Microsomal Prostaglandin E₂ Synthase-1 Deletion Attenuates Isoproterenol-Induced Myocardial Fibrosis in Mice^[S]

Shuang Ji,¹ Rui Guo,¹ Jing Wang, Lei Qian, Min Liu, Hu Xu, Jiayang Zhang, Youfei Guan, Guangrui Yang, and Lihong Chen

Advanced Institute for Medical Sciences, Dalian Medical University, China (S.J., R.G., J.W., L.Q., M.L., H.X., J.Z., Y.G., L.C.) and School of Bioengineering, Dalian University of Technology, China (G.Y.)

Received March 23, 2020; accepted July 10, 2020

ABSTRACT

Deletion of microsomal prostaglandin E₂ synthase-1 (mPGES-1) inhibits inflammation and protects against atherosclerotic vascular diseases but displayed variable influence on pathologic cardiac remodeling. Overactivation of β -adrenergic receptors (β -ARs) causes heart dysfunction and cardiac remodeling, whereas the role of mPGES-1 in β -AR-induced cardiac remodeling is unknown. Here we addressed this question using mPGES-1 knockout mice, subjecting them to isoproterenol, a synthetic nonselective agonist for β -ARs, at 5 or 15 mg/kg per day to induce different degrees of cardiac remodeling in vivo. Cardiac structure and function were assessed by echocardiography 24 hours after the last of seven consecutive daily injections of isoproterenol, and cardiac fibrosis was examined by Masson trichrome stain in morphology and by real-time polymerase chain reaction for the expression of fibrosis-related genes. The results showed that deletion of mPGES-1 had no significant effect on isoproterenol-induced cardiac dysfunction or hypertrophy. However, the cardiac fibrosis was dramatically attenuated in the mPGES-1 knockout mice after either low-dose or high-dose isoproterenol exposure. Furthermore, in vitro study revealed that overexpression of mPGES-1 in cultured cardiac

fibroblasts increased isoproterenol-induced fibrosis, whereas knocking down mPGES-1 in cardiac myocytes decreased the fibrogenesis of fibroblasts. In conclusion, mPGES-1 deletion protects against isoproterenol-induced cardiac fibrosis in mice, and targeting mPGES-1 may represent a novel strategy to attenuate pathologic cardiac fibrosis, induced by β -AR agonists.

SIGNIFICANCE STATEMENT

Inhibitors of microsomal prostaglandin E₂ synthase-1 (mPGES-1) are being developed as alternative analgesics that are less likely to elicit cardiovascular hazards than cyclooxygenase-2 selective nonsteroidal anti-inflammatory drugs. We have demonstrated that deletion of mPGES-1 protects inflammatory vascular diseases and promotes post-myocardial infarction survival. The role of mPGES-1 in β -adrenergic receptor-induced cardiomyopathy is unknown. Here we illustrated that deletion of mPGES-1 alleviated isoproterenol-induced cardiac fibrosis without deteriorating cardiac dysfunction. These results illustrated that targeting mPGES-1 may represent an efficacious approach to the treatment of inflammatory cardiovascular diseases.

Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs) are among the most widely used drugs in the world. Based on the inhibition of the production of inflammatory prostaglandin E₂ (PGE₂), traditional NSAIDs and cyclooxygenase (COX)-2 selective inhibitors have become the best choice of antipyretic and analgesic drugs (Chenet al., 2013a). However, studies have increasingly shown that long-term use of such drugs is accompanied by significant cardiovascular side effects, such as

hypertension, stroke, myocardial infarction, and so on (Grosser et al., 2017). There has been intense interest in developing new NSAIDs that might preserve the anti-inflammatory efficacy while limiting the cardiovascular risks.

Microsomal PGE₂ synthase-1 (mPGES-1), the inducible PGE₂ terminal synthase, which is usually coupled with COX-2 to mediate the production of PGE₂ in inflammatory states (Thoren and Jakobsson, 2000; Uematsuet al., 2002; Deng et al., 2019), has gained considerable attention as a preferable target for new generation of antipyretic and analgesic drugs (Yang and Chen, 2016; Bergqvist et al., 2020). It has been reported that, unlike COX-2 selective inhibitors, deletion of mPGES-1 in mice is protective to inflammatory vascular diseases; for example, it retards atherogenesis (Wang et al., 2006), suppresses abdominal aortic aneurysm formation (Wang et al., 2008a), and limits postinjury neointima hyperplasia

This research was funded by the National Natural Science Foundation of China (81670242, 81570643 and 31900142) and research awards from the National 1000-Talent Plan of China and Liaoning BaiQianWan Talents Program.

¹S.J. and R.G. contributed equally to this work.

<https://doi.org/10.1124/jpet.120.000023>

[S] This article has supplemental material available at jpet.aspetjournals.org.

ABBREVIATIONS: β -AR, β -adrenergic receptor; ANF, atrial natriuretic factor; BNP, brain natriuretic peptide; COX, cyclooxygenase; CTGF, connective tissue growth factor; EF, ejection fraction; FS, fractional shortening; IL, interleukin; ISO, isoproterenol; KO, knockout; LV, left ventricular; MCP-1, monocyte chemoattractant protein 1; mPGES1, microsomal PGE₂ synthase-1; NSAID, nonsteroidal anti-inflammatory drug; PCR, polymerase chain reaction; PGE₂, prostaglandin E₂; PGF₂ α , prostaglandin F₂ α ; PGI₂, prostaglandin I₂; siRNA, small interfering RNA; TNF- α , tumor necrosis factor α ; VEGF, vascular endothelial growth factor; WT, wild type.

(Wang et al., 2011). However, the role of mPGES-1 in pathologic cardiac remodeling and heart dysfunction is still in debate. Although studies had reported impaired postischemic heart function and cardiac remodeling in mPGES-1-deficient mice (Degousee et al., 2008; Zhu et al., 2019), we and others failed to observe any adverse influence on cardiac remodeling after mPGES-1 was deleted globally or selectively in myeloid cells (Wu et al., 2009b; Chen et al., 2019). Moreover, in a model of angiotensin II-mediated cardiac remodeling, although lack of mPGES-1 did not affect cardiac hypertrophy and fibrosis, nevertheless poor cardiac function was observed (Harding et al., 2011). These paradoxes suggest a complex role of mPGES-1 in cardiac repair, and more work is required to refine the role of mPGES-1 in mediating pathologic myocardial remodeling and heart dysfunction.

In the present study, by using an isoproterenol (ISO)-induced cardiac remodeling model to simulate the hyperactivation of β -adrenergic receptor (β -AR) under acute stress conditions, we found that, although deletion of mPGES-1 fails to improve ISO-induced cardiac hypertrophy and heart dysfunction, lack of mPGES-1 is protective to ISO-induced cardiac fibrosis. Although clinical application of β -blockers can eliminate the cardiac injury and its adverse consequences caused by sympathetic nervous system overactivation to a certain extent, since β -AR is essential to many physiologic cardiac function and metabolism as well, application of β -blockers is also associated with some adverse effects (Everly et al., 2004). Therefore, it is necessary to further explore the molecular mechanism of β -AR overactivation mediated pathologic cardiac remodeling so as to provide novel therapeutic approaches. Our results demonstrated that targeting mPGES-1 might be a novel approach to prevent deleterious cardiac fibrosis induced by β -ARs agonists.

Materials and Methods

Mice and Isoproterenol Treatment. mPGES-1 knockout mice in the C57BL/6 background were created as previously described (Wang et al., 2006). Male mPGES-1 knockout (KO) mice and their wild-type (WT) littermates at the age of 3 to 4 months were maintained under a 12:12-hour light/dark cycle and were subjected to isoproterenol (sigma) or saline subcutaneously injection at 5 or

15 mg/kg per day for seven consecutive days. The mice were euthanized 24 hours after the last injection of isoproterenol, and the hearts were removed and prepared for further analyses. All procedures were in accordance with the guidelines approved by the Dalian Medical University Animal Care and Use Committee. Female mice were used in the current study, which represents a limitation.

Echocardiography Measurements. Echocardiography was performed to evaluate cardiac systolic and diastolic function of WT and mPGES-1 KO mice before and 24 hours after the last ISO injection. Mice were anesthetized with 1% isoflurane. The heart rate was stabilized at 400–500 beats per minute. The Vevo 3100 high-resolution imaging system (Fujifilm VisualSonics Inc., Tokyo, Japan) was used to measure the cardiac function on a short axis view, and three frames were analyzed for each animal. The percentage of ejection fraction (EF%), fractional shortening (FS%), left ventricular wall thickness, left ventricular mass, left ventricular internal dimension, and interventricular septum were identified and calculated as described in previous studies (Collins et al., 2003; Gao et al., 2011; Xiao et al., 2018).

Histology and Morphometry. Mice were euthanized and heart tissues were harvested and fixed in 4% paraformaldehyde. Then the tissues were embedded in paraffin and sectioned at 5- μ m intervals. Masson trichrome staining was performed according to standard procedures. For immunohistochemistry, heart sections were stained with antibodies against macrophage marker Mac-2 (Santa Cruz, Dallas, TX) and T lymphocyte marker CD3 (GeneTex, Irvine, CA). Tissue morphometric features were evaluated by PerkinElmer Mantra tissue imaging analysis system (PerkinElmer Inc., Waltham, MA).

Enzyme-Linked Immunosorbent Assay. Plasma were harvested for measurement of the levels of PGE₂, prostaglandin I₂ (PGI₂), and prostaglandin F_{2 α} (PGF_{2 α}) by validated enzyme-linked immunosorbent assay according to manufacturer instructions (Elabscience, Wuhan, China).

Real-Time Polymerase Chain Reaction. Total RNA was extracted from cardiac cells or heart tissues using Trizol reagent and reverse transcribed according to the manufacturer's protocol. Quantitation polymerase chain reaction (PCR) was performed using SYBR Green to detect PCR products in real time with LightCycler96 Sequence Detection System (Roche, Basel, Switzerland). All mRNA measurements were normalized to 18S or actin levels. The primers are shown in Table 1.

Cell Culture. We used neonatal Sprague-Dawley rats, both genders, for the in vitro cell culture experiments. Cardiac fibroblasts and myocytes were isolated and cultured as described previously (Nuamnaichati et al., 2018). In brief, 1- or 2-day-old neonatal Sprague-

TABLE 1
Primers for quantitative real-time PCR

PCR production	Forward primer	Reverse primer
Mouse ANF	CACAGATCTGTGGTTTCAAGA	CCTCATCTTCTACCGGCATC
Mouse BNP	GAAGGTGCTGTCCCAGATGA	CCAGCAGCTGCATCTTGAAT
Mouse collagen I	GAGCGGAGAGTACTGGATCG	GTTCCGGGCTGATGTACCAGT
Mouse collagen III	TCCCCTGGAATCTGTGAATC	TGAGTCGAATTGGGGAGAAT
Mouse fibronectin	AAGGTTCCGGAAGAGGTTGT	GAGCTTAAAGCCAGCGTCAG
Mouse IL-1 β	CTTCCCCAGGGCATGTTAAG	ACCCTGAGCGACCTGTCTTG
Mouse IL-6	GCTACCAAACCTGGATATAATCAGGA	CCAGGTAGCTATGGTACTCCAGAA
Mouse MCP-1	CCTGGATCGGAACCAATGA	ACCTTAGGGCAGATGCAGTTTTA
Mouse TNF- α	ATGGCCTCCCTCTCATCAGT	CTTGGTGGTTTGCTACGACG
Mouse 18S	GAAACGGCTACCACATCCAAGG	GCCCTCCAATGGATCCTCGTTA
Mouse β -actin	GATCTGGCACCACACCTTCT	GGGGTGTGTAAGGTCTCAAA
Rat collagen I	GAGACAGGCGAACAAGGTGA	GGGAGACCGTTGAGTCCATC
Rat collagen III	AGAGGCTTTGATGGACGCAA	GGTCAACCTCACCCTTAGC
Rat fibronectin	CGTGAGATATGTGGTTAGTGCT	CTCAGGGCTTGAGTAGGTCA
Rat mPGES-1	TGTGAGGACCACGAGGAAATG	CGCAACGACATGGAGACGAT
Rat β -actin	TCCTAGCACCATGAAGATC	AAACGCAGCTCAGTAACAG
Rat CTGF	GGCAGGGCCAACCACTGTGC	CAGTGCACTTGCCTGGATGG
Rat VEGF	TGCCAAGTGGTCCAG	CGCACACCGCATTAGG

Fig. 1. Effect of mPGES-1 deletion on isoproterenol-induced cardiac dysfunction and hypertrophy. (A) Echocardiography of ejection fraction (EF%), fractional shortening (FS%), and heart rate (HR) in beats per minute (BPM) of mice after seven consecutive days of injection of ISO at 5 mg/kg per day (scale bar, 1 mm; vehicle $n = 7-9$; ISO $n = 13-14$). LVID;d, end-diastolic left ventricular internal dimension; LVID;s, systolic left ventricular internal dimension; LVPW;d, end-diastolic left ventricular posterior wall thickness; LVPW;s, systolic left ventricular posterior wall thickness. (B) Echocardiography of end-diastolic left ventricular posterior wall thickness (LVPW;d) and left ventricular mass (vehicle $n = 7-9$; ISO $n = 13-14$). (C) Quantitative real-time PCR analysis of ANF and BNP mRNA levels in the hearts (vehicle $n = 5$; ISO $n = 9$). (D) Heart weight to body weight (HW/BW) ratio (vehicle $n = 7-9$; ISO $n = 13-14$). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Data are represented as means $\pm$ S.D. The P values were obtained by one-way ANOVA plus a post hoc analysis using a Bonferroni test.

Dawley rats were euthanized, and the hearts were digested with collagenase I. Cells were pelleted and plated on 10-cm dishes and cultured in Dulbecco's modified Eagle's medium supplemented with 10% FBS plus 1% penicillin and streptomycin. Unattached cardiac myocytes were removed to a new dish 3 to 4 hours later, and cardiac fibroblasts were attached to the bottoms of the dish. Cells at passage one were used for the experiment and analysis. Both cardiac myocytes and fibroblasts were changed to serum-free Dulbecco's modified Eagle's medium for 12 hours before isoproterenol stimulation or other treatment.

For the conditioned medium collection, primary cultured neonatal rat cardiac myocytes were treated with or without 20 μ M ISO for 12 hours, and the supernatant were collected as conditioned medium.

TABLE 2

Echocardiographic analysis of cardiac function in mice with low-dose isoproterenol (5 mg/kg per day) treatment of seven consecutive days. Data are means $\pm$ S.D.

Genotype	Vehicle treatment		ISO treatment	
	WT ($n = 9$) mm	KO ($n = 7$) mm	WT ($n = 13$) mm	KO ($n = 14$) mm
LVID;d	4.18 $\pm$ 0.17	4.14 $\pm$ 0.26	4.31 $\pm$ 0.31	4.30 $\pm$ 0.30
LVID;s	2.86 $\pm$ 0.22	2.77 $\pm$ 0.39	3.17 $\pm$ 0.40*	3.05 $\pm$ 0.40
LVPW;s	1.34 $\pm$ 0.17	1.24 $\pm$ 0.09	1.23 $\pm$ 0.18	1.41 $\pm$ 0.19 [#]
IVS;d	0.92 $\pm$ 0.13	0.95 $\pm$ 0.08	0.99 $\pm$ 0.16	0.98 $\pm$ 0.11
IVS;s	1.43 $\pm$ 0.20	1.50 $\pm$ 0.13	1.43 $\pm$ 0.21	1.44 $\pm$ 0.19

IVS;d, end-diastolic interventricular septum; IVS;s, systolic interventricular septum; LVID;d, end-diastolic left ventricular internal dimension; LVID;s, systolic left ventricular internal dimension; LVPW;d, end-diastolic left ventricular posterior wall thickness; LVPW;s, systolic left ventricular posterior wall thickness.

* $P < 0.05$, compared with vehicle group.

[#] $P < 0.05$, compared with WT group (one-way ANOVA plus a post hoc analysis using a Bonferroni test).

Basically, 2 ml of conditioned medium was collected from 1×10^6 cardiac myocytes.

RNA Interference. Primary cultured neonatal rat cardiac myocytes were transfected with Opti-MEM and Lipofectamine 3000 (Invitrogen, Waltham, MA), along with double stranded small interfering RNA (siRNA) (80–150 nM) according to the manufacturer's protocol to validate the knockdown effect. The siRNA sequence targeting rat mPGES-1 is sense (5'-CACUGCUGGUCAAGAUU-3') and antisense (5'-AUCUUGAUGACCAGCAGUGTT-3'). For the conditioned medium experiment, cells were pretreated with 20 μ M ISO and 100 nM siRNA, and the conditioned medium was collected and applied to the fibroblasts for 24 hours.

Adenovirus Overexpression. Adenoviruses carrying full-length rat mPGES-1 were purchased from Vigene Biosciences (Shandong, China), and rat GFP adenoviruses were used as a negative control. After 24–48 hours, the infection efficiency was determined by real-time PCR and Western blot. Sham infection was not used in the current study, which might represent a limitation.

Statistical Analysis. In all cases, quantitative results were expressed as means $\pm$ S.D. Student's t test were used to analyze the statistical differences between two groups; one-way ANOVA plus a post hoc analysis using Tukey's or a Bonferroni test were used to analyze the statistical differences among multiple groups. All data were analyzed using GraphPad Prism (version 8.0; GraphPad Software, La Jolla, CA). $P < 0.05$ was considered statistically significant.

Results

mPGES-1 Deletion Does Not Affect ISO-Induced Cardiac Hypertrophy. To investigate the effect of mPGES-1 deletion on ISO-induced cardiac remodeling,

Fig. 2. Effect of mPGES-1 deletion on low-dose isoproterenol-induced cardiac fibrosis. (A) Masson's trichrome staining of myocardial fibrosis from mice after seven consecutive days of ISO injection at 5 mg/kg per day (vehicle $n = 7-9$; ISO $n = 13$ to 14). (B) Real-time PCR analysis of the mRNA expression levels of collagen I, collagen III, and fibronectin in the heart tissues (vehicle $n = 3-5$; ISO $n = 6$ to 7). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Data are represented as means $\pm$ S.D. The P values were obtained by one-way ANOVA plus a post hoc analysis using a Bonferroni test.

mPGES-1 KO and WT mice were initially subjected to isoproterenol at a relatively low dose, 5 mg/kg per day, and the cardiac structure and function were assessed by echocardiography before and after seven consecutive days of ISO injection. As shown, although the cardiac function (reflected by ejection fraction and fractional shortness) was not altered by low-dose ISO exposure (Fig. 1A), obvious cardiac hypertrophy [reflected by increased end-diastolic left ventricular posterior wall thickness and left ventricular (LV) mass] was clearly observed in both WT and KO groups (Fig. 1B). However, no obvious difference was observed between the two genotypes (Fig. 1B) Table 2. Similarly, lack of mPGES-1 did not alter the expression of cardiac hypertrophic marker atrial natriuretic factor (ANF), brain natriuretic peptide (BNP) (Fig. 1C), and the mouse heart weight (Fig. 1D).

mPGES-1 Deletion Protects ISO-Induced Cardiac Fibrosis. Fibrosis is the most important feature of ISO-induced cardiac remodeling (Wang et al., 2019). Although most studies have demonstrated obvious morphologic cardiac fibrosis with ISO treatment at 5 mg/kg per day for 7 days, we only observe marginal interstitial fibrosis by Masson's trichrome staining in either WT or mPGES-1 KO mice (Fig. 2A), which might reflect the resistance of C57/BL6 mice to ISO-induced cardiac fibrosis (Park et al., 2018). In any event, the expression of mRNA for types I and III collagen, the main components of extracellular

matrix for cardiac fibrosis, as well as other fibrosis-related genes, such as fibronectin, were all significantly increased in WT mice (Fig. 2B), suggesting the occurrence of fibrosis in these mice. Importantly, the KO mice had significantly reduced expression of collagen I, collagen III, and fibronectin when compared with the WT mice (Fig. 2B), implying a protective effect of mPGES-1 deficiency on ISO-induced fibrosis.

Given the lack of morphologic fibrosis in the low-dose ISO models in our mice, we further treated the mice with ISO at a relatively high dose (15 mg/kg per day), which was reported to induce heart failure and worse cardiac fibrosis (Oudit et al., 2003). As expected, unlike low-dose ISO models, the Masson's trichrome staining did display markedly accumulated collagen deposition in heart sections from WT mice, and most importantly, dramatically attenuated cardiac collagen deposition was observed in the KO sections (Fig. 3A). Similarly, the expression of collagen I, collagen III, and fibronectin were significantly reduced in the KOs as well (Fig. 3B).

Previous studies have proved that the cardioprotective properties of mPGES-1 deletion on atherogenesis and vascular injury may reflect both the suppressed PGE₂ production and the augmented biosynthesis of PGI₂ (Wang et al., 2006, 2011). Similarly, here we detected a significant reduction of serum PGE₂ concentration in the mPGES-1 KO mice, and this suppression was also concomitant with increased PGI₂

Fig. 3. Effect of mPGES-1 deletion on high-dose isoproterenol-induced cardiac fibrosis. (A) Masson's trichrome staining of myocardial fibrosis from mice after seven consecutive days of ISO injection at 15 mg/kg per day (left). Quantification of fibrosis area (right, WT $n = 8$; KO $n = 8$). (B) Real-time PCR analysis of the mRNA expression of fibrotic genes in the hearts (WT $n = 9$; KO $n = 6$). (C) Plasma concentrations of PGE2, PGI2, and PGF2 α in mPGES-1 WT and KO mice 7 days after ISO treatment (WT $n = 9$; KO $n = 6$). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Data are represented as means $\pm$ S.D. The P values were obtained by two-tailed Student's t test.

production, whereas PGF2 α was unaltered (Fig. 3C). Thus, both the depression of PGE2 and the increase of PGI2 may synergistically contribute to the beneficial effect of mPGES-1 deficiency on ISO-induced cardiac fibrosis.

mPGES-1 Deletion Does Not Affect ISO-Induced Heart Dysfunction. In addition to severe cardiac fibrosis, high-dose ISO evoked clearly impaired cardiac function, reflected by significantly decreased ejection fraction and fractional shortening. However, despite the profound protective effect on cardiac fibrosis, mPGES-1 deficiency failed to improve ISO-induced cardiac dysfunction (Fig. 4A). Cardiac hypertrophy, reflected by end-diastolic left ventricular posterior wall thickness, LV mass (Table 3), and the heart weight to body weight ratio, was not affected by mPGES-1 deletion either (Fig. 4B).

Inflammation Was Not Affected by mPGES-1 Deletion. It has been established that activation of β -AR by ISO may lead to cardiac inflammation, which is known to contribute to cardiac fibrosis and lead to progressive impairment of cardiac function. To determine whether mPGES-1 deletion might influence ISO-induced inflammatory responses, we compared the cardiac expression of several proinflammatory cytokines. Although variable effects were observed for interleukin (IL)-1 β , IL-6, monocyte chemoattractant protein 1

(MCP-1), and tumor necrosis factor α (TNF- α) gene expression (Fig. 4C), the overall inflammatory response did not differ between the two groups. Immunohistochemistry staining of the macrophage marker Mac-2 and lymphocyte marker CD3 displayed unaltered inflammatory cell infiltration in the KO heart sections (Fig. 4D).

Knockdown of mPGES-1 Decreased Fibrosis. Cardiac fibroblasts play a critical role in ISO-induced cardiac fibrosis. To identify the mechanism by which mPGES-1 deletion protects ISO-induced cardiac fibrosis, we first examined the effects of mPGES-1 knockdown on primary cultured neonatal rat cardiac fibroblasts upon ISO stimulation. Surprisingly, ISO failed to directly increase fibrotic gene expression in fibroblasts (Fig. 5A). However, when we applied the conditioned medium from ISO-pretreated neonatal rat cardiac myocytes to the fibroblasts, the expression levels of fibrosis-related genes, including collagen I, collagen III, and fibronectin, were all remarkably increased (Fig. 5B). This finding is consistent with previous reports that cardiomyocytes directly respond to ISO and secrete paracrine factors such as connective tissue growth factor (CTGF) and vascular endothelial growth factor (VEGF) and then activate fibroblasts to augment cardiac fibrosis (Nuamnaichati et al., 2018). Indeed, here we also observed increased expression of CTGF, VEGF, and

Fig. 4. Effect of mPGES-1 deletion on isoproterenol-induced heart dysfunction and inflammation. (A) Echocardiography of EF%, FS%, and heart rate (HR) of mice after seven consecutive days of injection of ISO at 15 mg/kg per day (scale bar, 1 mm; WT $n = 9$; KO $n = 6$). (B) Heart weight to body weight (HW/BW) ratio (WT $n = 9$; KO $n = 6$). (C) Real-time PCR analysis of the mRNA expression of inflammatory genes (IL-1 β , IL-6, MCP-1, and TNF- α) in the hearts (WT $n = 9$; KO $n = 6$). (D) Immunostainings of Mac-2 (macrophage marker) and CD3 (lymphocyte marker) in the heart sections (WT $n = 9$; KO $n = 6$). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Data are represented as means $\pm$ S.D. The P values were obtained by one-way ANOVA plus a post hoc analysis using a Bonferroni test for (A) and by two-tailed Student's t test for (B–D).

mPGES-1 in ISO-pretreated neonatal rat cardiac myocytes (Supplemental Fig. 1). Moreover, knockdown of mPGES-1 in neonatal rat cardiac myocytes with siRNA transfection reversed the conditioned medium-induced upregulation in cardiac fibroblasts (Fig. 5C). The expression of mPGES-1 was used to validate the efficiency of siRNA (Fig. 5D).

Overexpression of mPGES-1 Increased Fibrosis. Furthermore, we examined the effects of mPGES-1 overexpression on primary cultured neonatal rat cardiac fibroblasts. As expected, adenovirus overexpression of mPGES-1 dramatically promotes the synthesis of PGE₂ (Fig. 6A) and the expression of fibrosis-related genes in a dose-dependent

TABLE 3

Echocardiographic analysis of cardiac function in high-dose isoproterenol (15 mg/kg per day)-treated mice

Data are mean $\pm$ S.D.

Genotype	0 days after ISO		7 days after ISO	
	WT ($n = 9$)	KO ($n = 6$)	WT ($n = 9$)	KO ($n = 6$)
LV mass (mg)	140.0 $\pm$ 21.13	144.8 $\pm$ 32.11	151.8 $\pm$ 31.59	148.7 $\pm$ 14.44
LVID;d (mm)	3.93 $\pm$ 0.31	4.09 $\pm$ 0.35	3.95 $\pm$ 0.16	4.19 $\pm$ 0.28 [#]
LVID;s (mm)	2.62 $\pm$ 0.26	2.74 $\pm$ 0.32	2.86 $\pm$ 0.34	3.07 $\pm$ 0.25*
LVPW;d (mm)	0.98 $\pm$ 0.15	0.91 $\pm$ 0.07	1.01 $\pm$ 0.22	0.92 $\pm$ 0.06
LVPW;s (mm)	1.43 $\pm$ 0.15	1.25 $\pm$ 0.10	1.41 $\pm$ 0.20	1.26 $\pm$ 0.09
IVS;d (mm)	0.87 $\pm$ 0.08	0.89 $\pm$ 0.13	0.94 $\pm$ 0.11	0.87 $\pm$ 0.12
IVS;s (mm)	1.36 $\pm$ 0.15	1.52 $\pm$ 0.26	1.42 $\pm$ 0.19	1.37 $\pm$ 0.13

IVS;d, end-diastolic interventricular septum; IVS;s, systolic interventricular septum; LVID;d, end-diastolic left ventricular internal dimension; LVID;s, systolic left ventricular internal dimension; LVPW;d, end-diastolic left ventricular posterior wall thickness; LVPW;s, systolic left ventricular posterior wall thickness.

[#] $P < 0.05$, compared with 0-day KO group.

* $P < 0.05$, compared with 7-day WT group (one-way ANOVA plus a post hoc analysis using a Bonferroni test).

Fig. 5. Effect of mPGES-1 knockdown on fibrogenesis in primary cultured neonatal rat cardiac fibroblasts and myocytes. (A) Real-time PCR analysis of the mRNA expression levels of collagen I, collagen III, and fibronectin in primary cultured cardiac fibroblasts treated by ISO (20 μ M) ($n = 3$). (B) Primary cultured neonatal rat cardiac myocytes were treated with or without ISO (20 μ M) for 12 hours. After ISO stimulation, the supernatants were collected and added to the cardiac fibroblasts and further incubated for 24 hours. Real-time PCR analysis of the mRNA expression levels of fibrotic genes in cardiac fibroblasts ($n = 3$). (C) The neonatal rat cardiac myocytes were treated with ISO (20 μ M) for 12 hours and transfected with mPGES-1-specific siRNA or negative control siRNA (100 nM). After treatment, the supernatants were collected and added to cardiac fibroblasts and further incubated for 24 hours. Quantitative real-time PCR analysis of the mRNA levels of fibrotic genes in the treated cardiac fibroblasts ($n = 3$). (D) Western blot analysis of mPGES-1 in cardiomyocytes transfected with scrambled or mPGES-1 siRNA (80, 100, 150 nM). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Data are represented as means $\pm$ S.D. The P values were obtained by two-tailed Student's t test.

manner (Fig. 6B). The efficiency of adenovirus infection was evaluated as well (Fig. 6C).

Discussion

Growing evidence has illustrated that, functionally coupled to COX-2, mPGES-1 is induced in various models of inflammation and is the dominant source of PGE₂ production involved in inflammation and pain hypersensitivity; thus mPGES-1 has been suggested to be an anti-inflammatory drug target alternative to NSAIDs (Kamei et al., 2004; Wang et al., 2008b; Koeberle and Werz, 2015). Using genetic manipulated mouse models, we and others have proved that deletion of mPGES-1, especially in myeloid cells, restrains atherosclerosis (Wang et al., 2006; Chen et al., 2014), attenuates vascular injury responses (Wang et al., 2011; Chen et al., 2013b), and suppresses aortic aneurysm formation (Wang

et al., 2008a), without significantly affecting blood pressure or thrombogenesis (Cheng et al., 2006).

Although these results favor targeted delivery of mPGES-1 inhibitors for inflammatory vascular diseases, the role of mPGES-1 inhibition in pathologic remodeling in heart remains unclear. Wu et al. (2009a,b) found that, unlike COX-2 inhibition, loss of mPGES-1 avoided the postinfarction death and did not increase ischemic myocardial injury after coronary occlusion in mice. In contrast, Harding et al. (2011) demonstrated a deleterious effect of mPGES-1 deletion on cardiac function after stress with angiotensin II, including reduced ejection fraction and dilated left ventricle chamber, whereas unaltered cardiac hypertrophy and fibrosis were observed. Moreover, Degousee et al. (2008, 2012) showed that either global or myeloid cell mPGES-1 deletion adversely interfered with cardiac remodeling and cut down survival after experimental myocardial infarction in mice. However, in a sharp contrast, we recently demonstrated

Fig. 6. Effect of mPGES-1 overexpression on primary cultured neonatal rat cardiac fibroblasts in vitro. Cardiac fibroblasts were infected with mPGES-1 adenovirus virus for different doses [from 5 to 50 multiplicity of infection (MOI)]; GFP (10 MOI) adenovirus virus was used as control. (A) ELISA analysis of PGE2 level in cell culture supernatant ($n = 3$). (B) Real-time PCR analysis of the expression of collagen I, collagen III, and fibronectin ($n = 3$). (C) Real-time PCR and Western blot analysis of the expression of mPGES-1. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Data are represented as means $\pm$ S.D. The P values were obtained by one-way ANOVA plus a post hoc analysis using a Tukey's test.

that inhibition of mPGES-1, especially in macrophages, might be beneficial to survival without worsened post-myocardial infarction cardiac dysfunction (Chen et al., 2019). Although there are many possible explanations for the above discrepancies, the complexity of the role of mPGES-1 in myocardial remodeling requires further investigation.

In the current study, we further addressed this question using an isoproterenol cardiac remodeling model. Isoproterenol is a synthetic nonselective agonist for β -ARs. Overstimulation of β -ARs has been reported to cause cardiac remodeling, including cardiac hypertrophy and fibrosis, and lead to the deterioration of cardiac function (Lohse et al., 2003; Dunser and Hasibeder, 2009). Indeed, it has been documented that the pathophysiological alterations induced by isoproterenol in the heart tissues of experimental animals highly mimic those observed in infarcted myocardial tissues of humans (El-Armouche and Eschenhagen, 2009).

Unlike the insignificant or adverse role of mPGES-1 deficiency in post-myocardial infarction cardiac remodeling, here we found that, although the cardiac function did not significantly improve, the abnormal collagen deposition and fibrotic gene expression were clearly suppressed in mPGES-1 KO mice. These data clearly indicated that mPGES-1 is a crucial enzyme involved in isoproterenol-induced myocardial fibrosis. Moreover, substrate redirection resulting from mPGES-1 deletion was seen for PGI₂ after ISO challenge. Given that PGI₂ has been well characterized as a cardioprotective lipid mediator (Wang et al., 2008b), it is reasonable for us to expect that both the reduction of PGE2 and increased formation of PGI₂ may have jointly contributed to the

protective effect of mPGES-1 inhibition on ISO-induced cardiac fibrosis. Notably, here we only observed a small (16.4%) decrease in serum PGE2 levels of the mPGES-1 KO mice, whereas nearly 80% decrease was reported in other studies (Cheng et al., 2006). Differences in tissue sources (plasma vs. urine) or detection techniques (ELISA vs. mass spectrometry) may account for this discrepancy.

Mechanistically, cardiac fibroblasts are critical in the accumulation of extracellular matrix, secretion of collagen, and induction of fibrosis after ischemic or chemical injury (Porter and Turner, 2009). Herein we demonstrated that overexpression of mPGES-1, which accompanied with increased PGE2 production, significantly promoted the isoproterenol-induced fibrosis-related gene expression in primary cultured cardiac fibroblasts. Therefore, the observed attenuated fibrosis in the mPGES-1 KO mice might be largely attributable to the suppressed fibrogenesis in fibroblasts. However, the contribution of mPGES-1 in cardiomyocytes to ISO-induced cardiac fibrosis cannot be excluded. Indeed, here we found that the fibroblasts failed to respond to ISO stimuli directly in vitro. On the contrary, when we applied the conditioned medium from ISO-pretreated cardiac myocytes to the fibroblasts, the expression of fibrosis-related genes increased. This supports the idea that the cardiomyocytes directly respond to ISO and secrete paracrine factors such as CTGF and VEGF and then activate fibroblasts to augment cardiac fibrosis (Nuamnaichati et al., 2018). Our results demonstrated that inhibition of mPGES-1 in cardiac myocytes would reverse the conditioned medium-induced expression of fibrosis-related genes in fibroblasts. Thus, it is possible for

us to expect that, upon ISO stimulation, PGE₂ may also act as a paracrine factor secreted from cardiac myocytes by mPGES-1 to stimulate fibrogenesis in the adjacent fibroblasts. Nevertheless, more evidence is needed to illustrate the contribution of mPGES-1 in cardiomyocytes to ISO-induced cardiac remodeling. Moreover, it was previously reported that inflammatory cells are the likely major source of PGE₂ biosynthesis in the heart after myocardial infarction (Degousee et al., 2008). Overactivation of β -ARs also includes activation of inflammation (Murray et al., 2000), and blocking inflammation has displayed effective attenuation of cardiac fibrosis. According to the most recent publication (Xiao et al., 2018), the infiltration of macrophages in the heart was mostly detected 24 hours after ISO injection, which reached a peak at 72 hours and then gradually disappeared 7 days after ISO treatment. Thus, in the current study, although we did not see a clear effect of mPGES-1 deletion on ISO-induced cardiac inflammatory cell infiltration and proinflammatory cytokine expression 7 days after ISO treatment, we cannot completely exclude the contribution of inflammation to the protective effect of mPGES-1 deletion on ISO-induced cardiac fibrosis. Evidence from experiments at an earlier time point or using the myeloid-specific mPGES-1-deficient mice would shed light on this important issue. Identification of the cellular source of PGE₂ in the heart after β -AR activation warrants further investigation.

In conclusion, our results suggested that mPGES-1 deficiency alleviated β -adrenergic stress-induced cardiac fibrosis by decreasing the expression of fibrotic genes in fibroblasts. Cardiac fibrosis is a common feature and characteristic of many heart diseases; these findings further strengthen the evidence and pave the way for targeting mPGES-1 as a safe pharmacological approach for the cardioprotective NSAIDs.

Authorship Contributions

Participated in research design: Guan, Yang, Chen.
Conducted experiments: Ji, Guo, Wang, Qian, Liu, Xu, Zhang.
Performed data analysis: Ji, Guo, Yang, Chen.
Wrote or contributed to the writing of the manuscript: Ji, Chen.

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Address correspondence to: Dr. Lihong Chen, Advanced Institute for Medical Sciences, Dalian Medical University, Dalian, Liaoning, China 116044. E-mail: lihong@dmu.edu.cn