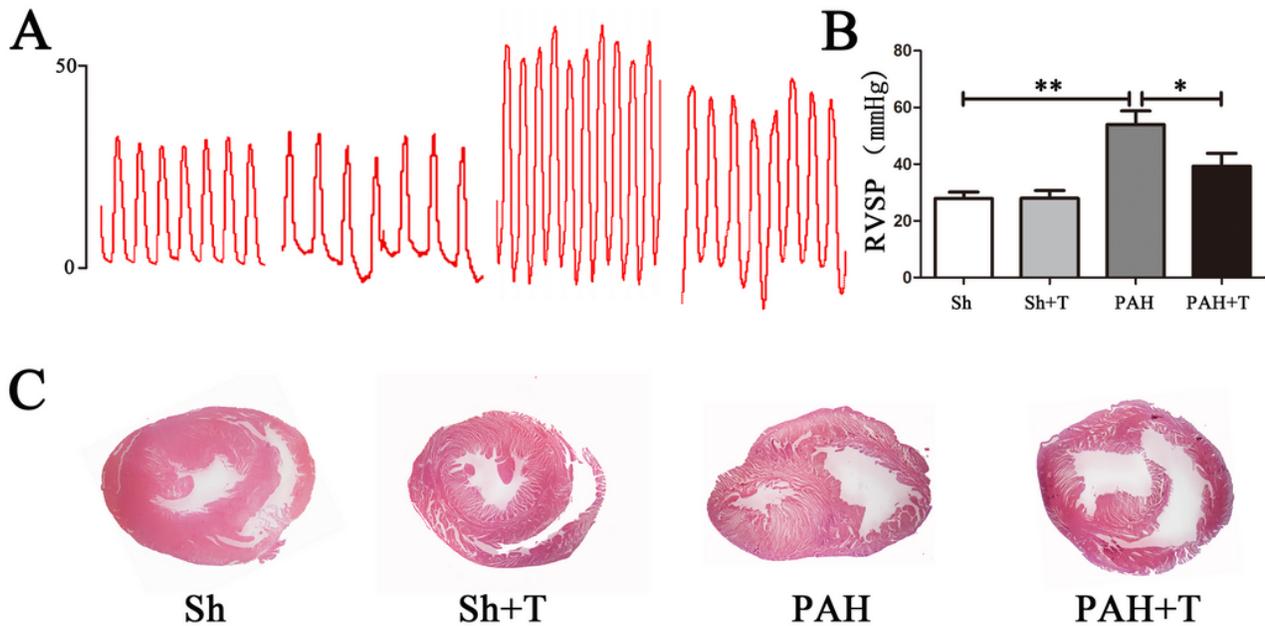


Figure 4

Ticagrelor administration prevented the pulmonary hypertension and improves RV function of PAH rats. (A) and (B) RVSP changes of PAH rats which were treated with ticagrelor. (C) The RV/LV+ S ratio of PAH rats. The representative visual shape of the RV is shown (D). * p <0.05 and * p < 0.05 mean that results had significant difference countered to sham and PAH group, respectively. RVSP= right ventricle systolic pressure.