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2,5-Dimethylcelecoxib prevents isoprenaline-induced cardiomyocyte hypertrophy and cardiac fibroblast activation by inhibiting Akt-mediated GSK-3 phosphorylation

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ABSTRACT

We previously reported that 2,5-dimethylcelecoxib (DM-celecoxib), a celecoxib derivative that is unable to inhibit cyclooxygenase-2, prevented cardiac remodeling by activating glycogen synthase kinase-3 (GSK-3) and prolonged the lifespan of heart failure mice with genetic dilated cardiomyopathy or transverse aortic constriction-induced left ventricular hypertrophy. However, it remained unclear how DM-celecoxib regulated structure and function of cardiomyocytes and cardiac fibroblasts involved in cardiac remodeling. In the present study, therefore, we investigated the effect of DM-celecoxib on isoprenaline-induced cardiomyocyte hypertrophy and cardiac fibroblast activation, because DM-celecoxib prevented isoprenaline-induced cardiac remodeling *in vivo*. DM-celecoxib suppressed isoprenaline-induced neonatal rat cardiomyocyte hypertrophy by the inhibition of Akt phosphorylation resulting in the activation of GSK-3 and the inhibition of β-catenin and mammalian target of rapamycin (mTOR). DM-celecoxib also suppressed the proliferation and the production of matrix metalloproteinase-2 and fibronectin of rat cardiac fibroblasts. Moreover, we found that phosphatase and tensin homolog on chromosome 10 (PTEN) could be a molecule to mediate the effect of DM-celecoxib on Akt. These results suggest that DM-celecoxib directly improves the structure and function of cardiomyocytes and cardiac fibroblasts and that this compound could be clinically useful for the treatment of β-adrenergic receptor-mediated maladaptive cardiac remodeling.

1. Introduction

Cardiac remodeling is essentially an adaptive reaction for overcoming various stresses created by pressure and/or volume overload, ischemic heart disease, neurohormonal abnormality or cardiomyopathies. However, long-lasting stresses cause maladaptive remodeling and eventually lead to heart failure by progressive ventricular dilation and impaired contractile function [1]. Although many processes are involved in the remodeling, the crucial ones that lead to heart failure are cardiac hypertrophy and fibrosis [2]. Cardiac stresses activate several hypertrophic signaling pathways in cardiomyocytes to increase their size and to stimulate fibroblast proliferation and the secretion of extracellular matrix (ECM) proteins and proinflammatory cytokines to induce tissue fibrosis [3]. Several medicines are currently administered to prevent maladaptive cardiac remodeling, such as β-adrenergic receptor antagonists, angiotensin-converting enzyme inhibitors, angiotensin receptor antagonists and mineralocorticoid receptor antagonists. However, their efficacy is limited and therefore novel strategies to inhibit maladaptive cardiac remodeling are urgently required.

2,5-Dimethylcelecoxib (DM-celecoxib) is a celecoxib derivative that is unable to inhibit cyclooxygenase-2. We previously reported that this compound prevented cardiac hypertrophy and fibrosis in mouse heart failure models [4,5]. In those studies, we found that the effect of DM-celecoxib was mediated by glycogen synthase kinase-3 (GSK-3), in which two subtypes GSK-3α and GSK-3β have been discovered. GSK-3 plays a key role in the regulation of cardiac hypertrophy and fibrosis by

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Table 1
The heart rate, systolic blood pressure and diastolic blood pressure of control or isoprenaline-treated mice receiving vehicle or DM-celecoxib.

Day	7									14								
Group	Control			ISO			ISO + DMC			Control			ISO			ISO + DMC		
N	7			5			6			7			5			6		
HR, b.p.m	625	±	55	775	±	46^{***}	772	±	29^{***}	640	±	39	740	±	$33^{\dagger\dagger\dagger}$	743	±	$21^{\dagger\dagger\dagger}$
SBP, mmHg	101	±	4	110	±	9	114	±	8^{**}	102	±	5	116	±	$6^{\dagger\dagger}$	125	±	$8^{\dagger\dagger\dagger}$
DBP, mmHg	65	±	4	70	±	14	61	±	5	67	±	6	68	±	6	72	±	10

Mice were administered isoprenaline or vehicle for 2 weeks and received feed containing DM-celecoxib (1000 ppm) or vehicle. The heart rate, systolic blood pressure, and diastolic blood pressure were measured on days 7 and 14. Values are the mean ± SD. $^{**}P < 0.01$, $^{***}P < 0.001$ vs control (day 7). $^{\dagger\dagger}P < 0.01$, $^{\dagger\dagger\dagger}P < 0.001$ vs control (day 14). N, number of mice; HR, heart rate; b.p.m, beats per minute; SBP, systolic blood pressure; DBP, diastolic blood pressure; ISO, isoprenaline; DMC, DM-celecoxib.

Table 2
Weight and echocardiography data of control or isoprenaline-treated mice receiving vehicle or DM-celecoxib.

	Control			ISO			ISO + DMC		
Weight									
Number of Mice		7			5			6	
BW, g	24.9	±	1.0	25.8	±	2.4	26.2	±	2.1
HW/TL, mg/mm	5.1	±	0.6	6.7	±	0.4^{***}	6.1	±	0.5^{*}
LW/TL, mg/mm	5.2	±	0.6	6.6	±	0.5^{***}	6.1	±	0.4^{*}
Echocardiography									
Number of Mice		5			5			5	
IVST, mm	0.72	±	0.08	1.02	±	0.08^{***}	0.86	±	$0.09^{\#}$
LVPWT, mm	0.68	±	0.05	0.98	±	0.05^{***}	0.90	±	0.07^{***}
LVAWT, mm	0.68	±	0.08	1.10	±	0.07^{***}	0.90	±	$0.07^{**\#\#}$
LVIWT, mm	0.70	±	0.07	1.04	±	0.06^{***}	1.04	±	$0.05^{***\#}$
LVEDD, mm	3.72	±	0.16	3.32	±	0.22^{*}	3.42	±	0.29
LVESD, mm	2.54	±	0.20	1.94	±	0.32^{*}	2.02	±	0.39
LVEF, %	66.2	±	2.9	78.6	±	6.3^{*}	77.6	±	7.9^{*}
FS, %	31.4	±	1.8	41.6	±	5.9^{*}	41.2	±	6.7^{*}
HR, b.p.m.	456.4	±	16.0	571.8	±	33.0^{***}	565.0	±	29.4^{***}

Mice were administered isoprenaline or vehicle for 2 weeks and received feed containing DM-celecoxib (1000 ppm) or vehicle. Transthoracic ultrasound sonography was performed under 2.0% isoflurane on day 14. The hearts and lungs were removed and weighed. Values are the mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs control. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs ISO.
b.p.m., beats per minute; BW, body weight; DMC, DM-celecoxib. FS, fractional shortening; HR, heart rate; HW/TL, heart weight-to-tibial length ratio; ISO, isoprenaline; IVST, interventricular septal wall thickness; LVAWT, left ventricular anterior wall thickness; LVEDD, left ventricular end-diastolic dimension; LVEF, left ventricular ejection fraction; LVESD, left ventricular end-systolic dimension; LVIWT, left ventricular inferior wall thickness; LVPWT, left ventricular posterior wall thickness; LW/TL, lung weight-to-tibial length ratio.

phosphorylating several transcription factors involved in the cellular functions of cardiomyocytes and fibroblasts [6–9]. It has been reported that GSK-3α and β show a preventive effect on cardiac remodeling and dysfunction [10–12]. And DM-celecoxib has been reported to inhibit Akt [13–15], which, in response to extracellular signals, phosphorylates GSK-3 to inhibit its activity [6–8]. Therefore, DM-celecoxib might prevent cardiac remodeling by releasing GSK-3 from the suppression by Akt. However, we have not yet clarified whether DM-celecoxib directly modulates structure and function of cardiomyocytes and cardiac fibroblasts and the mechanisms for DM-celecoxib-induced suppression of Akt.

Therefore, we attempted to examine whether DM-celecoxib directly acts on cardiomyocytes and cardiac fibroblasts under hypertrophic stimulation and to elucidate the mechanism of action of DM-celecoxib. In the present study, we used isoprenaline, a non-selective β-adrenergic receptor agonist, as a hypertrophic stimulus, because isoprenaline can mimic the sustained activation of the sympathetic nervous system, which underlies cardiac remodeling in clinical settings [16,17].

2. Materials and methods

2.1. Chemicals and antibodies

DM-celecoxib was synthesized as described previously [5,15]. Isoprenaline hydrochloride (ISO) and SB216763 were purchased from Sigma-Aldrich (St. Louis, MO, USA). PTEN inhibitor VO-OHpic trihydrate was from Chem Scene (Monmouth Junction, NJ, USA). Monoclonal anti-GSK-3β and anti-β-catenin antibodies were purchased from BD Biosciences (San Jose, CA, USA). Monoclonal anti-Akt, polyclonal anti-phospho-Akt (Ser473), polyclonal anti-GSK-3α, polyclonal anti-phospho-GSK-3α (Ser21), polyclonal anti-phospho-GSK-3β (Ser9), polyclonal anti-mammalian target of rapamycin (mTOR), polyclonal anti-phospho-mTOR (Ser2481), polyclonal anti-matrix metalloproteinasr-2 (MMP-2), and polyclonal anti-phosphatase and tensin homolog on chromosome 10 (PTEN) antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Polyclonal anti-fibronectin antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Monoclonal anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody was purchased from Abcam (Cambridge, UK).

2.2. Animal model

C57BL/6J mice (8–10 weeks old, male) were obtained from Japan SLC Inc. (Hamamatsu, Japan). Isoprenaline (20 mg/kg/day) or vehicle was continuously administrated to the mice using a subcutaneously implanted osmotic pump (ALZET; Cupertino, California, USA, Model 1002). The mice received feed containing 1000 ppm of DM-celecoxib for 2 weeks from the day of the implantation. Control mice received feed that did not contain the drug. Heart rate and blood pressure were measured on days 7 and 14 after the implantation by the tail-cuff

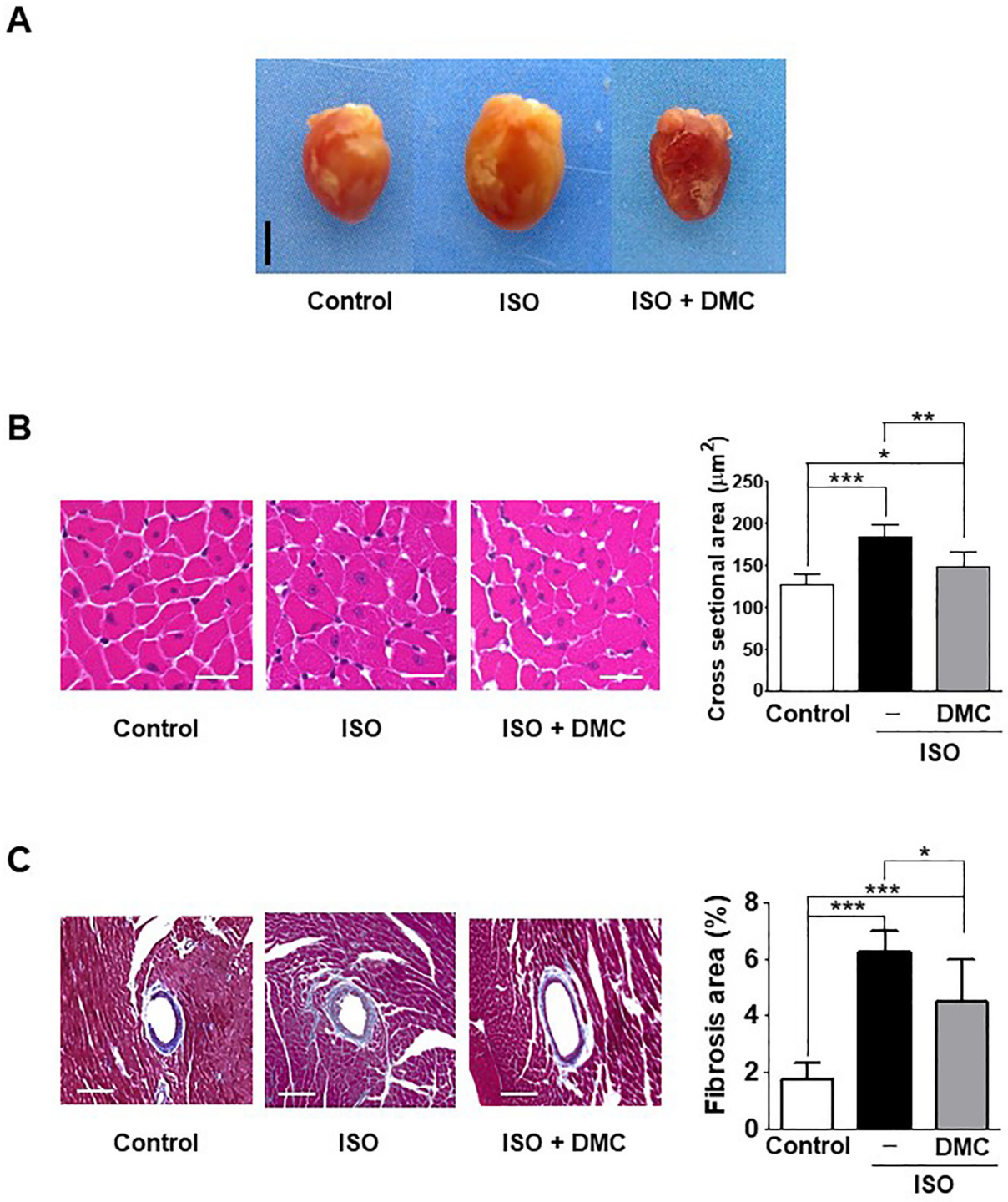

Fig. 1. DM-celecoxib alleviated cardiac hypertrophy and fibrosis induced by isoprenaline. Mice were administered isoprenaline or vehicle for 2 weeks and received feed containing DM-celecoxib (1000 ppm) or vehicle. Mice were euthanized and their hearts were removed for analysis. (A) Gross cardiac morphology. Scale bar, 3 mm. (B) Cardiomyocytes hypertrophy in the left ventricles (hematoxylin-eosin staining). Left, high-magnification views. Scale bar, 20 μm. Right, quantified cardiomyocyte cross-sectional areas. Values are the mean ± SD (control, n = 7; isoprenaline, n = 5; isoprenaline + DM-celecoxib, n = 6). (C) Fibrosis in the left ventricles (Masson trichrome staining). Left, high-magnification views. Scale bar, 100 μm. Right, quantified fibrotic areas in the left ventricle. Values are the mean ± SD (control, n = 7; isoprenaline, n = 5; isoprenaline + DM-celecoxib, n = 6). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. ISO, isoprenaline; DMC, DM-celecoxib.

method (BP-98A; Softron Co. Ltd., Tokyo, Japan), as previously reported [18]. Mice were euthanized on day 14 by cervical dislocation and their hearts and lungs were removed for analysis.

2.3. Echocardiography

On day 14 after the implantation, mice were anesthetized with 2.0% isoflurane, and their chest wall hair was removed with a hair remover and acoustic coupling gel was applied. Transthoracic ultrasound tomography (M-mode) was performed using a 14 MHz linear array probe

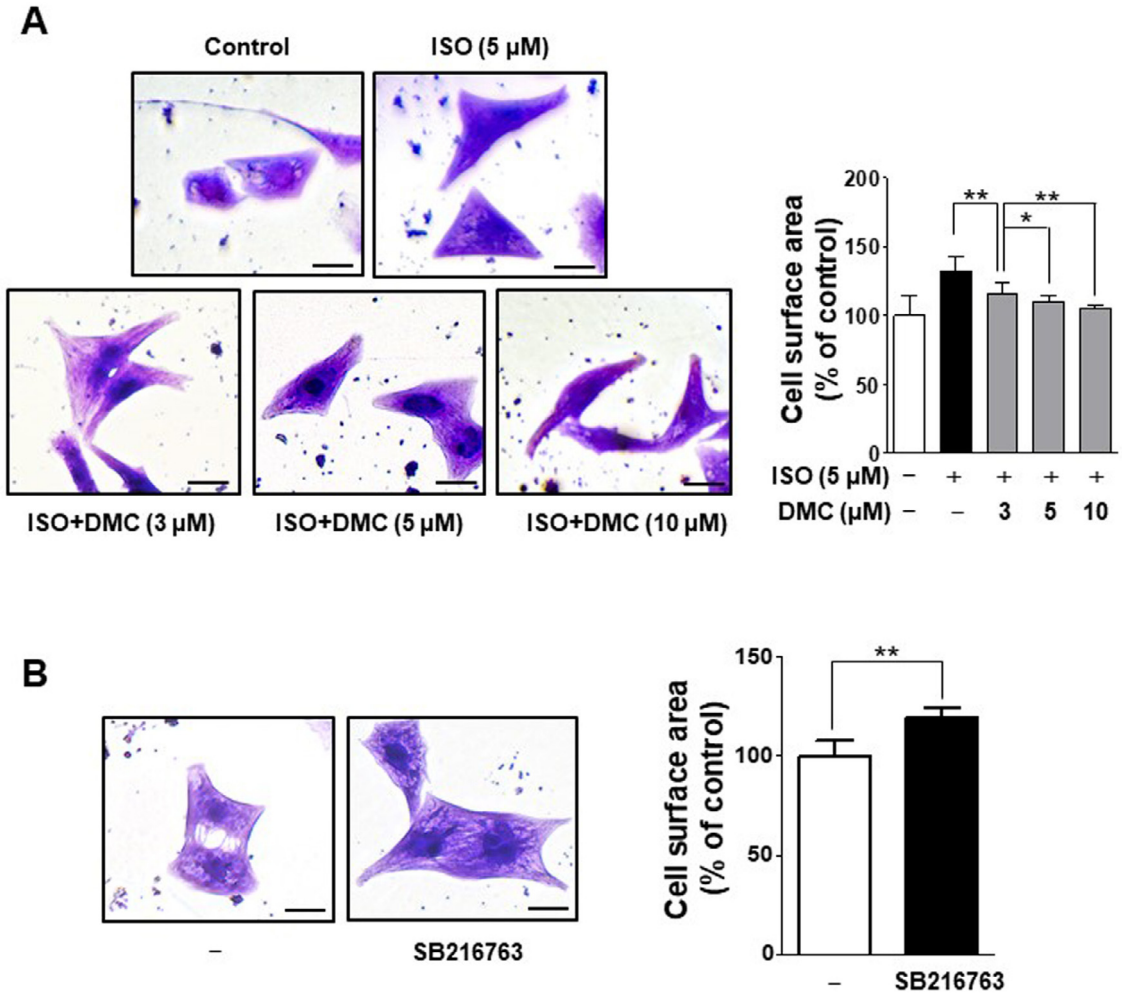

Fig. 2. DM-celecoxib alleviated isoprenaline-induced hypertrophy (A) Cell surface area measurements. Left, crystal violet staining of NRVCs. Scale bar, 25 μm. Right, relative cell surface area of NRVCs. Values are the mean ± SD (n = 4). (B) Effect of SB216763 on cell surface area. NRVCs were incubated with vehicle or SB216763 (20 μM) for 24 h. Left, crystal violet staining. Scale bar, 25 μm. Right, relative cell surface area. Values are the mean ± SD (n = 4). $^{*}P < 0.05$, $^{**}P < 0.01$. ISO, isoprenaline; DMC, DM-celecoxib. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

and a Nemio SSA-550A diagnostic ultrasound system (Toshiba, Tokyo, Japan).

2.4. Primary culture of neonatal rat ventricular cardiomyocytes

Primary neonatal rat ventricular cardiomyocytes (NRVCs) were isolated from the hearts of 1–2 days old Sprague-Dawley rats (SLC Inc.), as described previously with slight modifications [19]. Briefly, ventricles were cut into small pieces and digested five times with 0.1% collagenase type A (Roche Diagnostics; Indianapolis, IN, USA) in ADS buffer (pH 7.35, 20 mM Hepes-KOH, 116 mM NaCl, 5.4 mM KCl, 1 mM, NaH_2PO_4, 0.8 mM $MgSO_4$, and 5 mM glucose) at 37 °C for 6 min. The dispersed cells were washed once with ADS buffer and plated on 6-cm dishes. After 1 h incubation, unattached cardiac myocytes were collected and seeded on gelatin-coated 24-well plates (1×10^5 cells/well) for Western blotting and Akt activity analyses or 96-well plates (1×10^3 cells/well) for cell surface area measurements using Dulbecco's modified Eagle's medium (Wako Pure Chemical industries, Osaka, Japan) containing 10% fetal bovine serum (FBS; Sigma-Aldrich) and 1% penicillin-streptomycin (PS; 100 U/mL penicillin, 100 g/mL streptomycin) (Nacalai Tesque; Kyoto, Japan).

2.5. Primary culture of adult rat cardiac fibroblasts

Primary rat cardiac fibroblasts (RCFs) were isolated from the hearts of adult Sprague-Dawley rats (3–4 months old; Japan SLC Inc., Shizuoka, Japan) with the explant method, as described previously [20]. Briefly, the left ventricles were cut into small pieces and plated on 10-cm dishes. These small heart tissues were incubated in alpha-minimum essential medium (α-MEM; Nacalai Tesque) containing 10% FBS and 1% PS. Cardiac fibroblast obtained from the heart tissues (RCFs) were seeded on 24-well plates (5×10^4 cells/well) for Western blotting and Akt activity analyses or 96-well plates (3×10^3 cells/well) for cell proliferation assays.

2.6. Western blot analysis

The hearts were removed after the mice were euthanized and treated as described previously [21]. For the cultured-cell analysis, NRVCs or RCFs were pretreated with DM-celecoxib (3, 5, 10, and 20 μM) or vehicle for 1 h, and then stimulated with isoprenaline (5 μM) or vehicle for the indicated period.

The homogenized left ventricle samples or cell lysate samples were separated using 12% SDS-polyacrylamide gel electrophoresis. To separate high molecular-weight proteins, 7.5% tris-acetate gel electrophoresis was performed [22]. The samples were then transferred to

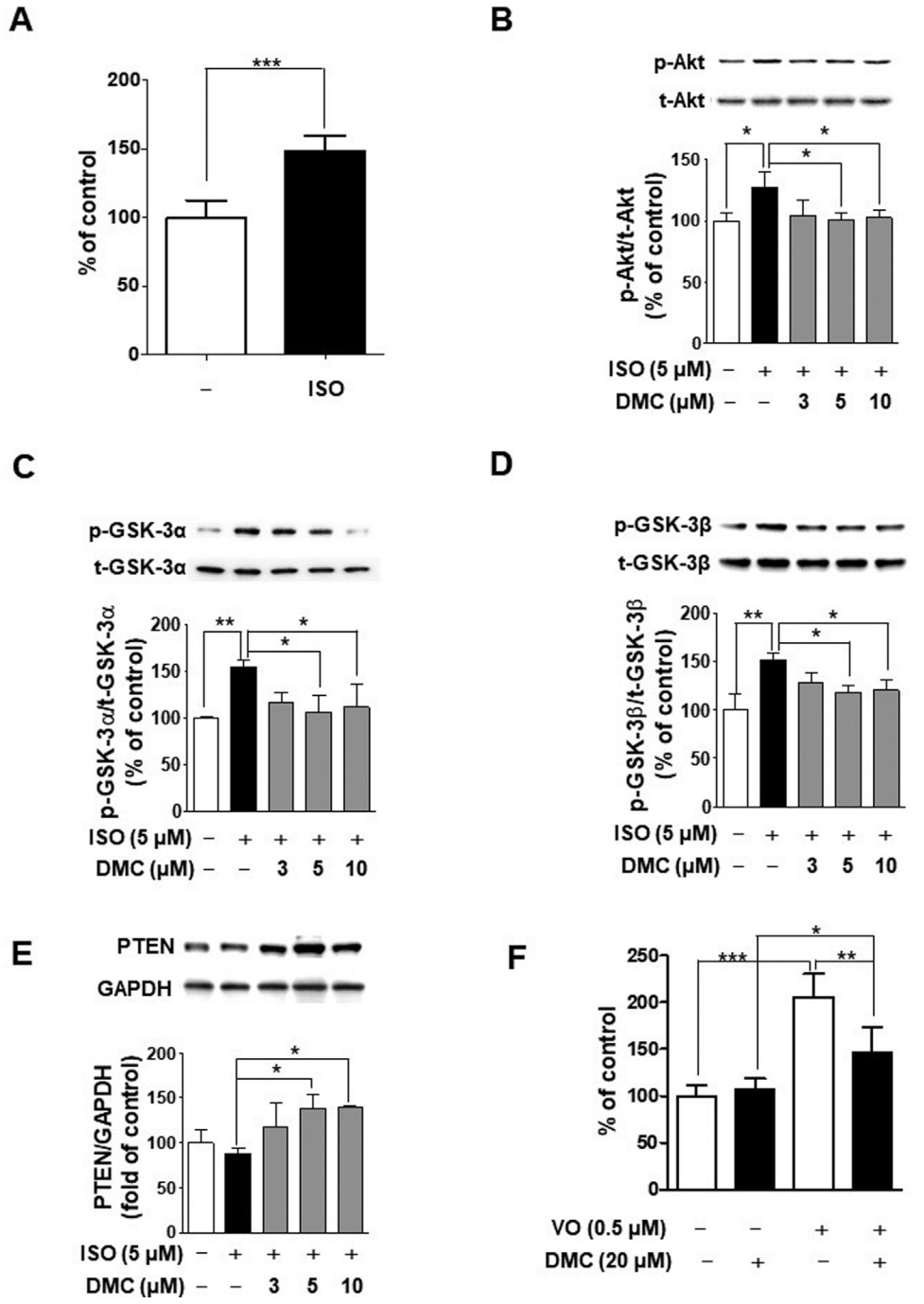

Fig. 3. DM-celecoxib modulated Akt/GSK-3 pathway in NRVCs. (A) Akt activity assay. The NRVCs were incubated with or without isoprenaline (5 μM) for 3 h. Values are the mean ± SD (n = 7). (B–E) Western blot analysis. After 1 h pretreatment of vehicle or DM-celecoxib, NRVCs were incubated with isoprenaline for 3 h. Protein samples were analyzed for (B) Akt, (C) GSK-3α, (D) GSK-3β, (E) PTEN. Quantified phosphorylated protein levels normalized to their total expression levels are expressed as percentages of the levels in control. The densities of the Western blots of total proteins normalized to those of GAPDH are shown as percentages of the level in control. Values are the mean ± SD (n = 3). (F) Akt activity assay. After 1 h pretreatment with vehicle or PTEN inhibitor VO-Ohpic (0.5 μM), NRVCs were stimulated with or without DM-celecoxib (10 μM) for 3 h. Values are the mean ± SD (n = 5–6). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. p-, phosphorylated; t-, total; ISO, isoprenaline; DMC, DM-celecoxib; VO, VO-OHpic.

polyvinylidene difluoride membranes using a semi-dry transfer system (Bio-Rad; Hercules, CA, USA). Western blot analysis was performed as described previously [23]. Optical densitometric scans were performed using the ImageJ software program (Ver. 1.49, National Institute of Health; Bethesda, MD, USA).

2.7. Histological analysis

The murine hearts were fixed in a neutral buffered 4% paraformaldehyde phosphate solution, cut transversely at the mid-ventricular level, and embedded in paraffin. The samples were then sectioned (thickness of 5 μm) and stained with hematoxylin-eosin or Masson trichrome, as described previously [21]. The extent of LV myocardial fibrosis was quantified using a BZ-X 700 and BZ-X Analyzer (KEYENCE, Osaka, Japan). One hundred cardiomyocytes in hematoxylin-eosin-stained samples from an LV cross section were randomly selected to measure the cardiomyocyte cross-sectional area using a BZ-X 700 and a BZ-X Analyzer, as described previously [24].

2.8. Determination of cell surface area in cardiomyocytes

After pretreatment with DM-celecoxib at the indicated concentrations for 1 h or with SB216763 (20 μM) for 3 h, NRVCs were stimulated with isoprenaline (5 μM) or vehicle for 24 h. NRVCs were fixed with 4% paraformaldehyde phosphate solution (Nacalai Tesque) and stained with 1% crystal violet (Wako Pure Chemical Industries; Osaka, Japan). The cross-sectional surface area of NRVCs was measured by a blinded investigator from microscopic images acquired from 10 or 5 random

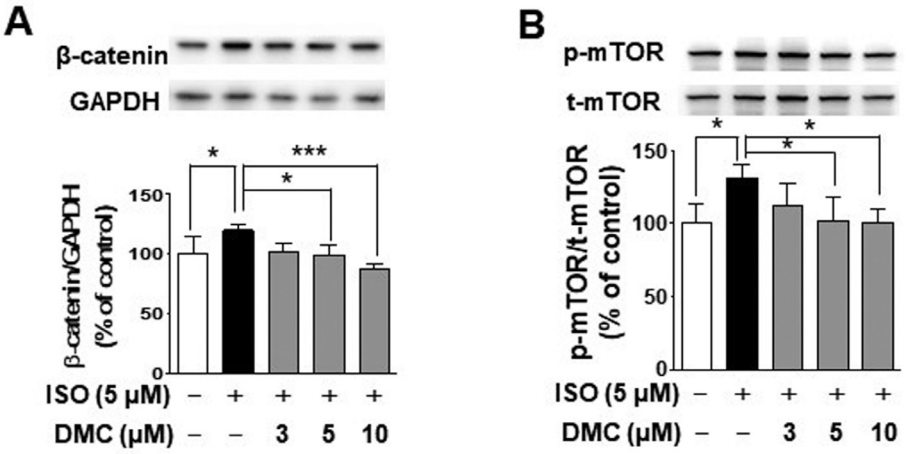

Fig. 4. DM-celecoxib suppressed Akt/GSK-3 pathway downstream molecules in NRVCs. Western blot analysis. After 1 h pretreatment of vehicle or DM-celecoxib, NRVCs were incubated with isoprenaline for 4 h. Protein samples were analyzed for (A) β-catenin and (B) mTOR. Quantified phosphorylated protein levels normalized to their total expression levels are expressed as percentages of the levels in control. The densities of the Western blots of total proteins normalized to those of GAPDH are shown as percentages of the level in control. Values are the mean ± SD (n = 4). $^{*}P < 0.05$, $^{***}P < 0.001$. p-, phosphorylated; t-, total; ISO, isoprenaline; DMC, DM-celecoxib.

fields (10 cells each) for DM-celecoxib treatment or SB216763 treatment, respectively, using a BZ-X 700 and a BZ-X Analyzer.

2.9. Akt kinase activity assay

Akt kinase activity was assessed using Akt kinase activity kit (Enzo Life Sciences, Farmingdale, NY, USA) according to the manufacturer's instructions. NRVCs were seeded on gelatin-coated 24-well plates and RCFs were seeded on non-coated 24-well plates. Twenty-four hours after seeding, NRVCs and RCFs were stimulated with isoprenaline (5 μM) or vehicle for 3 h. In the case of PTEN inhibitor pretreatment, NRVCs were treated with vehicle or VO-OHpic (0.5 μM) for 1 hr, then cells were stimulated with or without DM-celecoxib (10 μM) for 3 h. After PBS washing, cell lysates were collected by RIPA buffer (Wako Pure Chemical industries) containing protease inhibitor cocktail (Nacalai Tesque) and phosphatase inhibitor cocktail (Nacalai Tesque). The results were expressed as the percentage of the absorbance of control cells.

2.10. Cell proliferation assay

Cardiac fibroblasts proliferation was assessed using WST-8 (Nacalai Tesque, Kyoto, Japan) according to the manufacturer's instructions. Briefly, after pretreatment with DM-celecoxib at indicated concentrations for 1 h or SB216763 (10 μM) for 3 h, RCFs were stimulated with isoprenaline (5 μM) or vehicle for 72 h. The results were expressed as the percentage of the absorbance of control cells.

2.11. Statistics

Results are expressed as the mean ± SD. Mean values were compared using one-way analysis of variance with Tukey's post-hoc multiple comparison test. $P < 0.05$ was considered statistically significant.

2.12. Ethics

The study protocol was approved by the Ethics Committee of Animal Care and Experimentation at the University of Occupational and Environmental Health, Japan (approval protocol no. AE17-26) and Kyushu University (approval protocol no. A29-383-0). All animal handling and procedures were performed in compliance with the Institutional Guidelines for Animal Experiments and the Law (no. 105) and Notification (no. 6) of the Japanese Government and the NIH guidelines (Guide for the Care and Use of Laboratory Animals). All surgeries were performed under inhaled sevoflurane anesthesia and all efforts were made to minimize suffering.

3. Results

3.1. Effects of DM-celecoxib on isoprenaline-induced cardiac hypertrophy and fibrosis in vivo

First, we examined whether DM-celecoxib prevents cardiac remodeling in isoprenaline-induced cardiac hypertrophy. As shown in Table 1, the treatment with isoprenaline elevated heart rate and systolic blood pressure measured on days 7 and 14 in comparison with the treatment with vehicle, indicating that isoprenaline stimulated β-adrenergic receptor and thereby stimulated cardiac function. DM-celecoxib did not significantly influence isoprenaline actions on heart rate and blood pressure. Echocardiography showed that the treatment with isoprenaline significantly increased left ventricular (LV) wall thickness, LV ejection fraction (LVEF) and fractional shortening (FS) (Table 2). DM-celecoxib significantly attenuated the isoprenaline-induced thickening of LV wall, although it did not change LVEF or FS at this time point.

As shown in Table 2 and Fig. 1A, isoprenaline significantly increased the heart weight-to-tibia length ratio (HW/TL), the lung weight-to-tibia length ratio (LW/TL) and heart size. DM-celecoxib appeared to attenuate these effects by isoprenaline, although they were not statistically significant (HW/TL, $P = 0.106$; LW/TL, $P = 0.223$). DM-celecoxib prevented isoprenaline-induced cardiomyocyte hypertrophy and fibrous matrix deposition in the left ventricle (Fig. 1B and C).

These results suggested that DM-celecoxib prevented cardiac remodeling induced by β-adrenergic receptor stimulation as observed in previous studies that used other heart failure models [4,5].

3.2. Effect of DM-celecoxib on isoprenaline-induced cardiomyocyte hypertrophy in vitro

To examine whether DM-celecoxib directly acts on cardiomyocytes, we conducted *in-vitro* experiments using the primary culture of neonatal rat ventricular cardiomyocytes (NRVCs). As shown in Fig. 2A, DM-celecoxib significantly attenuated isoprenaline-induced hypertrophy of NRVCs. To confirm the role of GSK-3 in the regulation of cardiomyocyte hypertrophy, we examined the effect of SB216763, a GSK-3 inhibitor, on NRVCs. As shown in Fig. 2B, SB216763 significantly increased the cell surface areas, indicating that the inactivation of GSK-3 induced cardiomyocyte hypertrophy.

3.3. Effects of DM-celecoxib on the Akt-mediated signaling in cardiomyocytes

We previously reported that DM-celecoxib inhibited Akt, thereby

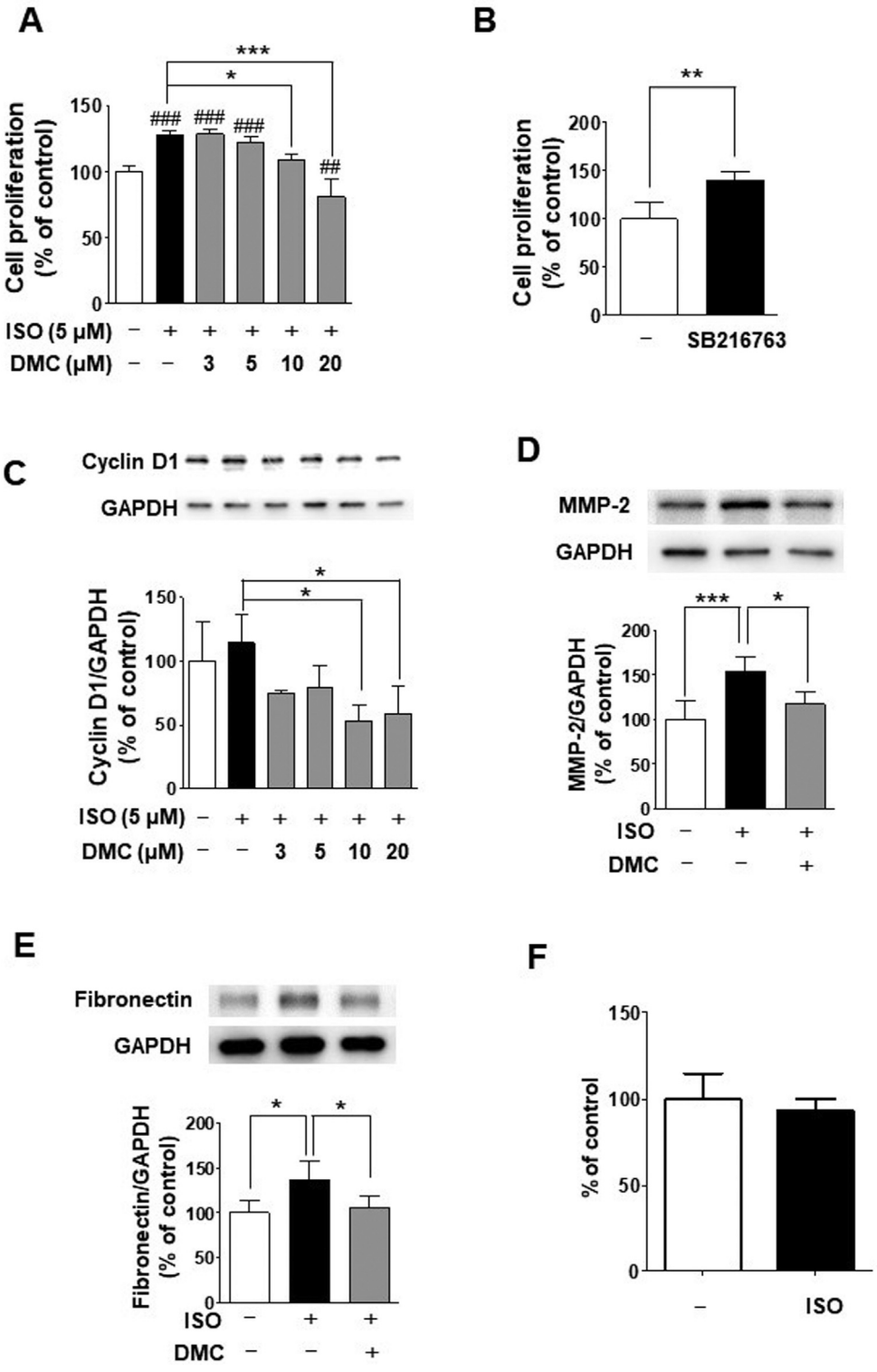

Fig. 5. DM-celecoxib suppressed proliferation and function of RCFs. (A) Cell proliferation assay. RCFs were incubated with ISO for 72 h after 1 h pretreatment with vehicle or DM-celecoxib. Values are the mean ± SD and expressed as percentage of control level. (n = 4–5). (B) Effect of SB216763 on cell proliferation. RCFs were incubated with vehicle or SB216763 (10 μM) for 72 h. Values are the mean ± SD and expressed as percentage of control level (n = 5). (C) Western blot analysis of cyclin D1. After 1 h pretreatment of vehicle or DM-celecoxib, RCFs were incubated with isoprenaline for 24 h. The densities of the Western blots of total proteins normalized to those of GAPDH are shown as percentages of the level in control. Values are the mean ± SD (n = 3). (D and E) Western blot analyses. After 1 h pretreatment with vehicle or DM-celecoxib (10 μM), RCFs were incubated with isoprenaline for 48 h. Protein samples were analyzed for (D) MMP-2, and (E) fibronectin. The densities of the Western blots of total proteins normalized to those of GAPDH are shown as percentages of the level in control. Values are the mean ± SD (n = 5). (F) Akt activity assay. The RCFs were incubated with or without isoprenaline (5 μM) for 3 h. Values are the mean ± SD (n = 6–7). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. p-, phosphorylated; t-, total; ISO, isoprenaline; DMC, DM-celecoxib.

activating GSK-3 in the hearts of dilated cardiomyopathy and pressure overload mouse models [4,5]. We first examined the effect of isoprenaline on Akt and found that it increased Akt activity in cardiomyocytes (Fig. 3A). As shown in Fig. 3B–D, isoplenaline elevated the phosphorylation levels of Akt, GSK-3α and GSK-3β, indicating that isoplenaline inhibited GSK-3 by the activation of Akt. However, DM-celecoxib suppressed the phosphorylation levels of these kinases, thereby activating GSK-3. Since celecoxib has been reported to inactivate Akt by upregulating the expression levels of PTEN in various cancer cell lines [25–27], we examined the effect of DM-celecoxib on this phosphatase. As shown in Fig. 3E, DM-celecoxib significantly increased the expression levels of PTEN, indicating that DM-celecoxib could inhibit Akt by increasing the amount of PTEN. Further, to clarify the involvement of PTEN in DM-celecoxib action, PTEN inhibitor VO-OHpic was employed. The PTEN inhibitor significantly increased Akt activity. DM-celecoxib alone did not show significant effect on Akt activity and this compound could reduce Akt activity elevated by PTEN inhibitor, although it did not reached to DM-celecoxib alone level

(Fig. 3F). These results indicated that PTEN inhibitor at least partially blocked an effect of DM-celecoxib on Akt.

β-catenin promotes cardiac hypertrophy and fibrosis [28] and its degradation by the ubiquitin system is accelerated by GSK-3. As shown in Fig. 4A, DM-celecoxib reduced the expression levels of β-catenin that had been elevated by isoprenaline. mTOR is activated by Akt and promotes cardiac hypertrophy [6,29]. Fig. 4B shows that DM-celecoxib reduced the phosphorylation levels of mTOR that had been elevated by isoprenaline.

These results suggested that DM-celecoxib seemed to activate GSK-3 by inhibiting Akt, thereby preventing isoprenaline-induced cardiomyocyte hypertrophy and that PTEN could mediate DM-celecoxib-induced suppression of Akt activity.

3.4. Effects of DM-celecoxib on isoprenaline-induced cardiac fibroblast activation

Proliferation of cardiac fibroblasts is a crucial event in the pathogenesis of cardiac fibrosis [1]. We and others reported that DM-celecoxib inhibited cell proliferation in several cell species [30–32]. Therefore, we examined the effect of DM-celecoxib on cardiac fibroblast proliferation using the primary culture of adult rat cardiac fibroblasts (RCFs). As shown in Fig. 5A, isoprenaline significantly accelerated RCF proliferation; however, DM-celecoxib attenuated this effect. Then, we examined the effect of SB216763, a GSK-3 inhibitor, on the proliferation of RCFs to determine the involvement of GSK-3. As shown in Fig. 5B, SB216763 promoted cell proliferation suggesting that the inactivation of GSK-3 stimulated RCF proliferation.

GSK-3 is deeply involved in the regulation of cell proliferation [33]; therefore, we measured the expression levels of cyclin D1, which is regulated by GSK-3 in the mRNA and protein levels [33]. As shown in Fig. 5C, DM-celecoxib significantly decreased the expression levels of cyclin D1, although the increase of cyclin D1 expression levels by isoprenaline was not statistically significant. These results indicated that DM-celecoxib suppressed RCF proliferation by suppressing cyclin D1 expression via activation of GSK-3.

Subsequently, we measured the expression levels of MMP-2 and fibronectin, which are implicated in extracellular matrix (ECM) deposition [34,35], to evaluate the effect of DM-celecoxib on fibrosis. The increases of fibronectin and MMP-2 induced by isoprenaline were reduced by the treatment with DM-celecoxib (Fig. 5D and E). Therefore, DM-celecoxib seemed to suppress cardiac fibroblast proliferation and ECM deposition. Although we examined the effect of isoprenaline on Akt activity in RCFs, it did not show significant effect (Fig. 5F).

4. Discussion

We previously reported that DM-celecoxib prevented cardiac hypertrophy and fibrosis induced by sarcomeric gene mutation and pressure overload in mice [4,5]. However, the direct effect of DM-celecoxib on cardiomyocytes and cardiac fibroblasts was not examined in those studies. In the present study, therefore, we conducted an *in-vitro* study and showed that DM-celecoxib directly prevented cardiomyocyte hypertrophy and cardiac fibroblast activation. Particularly in cardiomyocytes, we found that DM-celecoxib inhibited Akt-mediated signaling pathway and PTEN could be a candidate molecule to mediate the effect of DM-celecoxib on Akt. As inhibition of Akt resulted in GSK3 activation, our finding was in accordance with a previous study showing that the constitutive activation of GSK-3 protected the heart from remodeling induced by chronic β-adrenergic stimulation [12].

We used the β-adrenergic receptor agonist isoprenaline as a hypertrophic stimulus both for *in-vivo* and *in-vitro* studies. Isoprenaline non-selectively stimulates the G-protein coupled β-adrenergic receptors in cardiomyocytes and cardiac fibroblasts [19,36,37]. The human heart expresses β_1- and β_2-adrenergic receptors at a ratio of about 7:3 and both receptors increase heart rate and contractility. Although both receptors are coupled to Gs and thereby increase intracellular cAMP (classical signaling pathway), only β_2-adrenergic receptor is coupled to Gi and activates phosphodiesterase 2, mitogen-activated protein kinase (MAPK) and phosphoinositide-3-kinase (PI3K) (non-classical signaling pathways) [38]. Since stimulation of β_2-adrenergic receptor activates the PI3K/Akt signaling pathway and induces cardiomyocyte hypertrophy [39,40], DM-celecoxib may suppress cardiomyocyte hypertrophy by inactivating Akt. Isoprenaline also activates the cAMP/PKA pathway, MAPK pathway and Ca^{2+}-calcineurin pathway [36,40]. It has been shown that through these signaling pathways, transcriptional factors, such as nuclear factor of activated T-cell cytoplasmic 3 (NFATc3) and GATA4, are activated and translocate into the nucleus to stimulate transcription of several genes involved in cardiac hypertrophy [8,40]. GSK-3 directly inactivates NFATc3 and GATA4 by phosphorylating them [41]. Therefore, DM-celecoxib may also be able to suppress these hypertrophic signals evoked by isoprenaline by activating GSK-3.

Cardiac fibrosis results from complex pathological processes including a phenotypic change and excessive proliferation of cardiac fibroblasts and excessive deposition of extracellular matrix proteins. We reported previously that DM-celecoxib reduced proteins suggested to be involved in interstitial fibrosis, including fibronectin and MMP-2 [4], which promote cell attachment and collagen deposition [42,43]. Here, we showed that DM-celecoxib not only suppressed the expression of interstitial fibrosis related proteins but also directly suppressed cardiac fibroblast proliferation by the suppression of cyclin D1 expression. Cyclin D1 acts as a mitogenic signal sensor that is quickly expressed in response to many mitogenic signals for cell cycle progression through the activation of PI3K/Akt signaling pathway [33,44]. In cardiac fibroblasts, isoprenaline also has been shown to activate PI3K/Akt signaling pathway via β_2-adrenergic receptor and to stimulate fibroblast proliferation [45–47]. However we could not detect the activation of Akt by isoprenaline in RCFs. Therefore, further studies were required to clarify the mechanisms by which DM-celecoxib suppressed cardiac fibroblasts activation.

We and others have reported DM-celecoxib inhibits Akt [4,5,13–15], whereas the mechanisms are still not clarified. The tumor suppressor PTEN is a lipid phosphatase that dephosphorylates the D3 position of PIP_3 [48] and antagonizes PI3K mediated cellular signaling. It has been reported that the reduced PTEN level is associated with an elevated phosphorylation of Akt [49]. Indeed, we found that DM-celecoxib upregulated the expression of PTEN, whereas isoprenaline tended to decrease it. Since celecoxib has been shown to upregulate the expression levels of PTEN through transcription factor Sp1 in HeLa and SACC-83 cells [26], DM-celecoxib may act in the same way. Although further studies are required, PTEN could be a candidate molecule as a mediator of the effect of DM-celecoxib on Akt.

Results of the present study suggest that DM-celecoxib prevented β-adrenergic stimulation-induced cardiac remodeling by directly inhibiting cardiomyocyte hypertrophy and cardiac fibroblasts activation. Therefore, this compound could be a promising candidate as a new drug or a lead compound to develop a new drug to prevent cardiac remodeling and heart failure. However, there are two points that must be clarified in future studies. First, the mechanisms by which DM-celecoxib upregulate PTEN and suppress cardiac fibroblasts activation should be clarified. Second, we examined NRVCs and RCFs independently; however, interaction between these two cell species might exist. In fact, a previous study showed that paracrine factors, such as growth factors or cytokines, released from cardiomyocytes induced the proliferation of cardiac fibroblasts [19]. Therefore, examining the effect of DM-celecoxib on the interaction between the two cell species could provide further insight into the mechanism underlying the effect of DM-celecoxib on cardiac remodeling.

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Declaration of Competing Interest

The authors have no conflict of interest to declare.

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