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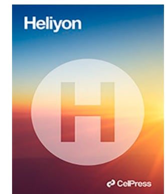

Pressure stress delays cyclooxygenase-2 expression induced by interleukin-1β in cultured human pulmonary artery smooth muscle cells

Sachiko Hiraide, Takuji Machida *, Shota Takihana, Mikoto Ohshita, Kenji Iizuka

Department of Pharmacological Sciences, School of Pharmaceutical Sciences, Health Sciences University of Hokkaido, Ishikari-Tobetsu, Hokkaido 061-0293, Japan

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A B S T R A C T

Introduction: Pulmonary artery smooth muscle cells (PASMCs) play an important role in the sequence of events leading to the formation of pulmonary hypertension (PH). However, little is known about the direct effects of high pressure on the function and intercellular signaling pathways of PASMCs. The aim of this study was to evaluate the effect of pressure stress that simulates PH on interleukin (IL)-1β- or angiotensin II-induced cyclooxygenase-2 (COX-2) expression in cultured human PASMCs.

Methods: Either 20 or 60 mmHg atmospheric pressure was applied to PASMCs by a pressure-loading apparatus. Protein expression and phosphorylation were analyzed by western blotting. mRNA expression was analyzed by quantitative real-time reverse transcription-polymerase chain reaction.

Results: IL-1β-induced COX-2 protein expression peaked at 6 h in non-pressurized cells, whereas COX-2 expression was delayed, peaking at 12 h, in 20 and 60 mmHg pressurized cells. Both pressures also delayed the time to peak *COX*-2 mRNA expression induced by IL-1β. In addition, pressure stress delayed the time to peak mitogen-activated protein kinase (MAPK) phosphorylation induced by IL-1β. In contrast, angiotensin II-induced transient *COX*-2 mRNA expression and MAPK phosphorylation were not affected by pressure stress.

Conclusion: These results suggest that pressure stress delays IL-1β-induced COX-2 expression via the delayed activation of MAPKs in PASMCs, and the effects of pressure stress differ according to the bioactive substance being stimulated. Our results demonstrate that the application of pressure stress to PASMCs directly alters cell function, which may provide a basic insight into our understanding of the pathogenesis of PH.

1. Introduction

Pulmonary hypertension (PH) is defined as mean pulmonary artery pressure ≥25 mmHg when measured at rest by right heart catheterization. Pathologically, PH is characterized by narrowing of the pulmonary artery lumen due to various factors [1], and continuously high pulmonary artery pressure results in right heart failure and even death. In particular, the overgrowth and thickening of pulmonary artery smooth muscle cells (PASMCs), caused by hypoxia, cytokines, and vasoconstrictors, play a crucial role in the

* Corresponding author.
E-mail address: tmachida@hoku-iryo-u.ac.jp (T. Machida).

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sequence of events leading to the formation and progression of PH [2–7]. Although elevated pulmonary arterial pressure occurs as a result of pulmonary vascular remodeling, the direct impact of elevated pulmonary arterial pressure on PASMCs remains unclear.

Cyclooxygenase (COX) is a key enzyme catalyzing the rate-limiting step in the synthesis of prostaglandins (PGs) and thromboxane. Two cyclooxygenase isozymes, COX-1 and -2, have been identified. COX-1 is constitutively expressed in various cells and has generally been shown to be unaltered in pathophysiological conditions. In contrast, COX-2 expression is induced by inflammatory cytokines such as interleukin (IL)-1β as well as some bioactive peptides such as angiotensin II and is responsible for the increased PG and/or thromboxane production seen in pathological conditions. In previous studies, the serum concentrations of inflammatory cytokines, including IL-1β, were shown to be higher in patients with pulmonary artery hypertension than in healthy controls [8,9] and these high concentrations are thought to play a supporting role in the development of PH. Elevated serum renin and angiotensin II levels have also been reported in patients with chronic thromboembolic pulmonary hypertension [10]. In PASMCs, PGI_2 is thought to be major product of COX-2, and it is likely to have a vasoprotective effect. In fact, COX-2-deficient mice undergo pulmonary vascular remodeling and PASMC hypertrophy under chronic hypoxia [11]. The Sugen 5416/chronic hypoxia rat model develops severe right ventricular systolic hypertension and has increased COX-2 expression in lung tissue [12]. Human PASMCs cultured at stiffness mimicking PH show increased proliferation, decreased apoptosis and *COX*-2 mRNA expression, and slightly reduced levels of COX-2-derived PGE_2 and the PGI_2 metabolite 6-keto $PGF_{1\alpha}$ compared with cells grown on substrates approximating normal PA stiffness [13]. Thus, alterations in COX-2 expression play an important role in the hyperplasia of PASMCs in PH pathological conditions. Furthermore, a previous study demonstrated decreased PGI_2 metabolite levels and increased thromboxane A_2 metabolite levels in the urine of patients with idiopathic and secondary forms of PH [14]. PGI_2 has potent vasodilatory, antiplatelet, antiproliferative, and dedifferentiating effects, thereby reducing pulmonary blood pressure as well as bronchial hyperresponsiveness within the lungs [15]. Regarding the treatment of PH, the advent of PGI_2 derivatives has improved outcomes [1], suggesting that PGI_2 has an important protective role against the development of PH pathophysiology.

We previously developed a pressure-loading apparatus that can apply arbitrary pressure to cultured cells [16–18]. Using this apparatus, we found that applying pulsatile pressure stress between 80 and 160 mmHg at a rate of 4 cycles/min in order to simulate systolic hypertension significantly suppresses IL-1β-induced COX-2 expression in rat aortic SMCs [19]. The inhibition of mitogen-activated protein kinase (MAPK) phosphorylation was involved in the mechanism for the pressure-induced suppression of COX-2 expression [19]. Therefore, it is possible that pressure stress simulating PH affects COX-2 expression in PASMCs. The aim of the present study was to clarify the effect of pure pressure stress simulating PH on IL-1β- and angiotensin II-induced COX-2 expression and its mechanism in cultured human PASMCs.

2. Materials and methods

2.1. Cell culture

Human PASMCs were purchased from Lonza (Cat. No. CC-2581; Basel, Switzerland) and maintained in SmGM™-2 containing 5 % fetal bovine serum, supplements, and growth factors (Lonza) and were used between passages 6 to 9. At 24 h before an experiment, the medium was replaced with serum-free medium containing 0.1 % bovine serum albumin to allow the cells to enter quiescence. The cells were then treated with IL-1β or angiotensin II. In some experiments, PASMCs were treated with MAPK inhibitors for 1 h before IL-1β stimulation.

2.2. Materials

IL-1β was obtained from PeproTech, Inc. (Rocky Hill, NJ). Angiotensin II was purchased from Peptide Institute, Inc. (Ibaraki, Osaka, Japan). (R)-1-(2-Oxo-2-(4-(4-(pyrimidin-2-yl)phenyl)piperazin-1-yl)ethyl)-N-(3-(pyridin-4-yl)-1H-indazol-5-yl)pyrrolidine-3-carboxamide (SCH 772984) was from Cayman Chemical (Ann Arbor, MI). 4-(4-Fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1H-imidazole (SB 203580), 1,9-pyrazoloanthrone (SP 600125), phosphorylated and total anti-extracellular signal-regulated kinase (ERK) rabbit polyclonal antibodies, phosphorylated and total anti-p38 MAPK rabbit polyclonal antibodies, and phosphorylated anti-*c*-Jun N-terminal kinase (JNK) rabbit polyclonal antibodies were from Calbiochem (San Diego, CA). An anti-COX-2 goat polyclonal antibody was from Santa Cruz Biotechnology (Dallas, TX). An anti-α-tubulin rabbit polyclonal antibody was from Medical & Biological Laboratories (Nagoya, Japan). A total anti-JNK rabbit polyclonal antibody was from R&D Systems, Inc. (Minneapolis, MN). Horseradish peroxidase-conjugated secondary antibodies were from Invitrogen (Carlsbad, CA) and Cell Signaling Technology (Danvers, MA).

2.3. Application of pressure stress

An original design pressure-loading apparatus was utilized in the experiments, as previously described [16,20]. In brief, culture dishes were placed in the humidified pressure-loading apparatus, and then exposed to atmospheric pressure of either 20 or 60 mmHg at 37 °C. IL-1β or angiotensin II was added to the culture medium at the beginning of pressurization.

2.4. Western blot analysis

Protein expression and phosphorylation were evaluated by Western blot analysis as previously described [19]. The cells were washed with ice-cold phosphate-buffered saline, and whole cell lysates were prepared in a mammalian protein extraction buffer (GE

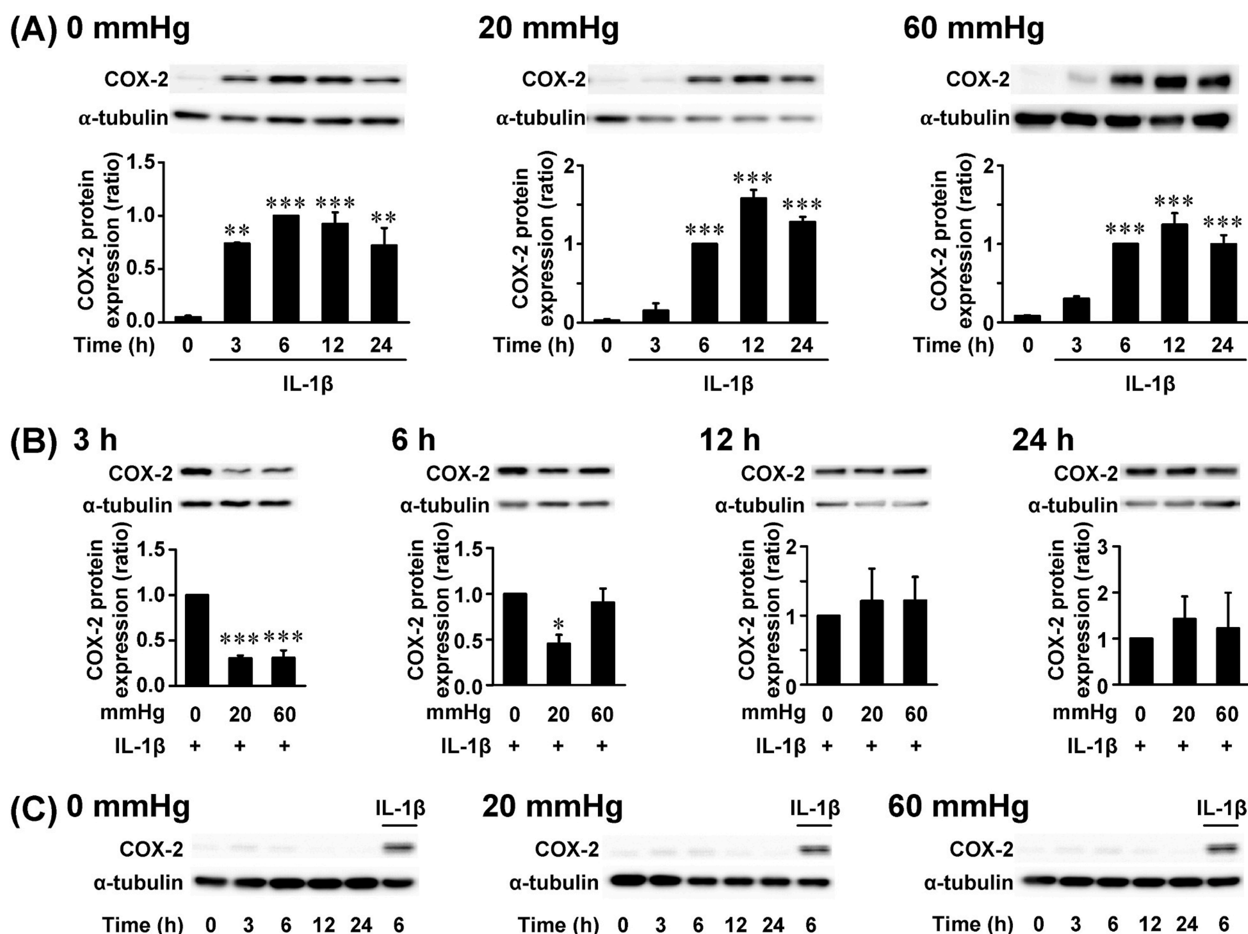

Fig. 1. Effect of pressure stress on IL-1β-induced COX-2 protein expression in human PASMCs. The cells were treated with IL-1β (3 ng/mL) for the indicated times (3, 6, 12, or 24 h) in the presence (20 or 60 mmHg) or absence (0 mmHg) of pressure. (A) Time-course changes. Upper panel: a representative blotting image. Lower panel: summary of densitometric analysis with COX-2 expression at 6 h set as 1. Each column represents the mean ± SEM of three experiments. Statistical significance was calculated using Dunnett's test (**$p < 0.01$ or ***$p < 0.001$ vs. 0 h). The uncropped versions of bands are shown in Supplementary Fig. 1. (B) Effect of pressure stress. Upper panel: a representative blotting image. Lower panel: summary of densitometric analysis with the non-pressurized condition (0 mmHg) set as 1. Each column represents the mean ± SEM of three experiments. Statistical significance was calculated using Dunnett's test (*$p < 0.05$ or ***$p < 0.001$ vs. 0 mmHg). The uncropped versions of bands are shown in Supplementary Figs. 2 and 3. (C) Effect of pressure stress on basal COX-2 protein expression. Representative blotting image. Protein samples after 6 h of IL-1β stimulation were used as positive controls. The uncropped versions of bands is shown in Supplementary Fig. 4.

Healthcare Life Sciences, Buckinghamshire, UK) containing a protease inhibitor mix (GE Healthcare Life Sciences). The cell lysates were separated by electrophoresis on either a 7.5 % or 10 % sodium dodecyl sulfate-polyacrylamide gel and then blotted onto a polyvinylidene fluoride membrane (Millipore, Bedford, MA). The blot was incubated for 2 h or overnight with a primary antibody. Subsequently, the immunoblot was incubated with a horseradish peroxidase-conjugated secondary antibody and visualized using an enhanced chemiluminescence kit. All bands were analyzed by densitometry in ImageJ software (National Institutes of Health, Bethesda, MD).

2.5. Real-time reverse transcription-polymerase chain reaction (RT-PCR)

The mRNA expression levels of *COX-2* and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) were determined by quantitative real-time RT-PCR with total RNA, as previously described [19]. Total RNA was extracted using the TRI reagent® (Sigma-Aldrich, St. Louis, MO). Real-time RT-PCR was carried out using a 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA) and a One Step TB Green® PrimeScript™ PLUS RT-PCR Kit (Takara Bio, Inc., Kusatsu, Japan). The sense primer for *COX-2* was 5′-TCACAGGCTTCCATTGACCAG-3′ and the antisense primer was 5′-CCGAGGCTTTTCTACCAGA-3′ [21]; the sense primer for *GAPDH* was 5′-GCCCAATACGACCAAATCC-3′ and the antisense primer was 5′-AGCCACATCGCTCAGACAC-3′ [22]. Data were analyzed using 7500 System SDS version 1.3.1 software (Applied Biosystems). Relative mRNA expression levels were calculated by the $\Delta\Delta^{CT}$ method. *GAPDH* expression was used as an endogenous control.

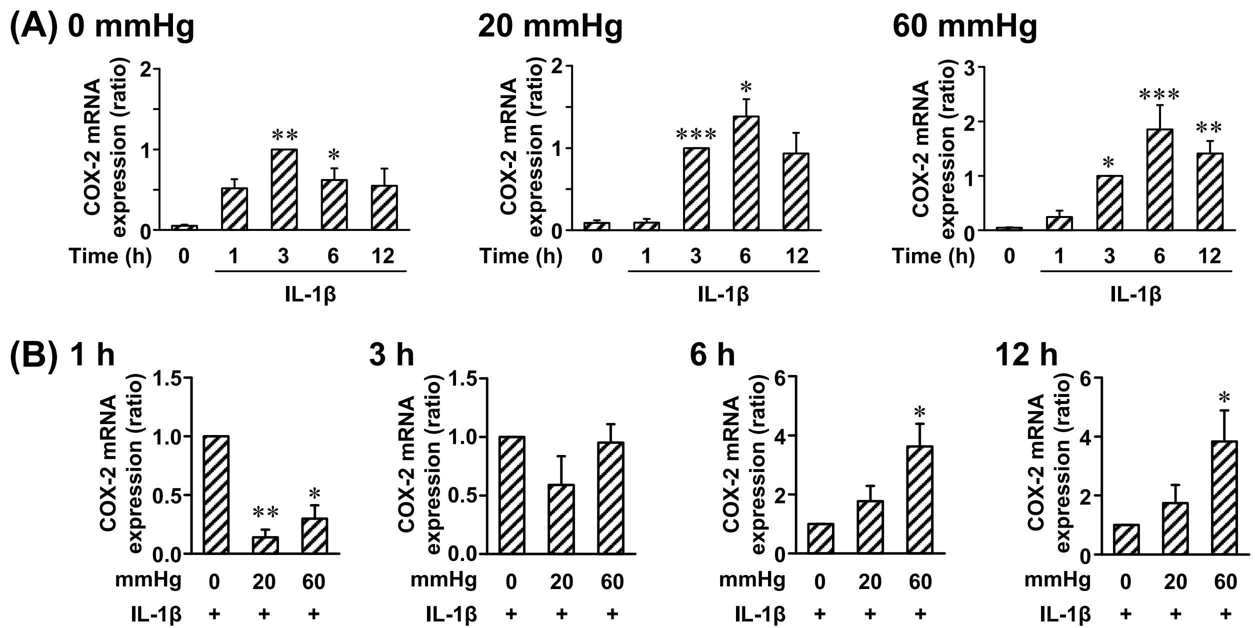

Fig. 2. Effect of pressure stress on IL-1β-induced *COX*-2 mRNA expression in human PASMCs. The cells were treated with IL-1β (3 ng/mL) for the indicated times (1, 3, 6, or 12 h) in the presence (20 or 60 mmHg) or absence (0 mmHg) of pressure. (A) Time-course changes. Summary of analysis expressed as a ratio with COX-2 expression at 3 h set as 1. Each column represents the mean ± SEM of four experiments. Statistical significance was calculated using Dunnett's test (*$p < 0.05$, **$p < 0.01$, or ***$p < 0.001$ vs. 0 h). (B) Effect of pressure stress. Summary of analysis expressed as a ratio with the non-pressurized condition (0 mmHg) set as 1. Each column represents the mean ± SEM of four experiments. Statistical significance was calculated using Dunnett's test (*$p < 0.05$ or **$p < 0.01$ vs. 0 mmHg).

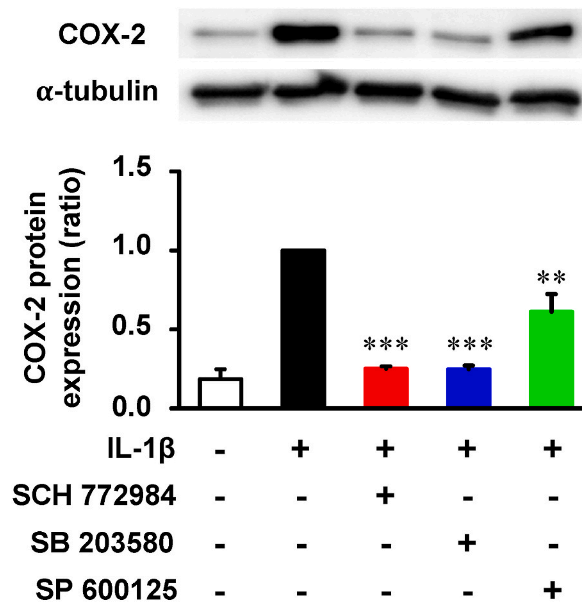

Fig. 3. Effects of MAPK inhibitors on IL-1β-induced COX-2 protein expression in human PASMCs. The cells were pretreated with SCH 772984 (1 μM), SB 203580 (1 μM), or SP 600125 (1 μM) for 60 min and then stimulated with IL-1β (3 ng/mL) for 6 h. Upper panel: a representative blotting image. Lower panel: summary of densitometric analysis under COX-2 expression by taking samples treated with IL-1β stimulation alone set as 1. Each column represents the mean ± SEM of three experiments. Statistical significance was calculated using Dunnett's test (**$p < 0.01$ or ***$p < 0.001$ vs. IL-1β alone). The uncropped versions of bands are shown in Supplementary Fig. 5.

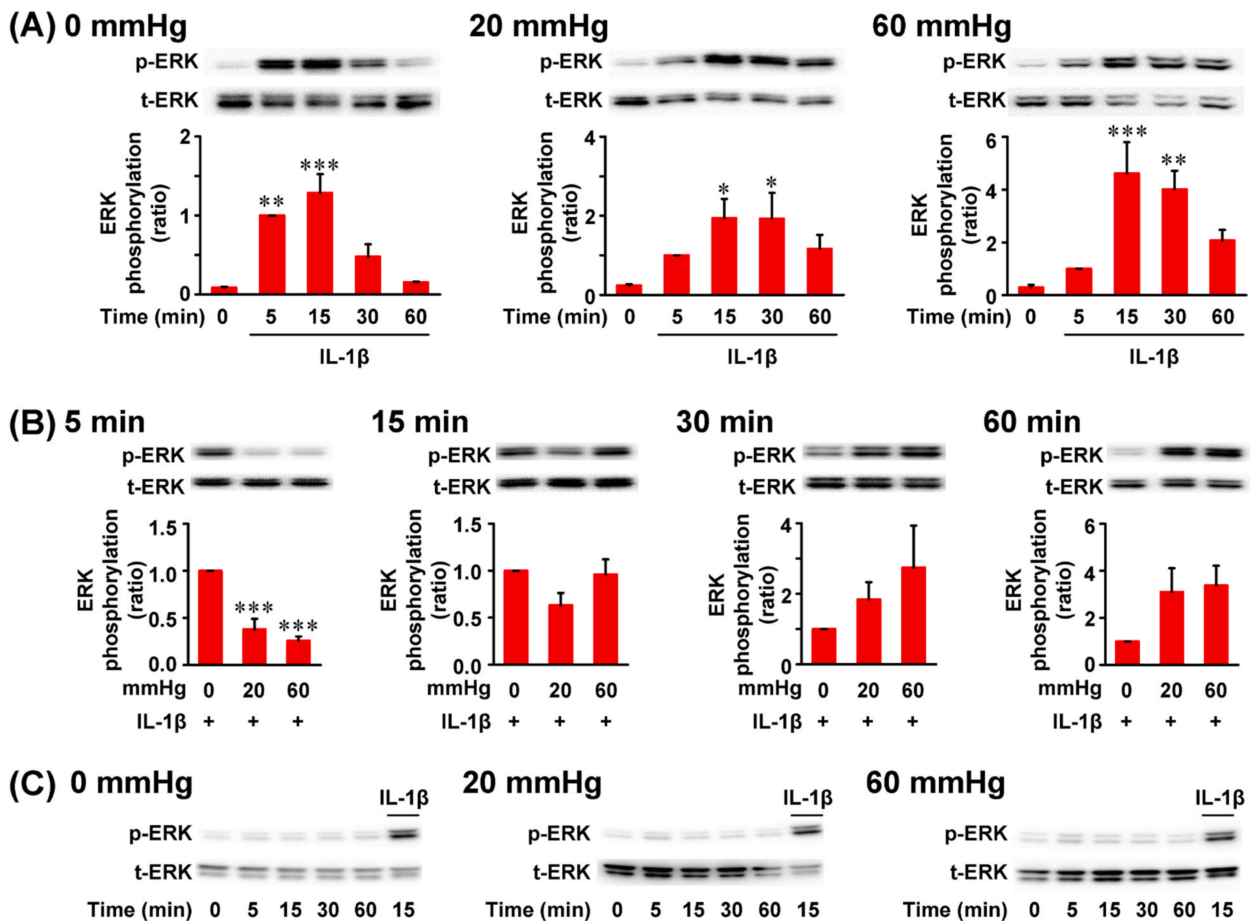

Fig. 4. Effect of pressure stress on IL-1β-induced ERK phosphorylation in human PASMCs. The cells were treated with IL-1β (3 ng/mL) for the indicated times (5, 15, 30, or 60 min) in the presence (20 or 60 mmHg) or absence (0 mmHg) of pressure. (A) Time-course changes. Upper panel: a representative blotting image. Lower panel: summary of densitometric analysis with the ERK phosphorylation ratio at 5 min set as 1. Each column represents the mean ± SEM of three (0 and 60 mmHg) or five (20 mmHg) experiments. Statistical significance was calculated using Dunnett's test (*$p < 0.05$, **$p < 0.01$, or ***$p < 0.001$ vs. 0 min). The uncropped versions of bands are shown in Supplementary fig. 6 (B) Effect of pressure stress. Upper panel: a representative blotting image. Lower panel: summary of densitometric analysis with the non-pressurized condition (0 mmHg) set as 1. Each column represents the mean ± SEM of three experiments. Statistical significance was calculated using Dunnett's test (***$p < 0.001$ vs. 0 mmHg). The uncropped versions of bands are shown in Supplementary Figs. 7 and 8. (C) Effect of pressure stress on basal ERK phosphorylation. Representative blotting image. Protein samples after 15 min of IL-1β stimulation were used as positive controls. The uncropped versions of bands are shown in Supplementary Fig. 9.

2.6. Statistical analysis

Results are expressed as the mean ± standard error of the mean (SEM) values of replicate experiments. Statistical analysis of the results was performed using one-way analysis of variance followed by Dunnett's test for multiple comparisons, if the differences in the variance of multiple groups were not significant. *P*-values less than 0.05 were considered significant.

3. Results

3.1. Pressure stress delays the induction of COX-2 expression by IL-1β stimulation in cultured PASMCs

In this study, we chose two pressures—20 mmHg, the upper end of the normal range of pulmonary arterial pressure [23], and 60 mmHg, which simulates severe PH—to investigate their effect on the COX-2 expression in PASMCs. As shown in Fig. 1A, IL-1β (3 ng/mL) significantly induced COX-2 protein expression within 3 h in non-pressurized cells. COX-2 protein expression peaked at 6 h after IL-1β stimulation. Pressure stress at 20 or 60 mmHg significantly induced COX-2 protein expression after 6 h of IL-1β stimulation, peaking at 12 h (Fig. 1A). When the effect of pressure stress on COX-2 expression was analyzed for each stimulation time, COX-2 protein expression was significantly lower at 3 h under the 20 and 60 mmHg pressurized conditions than under the non-pressurized condition (Fig. 1B). At 6 h, COX-2 protein expression was significantly lower under the 20 mmHg pressurized

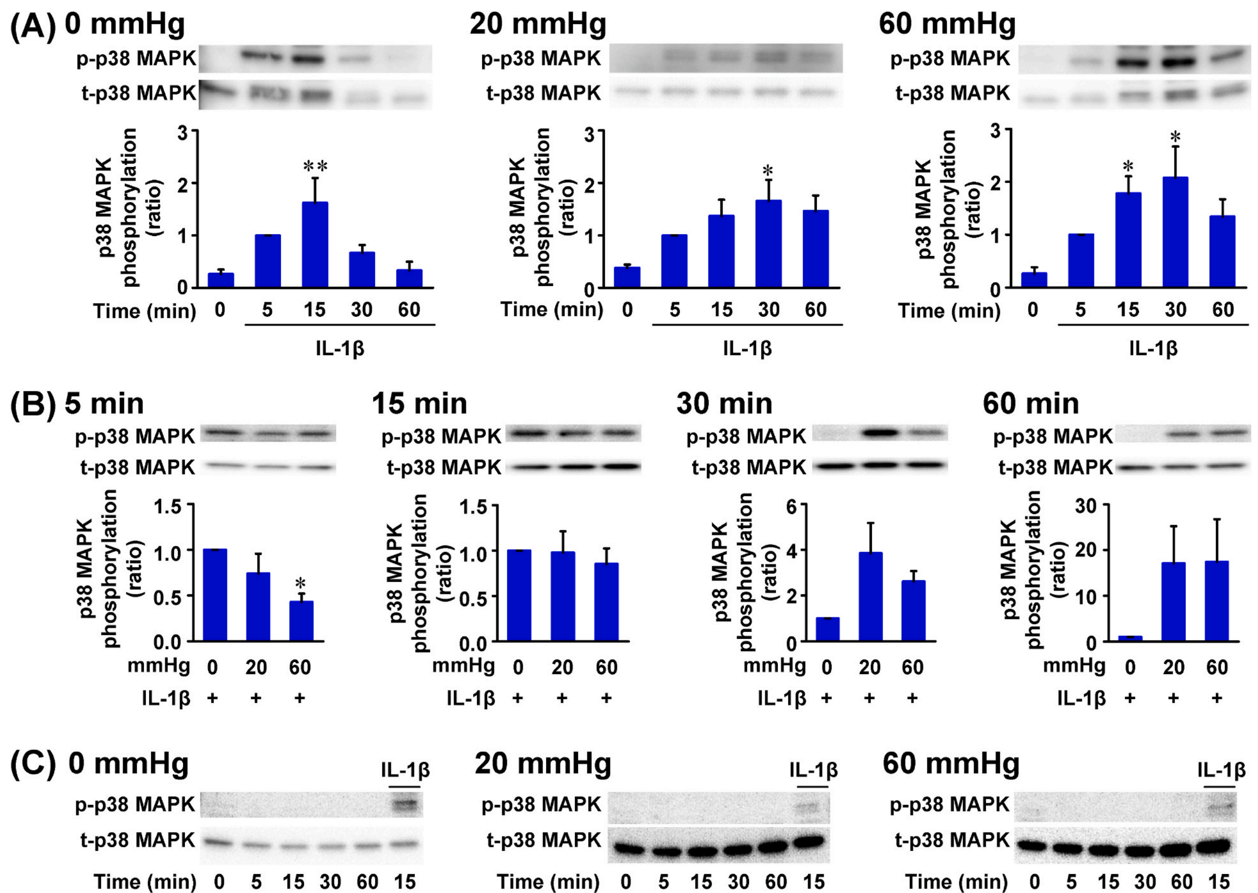

Fig. 5. Effect of pressure stress on IL-1β-induced p38 MAPK phosphorylation in human PASMCs. The cells were treated with IL-1β (3 ng/mL) for the indicated times (5, 15, 30, or 60 min) in the presence (20 or 60 mmHg) or absence (0 mmHg) of pressure. (A) Time-course changes. Upper panel: a representative blotting image. Lower panel: summary of densitometric analysis with the p38 MAPK phosphorylation ratio at 5 min set as 1. Each column represents the mean ± SEM of five (0 mmHg) or three (20 and 60 mmHg) experiments. Statistical significance was calculated using Dunnett's test (*$p < 0.05$ or **$p < 0.01$ vs. 0 min). The uncropped versions of bands are shown in Supplementary Fig. 10. (B) Effect of pressure stress. Upper panel: a representative blotting image. Lower panel: summary of densitometric analysis with the non-pressurized condition (0 mmHg) set as 1. Each column represents the mean ± SEM of three experiments. Statistical significance was calculated using Dunnett's test (*$p < 0.05$ vs. 0 mmHg). The uncropped versions of bands are shown in Supplementary Fig. 11. (C) Effect of pressure stress on basal p38 MAPK phosphorylation. Representative blotting image. Protein samples after 15 min of IL-1β stimulation were used as positive controls. The uncropped versions of bands are shown in Supplementary Fig. 12.

condition than under the non-pressurized condition (Fig. 1B). At 12 and 24 h, there was no significant difference in COX-2 protein expression in the presence or absence of pressure (Fig. 1B). Neither pressure stress at 20 nor 60 mmHg alone induced COX-2 protein expression (Fig. 1C).

IL-1β-induced transient *COX*-2 mRNA expression peaked at 3 h in non-pressurized cells (Fig. 2A). In contrast, *COX*-2 mRNA expression peaked at 6 h in pressurized cells at 20 and 60 mmHg (Fig. 2A). In fact, IL-1β-induced *COX*-2 mRNA expression was significantly lower at 1 h under the 20 and 60 mmHg pressurized conditions than under the non-pressurized condition (Fig. 2B). In contrast, at 6 and 12 h, IL-1β-induced *COX*-2 mRNA expression was increased in a pressure-dependent manner, with a significant increase at 60 mmHg (Fig. 2B).

3.2. Pressure stress delays transient MAPK phosphorylation induced by IL-1β stimulation in cultured human PASMCs

IL-1β-induced COX-2 protein expression was almost completely inhibited by SCH 772984 (1 μM), an ERK inhibitor, and by SB 203580 (1 μM), a p38 MAPK inhibitor, under the non-pressurized condition (Fig. 3). It was also significantly inhibited by SP 600125 (1 μM), a JNK inhibitor (Fig. 3). We next examined the effect of pressure stress on IL-1β-induced MAPK phosphorylation. IL-1β significantly increased ERK phosphorylation at 5 and 15 min in non-pressurized cells, whereas ERK phosphorylation was significantly increased at 15 and 30 min in pressurized cells (Fig. 4A). ERK phosphorylation was significantly lower at 5 min under both pressurized conditions than under the non-pressurized condition (Fig. 4B). In contrast, at 30 and 60 min, ERK phosphorylation tended to higher under the pressurized conditions than under the non-pressurized condition, although it was not significant (Fig. 4B). Neither pressure

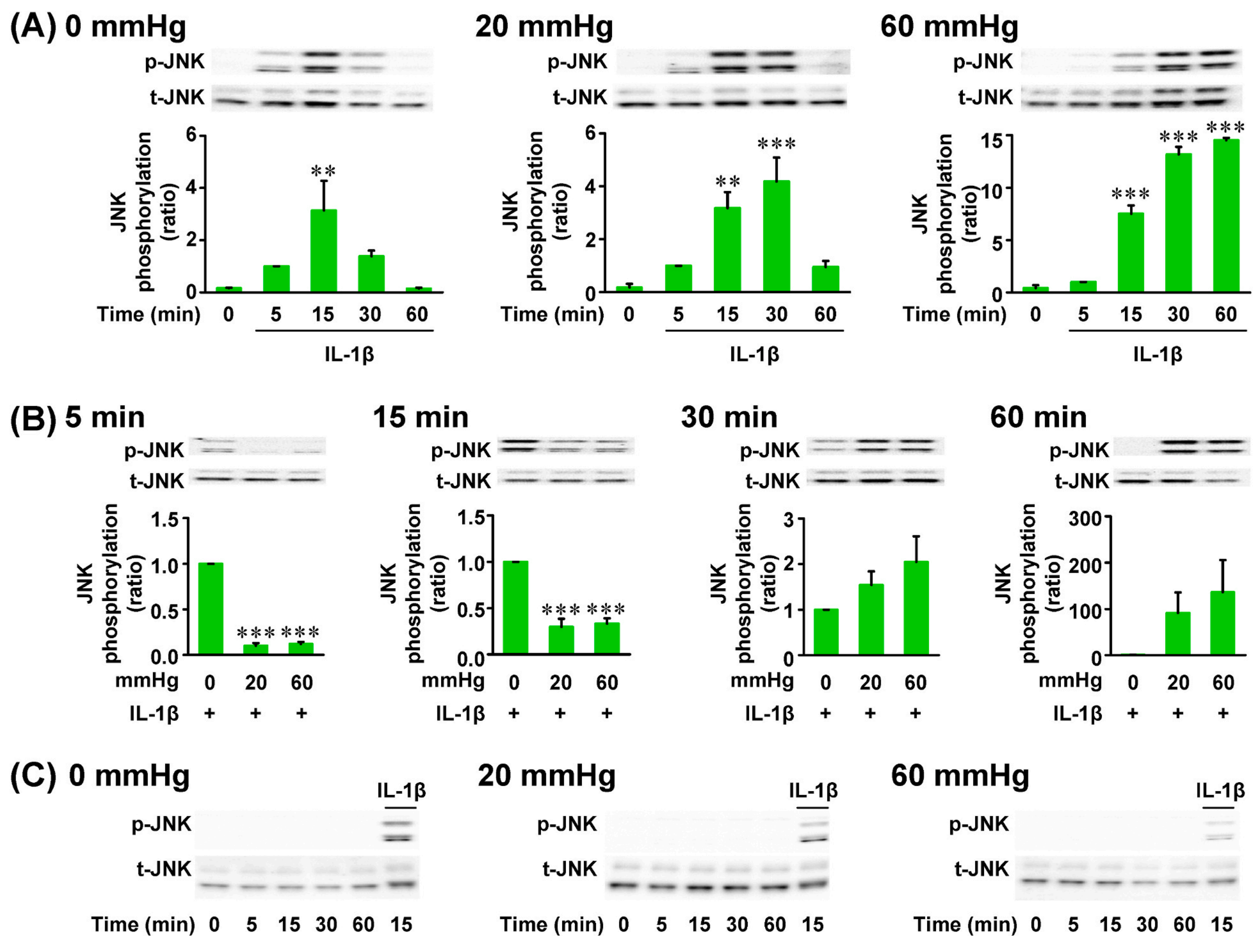

Fig. 6. Effect of pressure stress on IL-1β-induced JNK phosphorylation in human PASMCs. The cells were treated with IL-1β (3 ng/mL) for the indicated times (5, 15, 30, or 60 min) in the presence (20 or 60 mmHg) or absence (0 mmHg) of pressure. (A) Time-course changes. Upper panel: a representative blotting image. Lower panel: summary of densitometric analysis with the JNK phosphorylation ratio at 5 min set as 1. Each column represents the mean ± SEM of three (0 and 60 mmHg) or four (20 mmHg) experiments. Statistical significance was calculated using Dunnett's test (** $p < 0.01$ or *** $p < 0.001$ vs. 0 min). The uncropped versions of bands are shown in Supplementary Fig. 13. (B) Effect of pressure stress. Upper panel: a representative blotting image. Lower panel: summary of densitometric analysis with the non-pressurized condition (0 mmHg) set as 1. Each column represents the mean ± SEM of three experiments. Statistical significance was calculated using Dunnett's test (*** $p < 0.001$ vs. 0 mmHg). The uncropped versions of bands are shown in Supplementary Fig. 14. (C) Effect of pressure stress on basal JNK phosphorylation. Representative blotting image. Protein samples after 15 min of IL-1β stimulation were used as positive controls. The uncropped versions of bands are shown in Supplementary Fig. 15.

stress at 20 nor 60 mmHg alone induced ERK phosphorylation (Fig. 4C). IL-1β significantly increased transient p38 MAPK phosphorylation at 15 min in non-pressurized cells, whereas p38 MAPK phosphorylation peaked at 30 min in 20 and 60 mmHg pressurized cells (Fig. 5A). IL-1β-induced p38 MAPK phosphorylation was significantly lower at 5 min in pressurized cells at 60 mmHg than in non-pressurized cells (Fig. 5B). p38 MAPK phosphorylation tended to higher under the 20 and 60 mmHg pressurized conditions than under the non-pressurized condition, although it was not significant (Fig. 5B). Neither pressure stress at 20 nor 60 mmHg alone induced p38 MAPK phosphorylation (Fig. 5C). IL-1β significantly increased transient JNK phosphorylation with a peak at 15 min in non-pressurized cells (Fig. 6A). The peak time of JNK phosphorylation was at 30 min under 20 mmHg pressure, and at 60 min under 60 mmHg pressure (Fig. 6A). IL-1β-induced JNK phosphorylation was significantly lower at 5 and 15 min in pressurized cells than in non-pressurized cells (Fig. 6B). JNK phosphorylation tended to higher at 30 and 60 min in both pressurized conditions than in the non-pressurized condition, although it was not significant (Fig. 6B). Neither pressure stress at 20 nor 60 mmHg alone induced JNK phosphorylation (Fig. 6C).

3.3. *Pressure stress has no effect on transient MAPK phosphorylation and COX-2 mRNA expression induced by angiotensin II stimulation in cultured human PASMCs*

Angiotensin II (10 nM) significantly increased transient ERK phosphorylation at 15 min in non-pressurized cells (Fig. 7A), which returned almost completely to the basal level at 30 min. Pressure had no significant effect on the peak time of ERK phosphorylation,

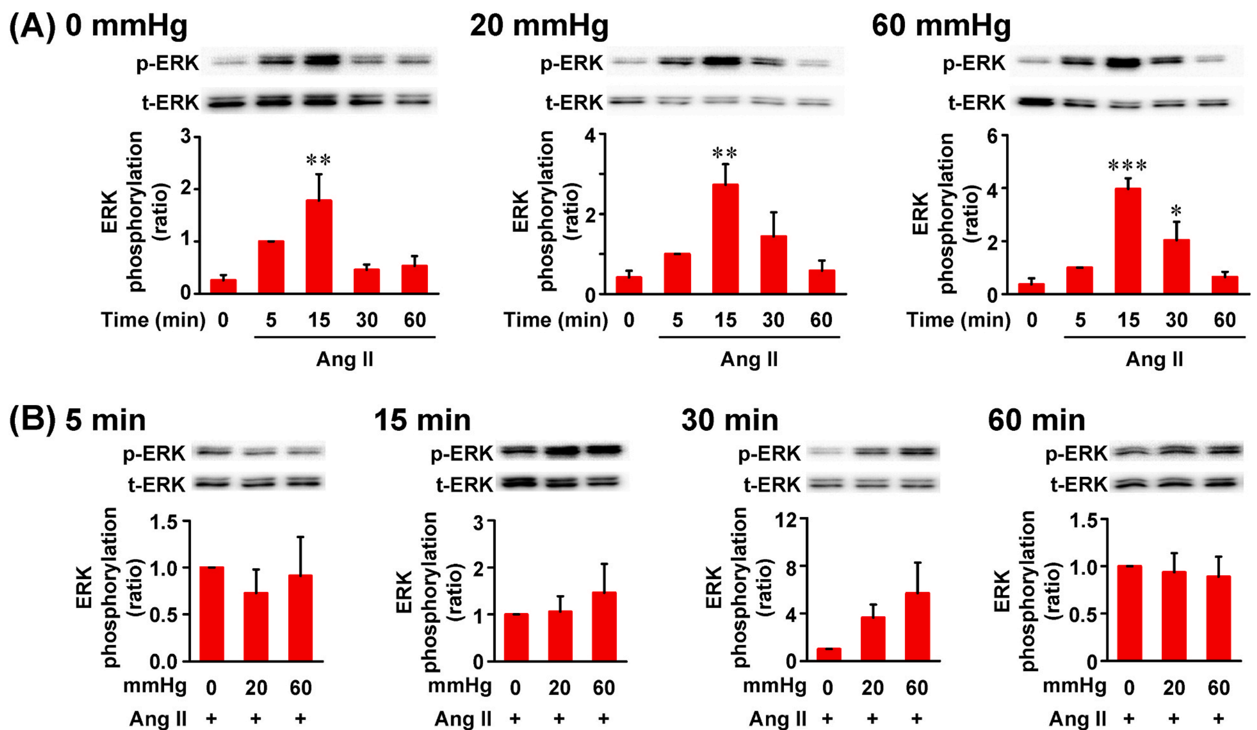

Fig. 7. Effect of pressure stress on angiotensin II-induced ERK phosphorylation in human PASMCs. The cells were treated with angiotensin II (10 nM) for the indicated times (5, 15, 30, or 60 min) in the presence (20 or 60 mmHg) or absence (0 mmHg) of pressure. (A) Time-course changes. Upper panel: a representative blotting image. Lower panel: summary of densitometric analysis with the ERK phosphorylation ratio at 5 min set as 1. Each column represents the mean ± SEM of three experiments. Statistical significance was calculated using Dunnett's test (*$p < 0.05$, **$p < 0.01$, or ***$p < 0.001$ vs. 0 min). The uncropped versions of bands are shown in Supplementary Fig. 16. (B) Effect of pressure stress. Upper panel: a representative blotting image. Lower panel: summary of densitometric analysis with the non-pressurized condition (0 mmHg) set as 1. Each column represents the mean ± SEM of three experiments. Statistical significance was calculated using Dunnett's test. The uncropped versions of bands are shown in Supplementary Figs. 17 and 18.

although ERK phosphorylation did not completely return to the basal level at 30 min (Fig. 7A). Both pressure conditions tended to increase ERK phosphorylation only at 30 min (Fig. 7B). Angiotensin II significantly increased transient p38 MAPK phosphorylation at 15 min in non-pressurized and pressurized cells (Fig. 8A). Pressure had no effect on p38 MAPK phosphorylation at any of the times tested (Fig. 8B). Angiotensin II (10 nM) did not induce JNK phosphorylation in non-pressurized and pressurized cells (data not shown).

Angiotensin II significantly induced transient *COX*-2 mRNA expression at 1 h in non-pressurized and pressurized cells (Fig. 9A). Pressure stress had no effect on *COX*-2 mRNA expression at any of the times tested (Fig. 9B).

4. Discussion

In this study, we first demonstrated that the pressure stress delayed the induction of COX-2 expression by IL-1β in cultured human PASMCs. The delayed expression of COX-2 was observed not only at 60 mmHg, which simulates severe PH, but also at 20 mmHg, which is near the upper end of the normal range of pulmonary arterial pressure. Thus, COX-2 induction occurs just before the development of PH, suggesting that it may be involved in the mechanism underlying the development and progression of PH. Although the delayed expression of COX-2 observed in this study might affect pathogenesis during acute elevation of pulmonary arterial pressure, the extent to which it is involved in pathogenesis during chronic elevation in pulmonary arterial pressure is unknown. Although pressure stress delayed COX-2 expression, its protein levels were not affected at 12 and 24 h. When pressure stress simulating systolic hypertension is applied to aortic SMCs, IL-1β-induced COX-2 protein expression is inhibited but not delayed [19]. These results suggest that the effect of pressure stress on COX-2 expression differs between PASMCs and aortic SMCs. Nevertheless, *COX*-2 mRNA expression was increased at 6 and 12 h by pressure at 60, but not 20, mmHg. Therefore, it is possible that pressure at 60 mmHg increases COX-2 expression after 24 h of IL-1β stimulation or delays the timing of the decrease in COX-2 expression, but further investigation is required.

Because PH occurs in response to chronic hypoxia [24], PASMCs exposed to hypoxic environments are normally used to study PH *in vitro*. COX-2 expression is not significantly increased in human PASMCs cultured under short-term (<24 h) hypoxia [3]. Chemical hypoxia induced by $CoCl_2$ for 24 h increases hypoxia-inducible factor-1α expression and decreases COX-2 expression and PGI_2 secretion in cultured human PASMCs [25]. However, in our experimental condition, pressure stress did not affect hypoxia-inducible

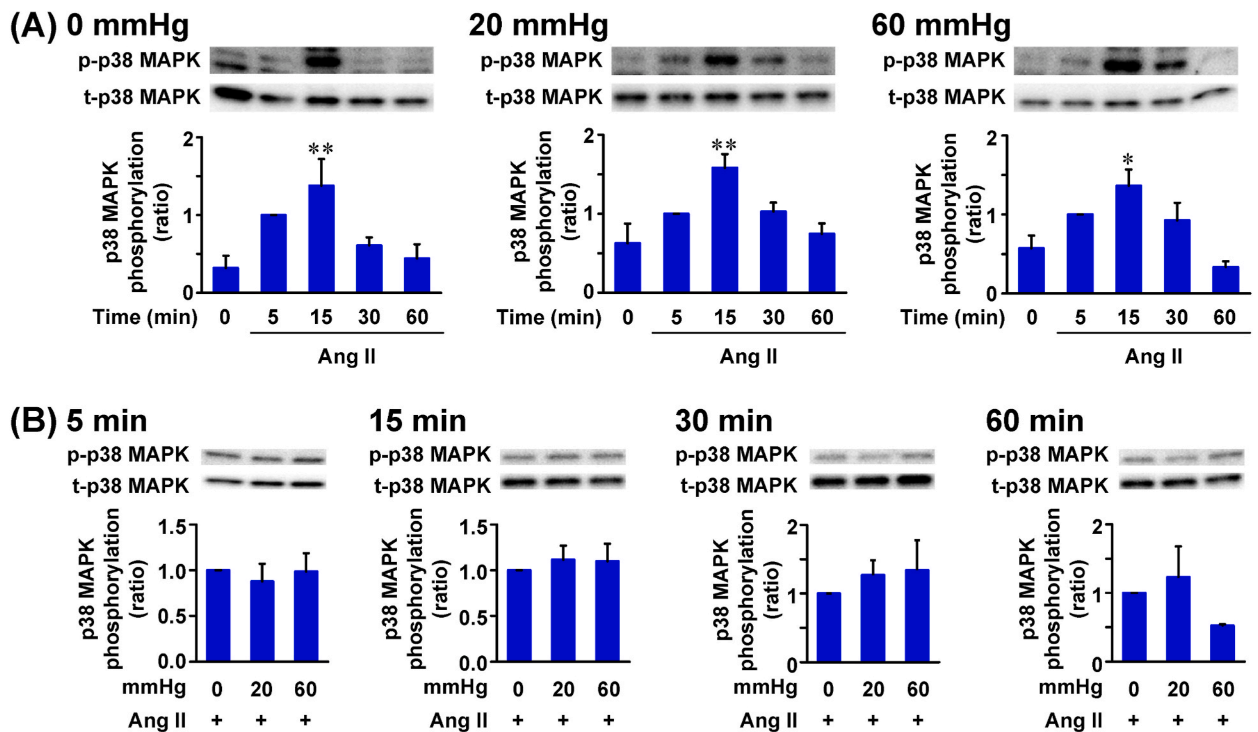

Fig. 8. Effect of pressure stress on angiotensin II-induced p38 MAPK phosphorylation in human PASMCs. The cells were treated with angiotensin II (10 nM) for the indicated times (5, 15, 30, or 60 min) in the presence (20 or 60 mmHg) or absence (0 mmHg) of pressure. (A) Time-course changes. Upper panel: a representative blotting image. Lower panel: summary of densitometric analysis with the p38 MAPK phosphorylation ratio at 5 min set as 1. Each column represents the mean ± SEM of three experiments. Statistical significance was calculated using Dunnett's test (*$p < 0.05$ or **$p < 0.01$ vs. 0 min). (B) Effect of pressure stress. Upper panel: a representative blotting image. Lower panel: summary of densitometric analysis with the non-pressurized condition (0 mmHg) set as 1. Each column represents the mean ± SEM of three experiments. Statistical significance was calculated using Dunnett's test. The uncropped versions of bands are shown in Supplementary Fig. 19.

factor-1α protein expression (data not shown). Thus, the reduction of COX-2 expression by IL-1β stimulation is suggested to be pressure-induced, independent of oxygen concentration.

Under our experimental conditions, pressure stress itself did not induce COX-2 expression in PASMCs. Thus, it is suggested that pressure stress may affect the signal transduction pathway of IL-1β. Several studies have reported that MAPK phosphorylation is involved in IL-1β-induced COX-2 expression in various cell types [19,26–28]. In fact, IL-1β-induced COX-2 protein expression was suppressed by an ERK inhibitor, a p38 MAPK inhibitor, and a JNK inhibitor in this study, indicating that these three MAPKs are involved in COX-2 expression. Pressure stress delayed the phosphorylation of all three MAPKs. It is worth noting that MAPK phosphorylation was affected within 5 min of exposure to pressure. We have found similar responses with rat aortic SMCs. Among the three MAPKs, only ERK is involved in IL-1β-induced COX-2 expression in rat aortic SMCs; pressure stress simulating systolic hypertension significantly inhibits ERK phosphorylation within 5 min [19]. Taken together, the pressure-stress response to IL-1β stimulation, which suppresses ERK phosphorylation within 5 min, is suggested to be a common response in vascular SMCs, both in the pulmonary artery and thoracic aorta. Pressure stress is one of the mechanical stimuli that vascular cells are exposed to. The response observed immediately after the onset of pressure stress may be related to mechanosensors on the cell membrane, such as piezo-type mechanosensitive ion channel component 1 (Piezo1), which is expressed in human PASMCs [29]. In pancreatic acinar cells, the pressure-induced activation of Piezo1 and secondary activation of transient receptor potential vanilloid 4 (TRPV4) are reported to be involved in pressure-induced pancreatitis [30,31]. Moreover, mechanical stimuli (cyclic tensile strain) are reported to inhibit the IL-1β-induced release of PGE_2 via a TRPV4-dependent pathway in isolated chondrocytes [32]. The IL-1β-induced release of PGE_2 and expression of COX-2 are completely inhibited by GSK1016790A, a TRPV4 agonist [32]. The involvement of the Piezo1–TRPV4 pathway via pressure stress and IL-1β stimulation in PASMCs needs to be explored in future studies.

Angiotensin II plays an important role in the pathogenesis of pulmonary vascular remodeling in PH. In fact, a previous study reported that treatment with angiotensin-converting enzyme inhibitors relieved pulmonary vascular remodeling in PH in a rat model [33]. Pressure stress did not affect the peak time of angiotensin II-induced MAPK phosphorylation or transient *COX*-2 mRNA expression. These results suggest that the effect of pressure stress on COX-2 expression depends on the type of bioactive substance. Pressure stress is suggested to affect specifically signal transduction between IL-1β receptor stimulation and MAPK phosphorylation. When IL-1β binds to the IL-1 receptor, it activates MAPK via IL-1 receptor-associated kinases, tumor necrosis factor receptor-associated factor 6, and transforming growth factor-β-activated kinase 1 [34]. In contrast, activation of the angiotensin II type 1 (AT_1) receptor by

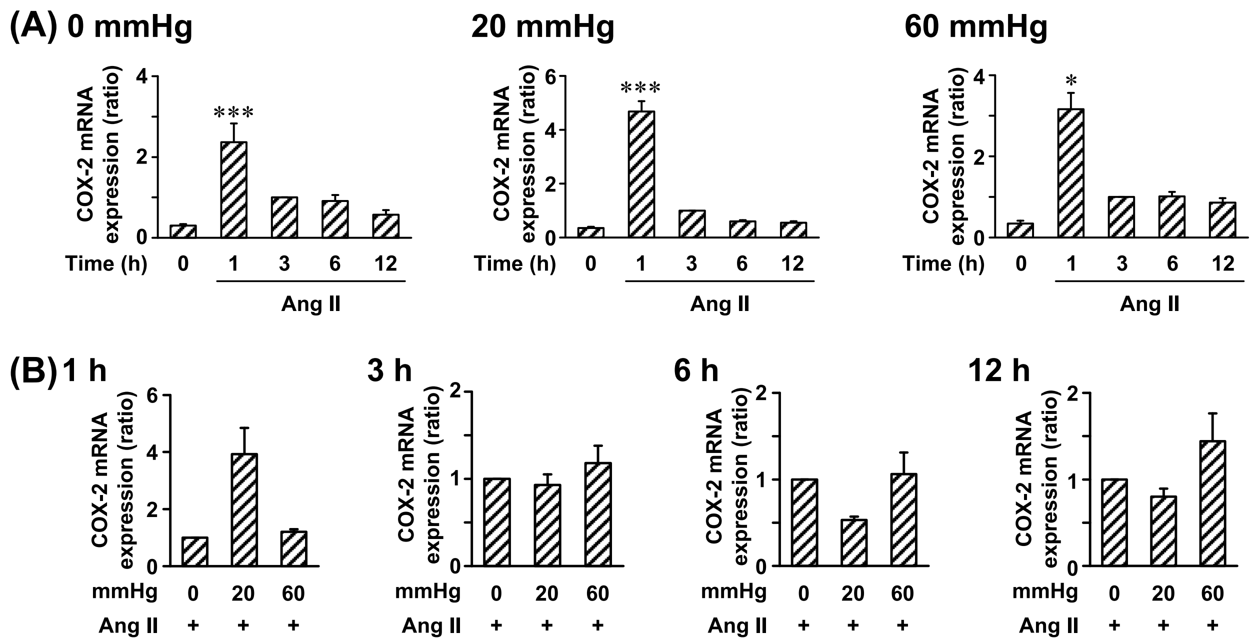

Fig. 9. Effect of pressure stress on angiotensin II-induced *COX*-2 mRNA expression in human PASMCs. The cells were treated with angiotensin II (10 nM) for the indicated times (1, 3, 6, or 12 h) in the presence (20 or 60 mmHg) or absence (0 mmHg) of pressure. (A) Time-course changes of *COX*-2 mRNA expression. Summary of analysis expressed as a ratio with COX-2 expression at 3 h set as 1. Each column represents the mean ± SEM of four experiments. Statistical significance was calculated using Dunnett's test ($*p < 0.05$ or $***p < 0.001$ vs. 0 h). (B) Effect of pressure stress on *COX*-2 mRNA expression. Summary of analysis expressed as a ratio with the non-pressurized condition (0 mmHg) set as 1. Each column represents the mean ± SEM of four experiments. Statistical significance was calculated using Dunnett's test.

angiotensin II activates phospholipase C, producing diacylglycerol and inositol-3-phosphate [35]. Angiotensin II-induced MAPK phosphorylation in vascular SMCs occurs through the activation of calcium-dependent tyrosine kinases via the elevation of intracellular calcium levels by inositol-3-phosphate receptor stimulation [36]. Interestingly, AT_1 receptor and TRPV4 form a constitutive heterodimer in the membrane [37]. It has been shown that angiotensin II stimulation causes a robust interaction between TRPV1 and β-arrestin 1 within 2 min, which triggers TRPV4 internalization in rat aortic SMCs [37]. Because TRPV4 is an important factor for Piezo1 signaling, the internalization of TRPV4 by angiotensin II may be related to the fact that pressure loading has no effect on COX-2 expression.

Although our findings reveal a unique regulation of COX-2 expression via pure pressure stress, there are several limitations in this study. For instance, the extent to which the effects of pressure stress on COX-2 expression revealed in this study impact production of PGI_2 remains to be determined. In addition, an apparatus that can apply pressure stress to cells under hypoxic conditions and can enable analysis of cellular responses in a more *in vivo*-like environment has yet to be developed. Resolution of these issues will further clarify the extent to which the events identified in this study contribute to pathogenesis during chronic elevation of pulmonary artery pressure.

In summary, this study attempted to reproduce an *in vitro* PH model by applying pure high atmospheric pressure to cultured human PASMCs. IL-1β-induced COX-2 expression was delayed by pressure stress, which was associated with the delayed phosphorylation of MAPKs. Angiotensin II-induced MAPK phosphorylation and COX-2 expression were not affected by pressure stress, indicating that the effect of pressure stress on MAPK phosphorylation depends on the type of local bioactive substance. Our results demonstrate that the application of pressure stress to PASMCs directly alters cell function, which may provide a basic insight into our understanding of the pathogenesis of PH and the development of therapy for PH.

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Data included in article/supp. material in article.

Additional information

No additional information is available for this paper.

CRediT authorship contribution statement

Sachiko Hiraide: Conceptualization, Data curation, Formal analysis, Investigation, Writing – original draft. **Takuji Machida:** Conceptualization, Formal analysis, Investigation, Writing – original draft, Writing – review & editing. **Shota Takihana:** Formal analysis, Investigation. **Mikoto Ohshita:** Formal analysis, Investigation. **Kenji Iizuka:** Conceptualization, Writing – review & editing.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:Takuji Machida reports a relationship with Japan Tobacco Inc that includes: funding grants.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e21008.

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