Abstract  Lysophosphatidylcholine (LPC) may accumulate in the heart to cause fibrotic events, which is mediated through fibroblast activation and collagen accumulation. Here, we evaluated the mechanisms underlying LPC-mediated collagen induction via mitochondrial events in human cardiac fibroblasts (HCFs), coupling application of the pharmacologic cyclooxygenase-2 (COX-2) inhibitor, celecoxib, and genetic mutations in FOXO1 on the fibrosis pathway. In HCFs, LPC caused prostaglandin E₂ (PGE₂) receptor 4 (EP₄)-dependent collagen induction via activation of transcriptional activity of forkhead box protein O1 (FoxO1) on COX-2 gene expression. These responses were mediated through LPC-induced generation of mitochondrial reactive oxygen species (mitoROS), as confirmed by ex vivo studies, which indicated that LPC increased COX-2 expression and oxidative stress. LPC-induced mitoROS mediated the activation of protein kinase C (PKC)α, which interacted with and phosphorylated dynamin-related protein 1 (Drp1) at Ser⁶¹⁶, thereby increasing Drp1-mediated mitochondrial fission and mitochondrial depolarization. Furthermore, inhibition of PKCα and Drp1 reduced FoxO1-mediated phosphorylation at Ser²⁵⁶ and nuclear accumulation, which suppressed COX-2/PGE₂ expression and collagen production. Moreover, pretreatment with celecoxib or COX-2 siRNA suppressed WT FoxO1; mutated Ser²⁵⁶-to-Asp²⁵⁶ FoxO1-enhanced collagen induction, which was reversed by addition of PGE₂. Our results demonstrate that LPC-induced generation of mitoROS regulates PKCα-mediated Drp1-dependent mitochondrial fission and COX-2 expression via a PKCα/Drp1/FoxO1 cascade, leading to PGE₂/EP₄-mediated collagen induction. These findings provide new insights about the role of LPC in the pathway of fibrotic injury in HCFs.

Cardiac fibrosis is characterized by activation of cardiac fibroblasts (CFs), persistence of differentiated myofibroblasts, and synthesis of excessive extracellular matrix (ECM) triggered by various factors (1, 2). Lysophosphatidylcholine (LPC) is hydrolyzed by phospholipase A₂ that is generated from cell membrane-derived phosphatidylcholine and accumulates in ischemic and injured myocardium, associating with cardiomyocyte apoptosis in fibrotic hearts (3, 4). LPC acts as a pro-inflammatory mediator and induces interleukin-6 (IL-6) expression (5), whereas IL-6 is involved in fibroblast activation (6). Therefore, we investigated to...
determine whether LPC-regulated fibrotic events resulted from collagen production in human CFs (HCFs).

Mitochondrial fission increases mitochondrial fragmentation that reflects mitochondrial membrane depolarization (7) and excessive mitochondrial reactive oxygen species (mitoROS) production (8–10). Dynamin-related protein 1 (Drp1) serves as an initiator of mitochondrial fission when it is translocated from the cytosol to the mitochondrial outer membrane (10, 11). Drp1 is regulated by posttranslational modifications, including phosphorylation and dephosphorylation (12). The phosphorylation of Drp1 at different amino acid residues plays opposite roles in mitochondrial fission; phosphorylation at Ser616 and Ser637 is responsible for the initiation and inhibition of fission, respectively (10, 13). In contrast, overproduction of ROS also contributes to Drp1-mediated mitochondrial fission (13). LPC has been reported to facilitate mitoROS generation (14, 15) and mitochondrial membrane depolarization (14). Nonetheless, whether LPC impairs mitochondrial function, in particular, via Drp1-mediated mitochondrial fission in HCFs, remains unknown. Protein kinase Cs (PKCs) consist of a catalytic domain (16), which requires phospholipids, such as LPC, to promote PKCa activation (17). However, the role of ROS in interaction between PKCa and Drp1 is not defined in HCFs. Therefore, we investigated the interaction between PKCa and Drp1 leading to Drp1-mediated mitochondrial fragmentation.

Cyclooxygenase-2 (COX-2) is responsible for the synthesis of prostaglandins (PGs), including prostaglandin E2 (PGE2) (18). The COX-2/PGE2 axis is involved in various pathophysiological processes, including inflammation, tumorigenesis, and proliferation (18–20). The upregulation of COX-2 in the myocardium is associated with heart failure (21). The levels of PGE2 at tissue sites are accompanied by collagen deposition (22, 23). Although the contribution of the LPC-induced COX-2/PGE2 axis in collagen production is not well-established, induction of PGE2 can auto-regulate PGE2 receptors (EPs), including EP2, EP3, and EP4, have been shown to inhibit collagen synthesis (24–26). In contrast, activation of EP4 can increase collagen synthesis (23). However, whether LPC-induced COX-2/PGE2-dependent IL-6 expression could promote collagen induction is not completely elucidated in HCFs.

Here, we demonstrated that LPC-induced mitoROS generation contributed to PGE2/EP2-dependent collagen induction. Mechanistically, mitoROS, induced by LPC, were found to regulate activation of PKCa that interacted with Drp1, leading to mitochondrial fragmentation and depolarization. In addition, LPC-regulated COX-2 expression was mediated via the mitoROS/PKCa/Drp1 cascade in HCFs. Our study provided new insights into a relationship between mitochondrial events and COX-2-dependent collagen induction in HCFs exposed to LPC.

METHODS

Reagents and antibodies

Anti-phospho-forkhead box protein O1 (FoxO1) (Ser256) (rabbit polyclonal antibody, Cat# 9461), anti-phospho-JNK1/2 (rabbit polyclonal antibody, Cat# 9461), anti-phospho-Drp1 (Ser616) (rabbit monoclonal antibody, Cat# 2880), anti-phospho-drp1 (Ser637) (rabbit monoclonal antibody, Cat# 6319), anti-Drp1 (rabbit monoclonal antibody, Cat# 5391), and anti-FoxO1 (rabbit monoclonal antibody, Cat# 2880) antibodies were obtained from Cell Signaling (Danvers, MA). Anti-COX-2 (rabbit monoclonal antibody, Cat# ab62331), anti-phospho-PKCa (rabbit monoclonal antibody, Cat# ab180848), and anti-TOM20 (mouse monoclonal antibody, Cat# ab56783) antibodies were obtained from Abcam (Cambridge, UK). Anti-JNK1/2 (mouse monoclonal antibody, Cat# sc-137020), anti-PKCa (rabbit polyclonal antibody, Cat# sc-2058), and anti-lamin A (rabbit polyclonal antibody, Cat# sc-20580) antibodies were obtained from Santa Cruz (Santa Cruz, CA). Anti-GAPDH (mouse monoclonal antibody, Cat# MCA-1D4) antibody was obtained from EnCor Biotechnology (Gainesville, FL). LPC (L-0906) was obtained from Sigma-Aldrich (St. Louis, MO). LPC was dissolved in 0.5% ethanol and filtered through a 0.22 μm syringe filter. A final concentration of 0.5% ethanol was used for the experiments. NS-398, Gö 6976, SP600125, and celecoxib were obtained from Biomol (Plymouth Meeting, PA). MitoQ and Gö 6983 were obtained from Cayman Chemicals (Ann Arbor, MI). AS1842856 was obtained from EMD Millipore (Billerica, MA). Mitotempo, dynasore, and mdivi-1 were obtained from Santa Cruz. The pharmacological inhibitors were dissolved in DMSO at a working concentration of 0.5% DMSO used for all experiments. SDS-PAGE reagents were obtained from MBio Inc. (Taipei, Taiwan).

Animal care and experimental procedures

All animal care and experimental procedures complied with the UK Animals (Scientific Procedures) Act (1986, Directive 2010/63/EU) of the European Parliament and the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (National Institutes of Health Publication No. 85-23, revised 1996). Animal studies are reported in compliance with the ARRIVE. Male Institute of Cancer Research mice (25–30 g, 8 weeks old) were purchased from the National Laboratory Animal Centre (Taipei, Taiwan) and randomly assigned to standard cages, with five animals per cage and kept in standard housing conditions with food and water ad libitum, according to the guidelines of Animal Care Committee of Chang Gung University (Approval Document No. CGU 16/046) and National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Institute of Cancer Research mice were anesthetized with one injection of Zoletil (40 mg/kg ip) and xylazine (10 mg/kg ip). After anesthesia, mice were withdrawn with lined forceps on the paws, and then their chests were opened and the hearts were quickly removed for the experiments. The cardiac apaxes of the mice were sliced into three segments and assigned randomly into three groups: vehicle (containing 0.5% (v/v) ethanol and 0.5% (v/v) DMSO), LPC treatment (containing 40 μM LPC with 0.5% (v/v) ethanol and 0.5% (v/v) DMSO), and Mitotempo plus LPC treatment (containing 10 μM Mitotempo plus 40 μM LPC with 0.5% (v/v) ethanol and 0.5% (v/v) DMSO); five slices were chosen from each group. The slices of cardiac apaxes were pretreated with the inhibitors for 1 h, and then incubated with LPC for 6 h in Krebs solution (pH 7.4 at 37°C). The homogenates of cardiac apaxes were prepared and lysed in a lysis buffer and subjected to Western blot analysis and RT-quantitative (q)PCR, as previously described (5).

Measurement of GSH/GSSG ratio

The ex vivo heart apaxes, with or without respective inhibitor treatment for 1 h, were incubated with 40 μM LPC for 6 h. The homogenates were used to measure the ratio of GSH/GSSG as the marker of oxidative stress in the heart tissues, which
was determined using a glutathione detection kit according to the manufacturer’s instructions (Enzo Life Sciences, Farmingdale, NY).

Cell cultures
HCFs were purchased from ScienCell Research Laboratories (San Diego, CA) and maintained in DMEM/nutrient mixture F-12 (DMEM/F-12) medium supplemented with 10% FBS, as previously described (27).

Preparation of samples and Western blot analysis
Growth-arrested HCFs were incubated without or with different concentrations of LPC at 37°C for the indicated time intervals. When pharmacological inhibitors were used, they were added 1 h prior to the exposure to LPC. After incubation, the cells were rapidly washed with ice-cold PBS and lysed with a sample buffer containing 125 mM Tris-HCl, 1.25% SDS, 6.25% glycerol, 3.2% β-mercaptoethanol, and 7.5 nM bromophenol blue with pH 6.8. Samples were denatured, subjected to SDS-PAGE using a 10% (w/v) running gel, and transferred to nitrocellulose membrane (BioTracTM NT membrane, Pall Life Sciences, Ann Arbor, MI). The membranes were immunoblotted with one of the primary antibodies (1:1,000 dilution) overnight at 4°C, followed by incubation with a peroxidase-conjugated secondary antibody at room temperature for 2 h. The immunoreactive bands were visualized by enhanced chemiluminescence reagent (Western Lightning Plus; Perkin Elmer, Waltham, MA). The images of the immunoblots were acquired using a UVP BioSpectrum 500 imaging system (Upland, CA), and densitometric analysis was conducted using UN-SCAN-IT gel software (Orem, UT).

RT-PCR and qPCR analyses
Total RNA was extracted with TRIzol (Sigma-Aldrich) according to the manufacturer’s instructions. First-strand cDNA synthesis was performed with 5 μg of total RNA using Oligo(dT)15 as primer in a final volume of 20 μl [25 ng/μl Oligo(dT)15, 0.5 mM dNTPs, 10 mM DTT, 2 units/μl RNase inhibitor, and 10 units/μl of Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA)]. The synthesized cDNAs were used as templates for PCR reaction using Q-Amp™ 2x Screening Fire Taq Master Mix (Bio-Genesis Technologies, Taipei, Taiwan) and primers for the target genes. qPCR was performed using Luna Universal probe qPCR Master Mix (M3004; New England BioLabs, Beverly, MA) on a StepOnePlus™ real-time PCR system (Applied Biosystems, Foster City, CA). The relative amount of the target gene was calculated using 2^ΔΔCt formula (ΔCt = threshold cycle). The sequences of the primers used are shown in supplemental Table S1.

Measurement of soluble collagen secretion
HCFs were seeded in 6-well culture plates. After reaching 90% confluence, the cells were transferred to serum-free DMEM/F-12 medium overnight. On the next day, the cells were transfected with siRNA and then treated with 40 μM of LPC for the indicated time intervals in either the presence or the absence of pharmacological inhibitors. The media were collected and the levels of soluble collagen (types I–V collagen) were analyzed using a Sircol collagen assay kit (Biocolor, Northern Ireland, UK).

Determination of mitoROS
HCFs were seeded in 6-well culture plates with coverslips. After the cells reached 90% confluence, they were transferred to serum-free DMEM/F-12 overnight. When pharmacological inhibitors were used, they were added 1 h prior to the exposure to LPC. After treatment with LPC, the cells were washed with media, and then incubated in DMEM/F12 medium containing 500 nM MitoTracker Green (Invitrogen) for 10 min. To visualize mitochondrial morphology, the cells were washed thrice with media, and then observed using a fluorescence microscope with a FITC filter (Axiovert 200M; Carl Zeiss).

Analysis of mitochondrial membrane potential
HCFs were seeded in 6-well culture plates with coverslips. After the cells reached 90% confluence, they were cultured in serum-free DMEM/F-12, and then stimulated with LPC for the indicated time intervals in either the presence or the absence of pharmacological inhibitors for 1 h prior to LPC exposure. To detect mitochondrial membrane potential (∆Ψm), cells were subjected to a mitochondria staining kit (CS0390; Sigma-Aldrich). Cells were cultured in 10 μg/ml 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolocarbocyanine iodide (JC-1) in DMEM/F-12 containing 10% FBS at 37°C for 10 min. In healthy cells, JC-1 monomers accumulated as aggregates in the mitochondria due to existing mitochondrial polarization. These aggregates were visible on the red channel (rhodamine) when viewed with a fluorescence microscope. The JC-1 exists as a monomer and was visible on the green channel (FITC) when mitochondria were depolarized. The fluorescent images were captured with a fluorescence microscope (Axiovert 200M; Carl Zeiss) and quantified using ImageJ software (1.41v; US National Institutes of Health). Moreover, JC-1 fluorescence was also measured in a fluorescence plate reader (Synergy™2, H1 Hybrid Reader; BioTek). The fluorescence intensities of JC-1 monomer were measured by excitation at 490 nm and emission at 530 nm, and fluorescence intensities of JC-1 aggregates were measured by excitation at 520 nm and emission at 590 nm.

Transient transfection with siRNAs
HCFs were plated in 12-well plates, 6-well plates, or 10 cm dishes and after reaching about 90% confluence, were transfected with fresh serum-free DMEM/F-12 medium before transfection. The siRNAs of COX-2 (SASI_Hs01_00152843), EP2 (SASI_Hs01_00158176), EP3 (SASI_Hs02_00303570), EP4 (SASI_Hs02_00105505), FOXO1 (SASI_Hs01_0076732), and scrambled siRNA were obtained from Sigma-Aldrich. The sequences of siRNAs are shown in supplemental Table S2. Transient transfection of siRNA was conducted using GenMute™ siRNA transfection reagent according to the manufacturer’s instructions (SignaGen Laboratories, Gaithersburg, MD). The siRNA (100 nM) was added to each well, and then the cells were incubated at 37°C for 6 h. The cells were transferred to DMEM/F-12 medium containing 10% FBS for an additional 6 h, washed twice with PBS, and then maintained in serum-free DMEM/F-12 medium for 24 h before treatment with LPC.

Construction of FoxO1 plasmid DNA
Ser256→Ala256 (S256A) FoxO1 mutant and Ser256→Asp256 (S256D) FoxO1 mutant were cloned into the EcoRV-HindIII site of the pCMV-Tag2B vector, as previously described (5).
Transient transfection of plasmid DNA
HCFs were seeded in 6-well plates or 10 cm dishes and after they reached 90% confluence, they were transfected to serum-free DMEM/F-12 medium and transiently transfected with plasmid DNA using an X-tremeGENE™ HP DNA transfection reagent (Roche Applied Science, Indianapolis, IN), as previously described (5).

Measurement of COX-2 promoter activity
For construction of the COX-2-luc plasmid, a human COX-2 promoter, a region spanning from ∼484 to +37 was cloned into pGL3-basic vector, as previously described (5). HCFs were co-transfected with pGL3b-cox-2 and pCMV-β-gal plasmid (as an internal control). Promoter activities of COX-2 were determined using a luciferase assay HIT kit (BioThema, Handen, Sweden) and normalized with β-Gal reporter gene as determined by using a Galacto-Light Plus™ system (Applied Biosystems, Bedford, MA).

Immunofluorescence staining
HCFs were seeded on coverslips in 6-well culture plates, and after they reached 90% confluence, they were transferred to serum-free DMEM/F-12 medium overnight, and then stimulated with 40 μM of LPC for the indicated time intervals. After washing twice with ice-cold PBS, the cells were fixed with 4% (w/v) paraformaldehyde in PBS for 30 min, and then permeabilized with 0.1% Triton X-100 in PBS for 15 min. The staining was performed by incubating with 5% BSA for 2 h at 37°C, followed by incubation with a primary anti-phospho-Drp1S616 rabbit polyclonal antibody (1:100 dilution) and anti-TOM20 mouse monoclonal antibody (1:1,000) overnight in PBS containing 1% BSA. The cells were washed thrice with PBS and incubated for 2 h with a FITC-conjugated goat anti-rabbit antibody and rhodamine-conjugated goat anti-mouse antibody (1:100 dilution; Jackson ImmunoResearch, West Grove, PA) in PBS containing 1% BSA. Finally, cells were washed thrice with PBS, and then mounted with aqueous mounting medium containing DAPI (H1200; Vector Laboratories, Burlingame, CA). Images were captured with a fluorescence microscope (Axiovert 200 M; Carl Zeiss).

Chromatin immunoprecipitation assay
To detect the association of transcription factors with human COX-2 promoter, chromatin immunoprecipitation analysis was performed. Protein-DNA complexes were fixed by 1% formaldehyde in DMEM/F-12 medium and the reaction was terminated with 125 mM glycine. The sample was lyzed, immunoprecipitated, washed, and eluted, as previously described (5). The enrichment of specific DNA and input DNA (as an internal control) were subjected to PCR amplification. The primer sequences were: FoxO1 forward primer 5′-AAGACATCTGGCGGAAACC-3′ and reverse primer 5′-ACAATTTGTCGGTACCCGAG-3′, which were specifically designed from the COX-2 promoter region (~300 to +2). qPCR was performed using Luna Universal qPCR master mix kit (M3003; New England BioLabs) on a StepOnePlus™ real-time PCR system (Applied Biosystems, Foster City, CA).

Isolation of subcellular fractions
HCFs were seeded in 10 cm dishes, and after they reached 90% confluence, they were transfected to serum-free DMEM/F-12 medium for 24 h, and then incubated with LPC for the indicated time intervals. The subcellular fractions were prepared using a NE-PER nuclear and cytoplasmic extraction kit according to the manufacturer’s instructions (Thermo Scientific, Rockford, IL), as previously described (5). The protein concentrations of sample were determined by BCA assay and Western blotting.

Data and statistical analysis
All the data were estimated using GraphPad Prism Program 5 (GraphPad, San Diego, CA). Quantitative data were expressed as the mean ± SEM of at least three individual experiments (n ≥ 3), and analyzed with a one-way ANOVA followed by Tukey’s post hoc test at a *P< 0.05 or #P< 0.01 level of significance. Error bars were omitted when they fell within the dimensions of the symbols.

RESULTS
LPC-induced mitoROS generation mediates COX-2-dependent collagen secretion
LPC has been shown to stimulate mitoROS production as a result of proton leakage from the electron respiratory chain in various cell types (14, 15). With respect to the effect of LPC treatment on mitoROS production in HCFs, the data revealed that mitoROS were generated (Fig. 1A, B) and were attenuated by mitochondrial antioxidants (MitoTEMPO and MitoQ). mitoROS have been demonstrated to mediate COX-2 expression in various cell types (28–30). Thus, we determined whether mitoROS regulated the LPC-induced COX-2 expression in HCFs. Pretreatment with MitoTEMPO or MitoQ attenuated COX-2 protein induction in a concentration-dependent manner (Fig. 1C, D). In addition, scavenging of mitoROS by MitoTEMPO or MitoQ reduced the COX-2 mRNA expression and promoter activity (Fig. 1E), suggesting that mitoROS are key players in the induction of COX-2 by LPC in HCFs. GSH protects against cellular ROS and forms GSSG when GSH is oxidized; thereby, the ratio of GSH:GSSG is used as a marker of oxidative stress (31). We further confirmed that LPC decreased the ratio of GSH:GSSG (Fig. 1F) and increased COX-2 protein and mRNA expression (Fig. 1G) in ex vivo mouse heart apexes, which were reduced by pretreatment with MitoTEMPO (Fig. 1F, G). These results suggested that the LPC-induced increase in mitoROS production is associated with COX-2 expression.

CFs act as central modulators, maintaining the heart structure and function. The imbalance between the synthesis and degradation of ECM components leads to cardiac fibrosis (32). Here, HCFs were used as a model that mimicked cardiac fibrosis in vitro. The levels of soluble collagen were markedly increased in the cultured media of HCFs treated with LPC (Fig. 1H). COX-2 metabolites, such as PGs, have been shown to mediate tissue fibrosis, including collagen deposition (22). Therefore, we determined whether LPC-induced COX-2 expression contributed to the increase in extracellular collagen content in HCFs. The levels of collagen content were upregulated after LPC treatment, which was significantly alleviated after pretreatment with celecoxib and NS-398, inhibitors of COX-2, and MitoTEMPO (Fig. 1H), suggesting that the levels of extracellular collagen are associated with mitoROS-mediated COX-2 expression in HCFs upon LPC exposure. Moreover, we also found that addition of PGE2 induced collagen production from HCFs (Fig. 1H), suggesting that the COX-2/PGE2 axis could be involved in LPC-induced collagen secretion.
Fig. 1. LPC-induced mitoROS generation is involved in COX-2-dependent collagen secretion. A: HCFs were pretreated with either Mito-TEMPO (1 μM) or MitoQ (100 nM) for 1 h, and then treated with either 0.5% EtOH (vehicle control) or LPC (40 μM) for the indicated time intervals (0, 15, 30, 60 min). mitoROS generation was detected under a fluorescence microscope with MitoSOX Red. Representative images are shown. Scale bar indicates 100 μm, n = 5. B: HCFs were pretreated with either MitoTEMPO or MitoQ for 1 h, and then incubated with either 0.5% EtOH (vehicle control) or LPC for the indicated time intervals. mitoROS production was determined by MitoSOX Red staining. The fluorescence unit of MitoSOX Red was measured using a fluorescent microplate reader. Values are shown as fold change, n = 5. C, D: HCFs were pretreated with either MitoTEMPO or MitoQ for 1 h followed by LPC for 6 h. The protein levels of COX-2 and GAPDH were determined by Western blotting. Densitometry analyses of COX-2 protein levels were normalized to GAPDH and relative to control (0.5% DMSO and 0.5% EtOH), n = 5. E: HCFs were pretreated with either MitoTEMPO or MitoQ for 1 h followed by LPC for 4 h. The mRNA levels of COX-2 and GAPDH were determined by using RT-qPCR (open bars). Results were normalized to GAPDH and relative to control (0.5% DMSO and 0.5% EtOH), n = 6. F: Mouse heart segments were pretreated with MitoTEMPO for 1 h followed LPC treatment for 6 h in Krebs solution bubbled with 5% O2 and 95% CO2 at 37°C. F: The heart tissues were homogenized and subjected to glutathione detection assay. The oxidative stress of heart was reported as the ratio of GSH to GSSG, n = 5. G: Western blot analysis or RT-qPCR were performed to determine the levels of COX-2 protein (open bars; n = 5) and mRNA
Drp1 is involved in mitochondrial fission and depolarization and COX-2 expression induced by LPC

mitoROS have been shown to promote mitochondrial fragmentation and promptly induce mitochondrial depolarization (7, 13). LPC has been demonstrated to stimulate mitochondrial membrane depolarization (14). We therefore examined whether LPC impaired Δϕm using JC-1 staining. The green intensity of JC-1 monomers was increased in HCFs stimulated by LPC, while the red intensity of JC-1 aggregates was decreased (Fig. 2A, supplemental Fig. S1A), indicating that Δϕm of HCFs was depolarized after exposure to LPC. Furthermore, LPC induced changes in mitochondrial morphologies from tube-shaped to fragmented mitochondria (Fig. 2B). Treatment of HCFs with 0.5% ethanol (EtOH) alone had no effect on Δϕm and mitochondrial morphology. Mitochondrial fragmentation has been shown to be regulated by cytosolic Drp1, which is recruited to the mitochondrial outer membrane via posttranslational modification (10, 12, 13). To determine the levels of phosphorylated-Drp1 protein upon LPC treatment in HCFs, the results revealed that LPC significantly increased phosphorylation of Drp1 at Ser616 but decreased its phosphorylation at Ser637 (Fig. 2C). Fluorescent images further supported that phosphorylation of Drp1 at Ser616 was also increased, which consequently led to its translocation into mitochondria (Fig. 3A, supplemental Fig. S1B). Together, these results indicated that LPC-induced mitochondrial fragmentation was mediated via Drp1-dependent mitochondrial fission in HCFs. Drp1 has been found to be involved in the expression of proinflammatory genes via ROS-mediated NF-κB activation (33). Our recent study also demonstrated that LPC-induced NF-κB activation regulates transcriptional activity of COX-2 in HCFs (5). Here, pretreatment with the inhibitor of Drp1 (mdivi-1) or GTPase (dynasore) attenuated LPC-induced COX-2 protein (Fig. 3B, C) and mRNA (Fig. 3D) expression and promoter activity (Fig. 3D). Furthermore, we corroborated our findings that Drp1 was involved in COX-2-mediated collagen induction upon LPC treatment in HCFs, which was attenuated by either mdivi-1 or dynasore (Fig. 3E).

PKCα is involved in LPC-induced COX-2 expression and collagen secretion

JNK1/2 have been shown to be involved in LPC-induced COX-2 expression, and also to be activated by PKCα (34). Therefore, we determined the role of the interaction between PKCα and JNK1/2 in the LPC-induced COX-2 expression in HCFs. Pretreatment with G6 6976 (an inhibitor of PKCα) or G6 6983 (a pan PKC inhibitor) attenuated COX-2 protein expression in a concentration-dependent manner (Fig. 4A, B), suggesting that PKCα is involved in the LPC-induced COX-2 expression in HCFs. Furthermore, LPC-induced COX-2 mRNA expression and promoter activity was attenuated by pretreatment with either G6 6976, G6 6983, or SP600125 (an inhibitor of JNK1/2) (Fig. 4C). Moreover, pretreatment with either G6 6976 or SP600125 significantly reduced collagen induction by LPC in HCFs (Fig. 4D), indicating that both PKCα and JNK1/2 participate in COX-2-mediated collagen induction by LPC in HCFs.

mitoROS initiates PKCα and Drp1 activation

Redox signaling pathways are regarded as activators for JNK1/2 (27) and PKCα (35) in various cell types. We further investigated the roles of mitoROS, PKCα, and JNK1/2 in the LPC-induced COX-2 expression in HCFs. Pretreatment with mdivi-1, G6 6976, or SP600125 had no effect on mitoROS production stimulated by LPC for 30 min; however, treatment with either mdivi-1 or G6 6976 significantly attenuated the mitoROS generation induced by LPC for 60 min (Fig. 4E). In addition, Western blot analyses revealed that the levels of PKCα and JNK1/2 phosphorylation were attenuated by MitoTEMPO (Fig. 4F, supplemental Fig. S2A). Furthermore, LPC-stimulated PKCα phosphorylation was attenuated by G6 6976, but not by SP600125 (Fig. 4F). Moreover, the levels of PKCα and JNK1/2 phosphorylation were reduced by transfection with siPKCα (supplemental Fig. S3). Taken together, mitoROS signaling is an upstream component of the PKCα-JNK1/2 cascade in HCFs stimulated by LPC.

mitoROS have been shown to activate Drp1 and, consequently, contribute to mitochondrial fission (13). In contrast, inhibition of mitochondrial fission could diminish mitoROS generation (8, 9). Therefore, we determined the role of mitoROS in Drp1 activation. mitoROS were not significantly altered by pretreatment with mdivi-1 upon LPC stimulation for 30 min (Fig. 4E). Moreover, phosphorylation of Drp1 at Ser616 was inhibited by pretreatment with MitoTEMPO and mdivi-1 upon LPC treatment for 60 min (Fig. 4F, supplemental Fig. S2A), suggesting that mitoROS are an upstream regulator of Drp1 activation. Pretreatment with G6 6976 attenuated phosphorylation of Drp1 at Ser616, which was further confirmed by transfection with siPKCα (Fig. 4F; supplemental Figs. S2A, S3). Interestingly, mdivi-1 also reduced PKCα phosphorylation (Fig. 4F, supplemental Fig. S2A), indicating that PKCα and Drp1 reciprocally regulate each other upon LPC treatment for 60 min. In addition, both mdivi-1 and G6 6976 reduced mitoROS generation upon LPC stimulation for 60 min (Fig. 4E, supplemental Fig. S2B). These data suggested that mitoROS may mediate Drp1 and PKCα activation at an early stage in response to LPC treatment, whereas blocking of mitochondrial fission could ameliorate mitoROS generation and, consequently, reduce positive feedback of Drp1 and PKCα activation in HCFs.
mitoROS enhance the interaction between PKCα and Drp1 leading to mitochondrial fission stimulated by LPC

Drp1 is recognized as a substrate for PKCs in various cell types (11, 36). Therefore, we investigated to determine whether PKCα directly bound to Drp1, which was a downstream component of PKCα in HCFs. We first found that LPC enhanced the interaction between PKCα and Drp1 in the PKCα- or Drp1-immunoprecipitated complexes in a time-dependent manner (Fig. 5A, B). Furthermore, the levels of phosphorylated Drp1 Ser616 were increased in the PKCα-immunoprecipitated complexes (Fig. 5A). We further investigated the involvement of mitoROS, PKCα, Drp1, and JNK1/2 in the interaction between PKCα and Drp1 stimulated by LPC. Pretreatment with MitoTEMPO or Gö6976 attenuated mitoROS-dependent PKCα-Drp1 activation (Fig. 5A, B) and restored the Δψm (supplemental Fig. S4A–C). Moreover, the phosphorylation of Drp1 Ser616 was inhibited after pretreatment with MitoTEMPO, mdv1-1, or Gö6976, but not with SP600125 (Fig. 5C, D). Together, these results suggested that LPC-induced mitoROS-dependent PKCα-Drp1 activation and, consequently, contributed to mitochondrial depolarization that was associated with mitochondrial fission in HCFs.

LPC-induced COX-2 expression is mediated via a mitoROS-PKCα-Drp1-JNK1/2-dependent FoxO1 pathway

LPC-stimulated FoxO1 Ser256 phosphorylation results in FoxO1 nuclear accumulation and enhances its binding to the regions of the COX-2 promoter via ROS-mediated JNK1/2 activation (5). We confirmed that FoxO1 was involved in the LPC-induced COX-2 expression, while pretreatment with AS1842856 (an inhibitor of FoxO1) enhances the phosphorylation at Drp1 Ser616 in HCFs stimulated by LPC.

Next, we investigated to determine whether Drp1-mediated fission and mitochondrial depolarization were mediated via a mitoROS-PKCα-Drp1 pathway in HCFs challenged with LPC. Pretreatment with MitoTEMPO, mdv1-1, or Gö6976, but not with SP600125, attenuated mitochondrial fragmentation (Fig. 5E) and restored the Δψm (supplemental Fig. S4A–C). Moreover, the phosphorylation of Drp1 Ser616 was inhibited after pretreatment with MitoTEMPO, mdv1-1, or Gö6976, but not with SP600125 (Fig. 5F, G). Together, these results suggested that LPC induced mitoROS-dependent PKCα-Drp1 activation and, consequently, contributed to mitochondrial depolarization that was associated with mitochondrial fission in HCFs.
decreased COX-2 protein expression (in a concentration-dependent manner), mRNA expression, and promoter activity (Fig. 6A, B) in HCFs. Furthermore, LPC-stimulated phosphorylation of FoxO1 Ser256 in the nuclear fraction was reduced in the presence of MitoTEMPO, Gö 6976, SP600125, mdivi-1, or AS1842856 (Fig. 6C). In addition, the transcriptional activity of p-FoxO1 Ser256 was also attenuated by these inhibitors (Fig. 6D). These results suggested that LPC-enhanced FoxO1 transcriptional activity on COX-2 expression is mediated via a mitoROS-PKC-Drp1-JNK1/2-dependent cascade in HCFs.

The role of FoxO1 in LPC-induced COX-2-dependent collagen production

FoxO1 plays an important role in the process of fibrosis, including collagen expression (37). Thus, we determined whether FoxO1 was involved in the LPC-induced COX-2-dependent collagen induction in HCFs. The induction of collagen by LPC was reduced by pretreatment with AS1842856 (Fig. 7A). The involvement of FoxO1-dependent COX-2 activation in collagen production was further confirmed by transfection with either FoxO1 or COX-2 siRNA and subsequently attenuated the LPC-induced collagen production (Fig. 7B). In addition, our data revealed that the decrease of collagen content was reversed by addition of PGE2 (Fig. 7B). These results suggested that LPC-induced collagen production is mediated through the FoxO1-dependent COX-2/PGE2 axis in HCFs.

Our previous report revealed that overexpression of WT FoxO1 or S256D FoxO1 (a phospho-mimic mutant), but not S256A FoxO1 (a phospho-silencing mutant), enhances the binding activity of FoxO1 with the COX-2 promoter, leading to COX-2 expression (5). We further investigated to determine whether FoxO1 was involved in COX-2-dependent collagen production through the overexpression of WT FoxO1, S256A FoxO1, or S256D FoxO1 in HCFs. We
Fig. 4. mitoROS/PKCα/JNK is involved in LPC-induced COX-2 expression and collagen secretion. A, B: HCFs were pretreated with various concentrations of Gö 6976 or Gö 6983 for 1 h, and then incubated with LPC for 6 h. The protein levels of COX-2 and GAPDH were determined by Western blot analysis. Densitometry analyses of COX-2 protein levels were normalized to GAPDH and relative to control (0.5% DMSO and 0.5% EtOH), n = 6. C: HCFs were pretreated with Gö 6976 (100 nM), Gö 6983 (30 nM), or SP600125 (1 μM) for 1 h, and then treated with LPC for 4 h. RT-qPCR was performed to measure COX-2 expression (open bars), n = 6. A dual luciferase activity assay was conducted to evaluate COX-2 promoter activity (gray bars), n = 5. Values are relative to control (0.5% DMSO and 0.5% EtOH). D: HCFs were pretreated with either Gö 6976 (100 nM) or SP600125 (1 μM) for 1 h followed by LPC treatment for 48 h. The conditioned media were subjected to evaluation of collagen content, n = 5. E, F: HCFs were pretreated with mdivi-1 (50 nM), Gö 6976 (100 nM), or SP600125 (1 μM) for 1 h, and then treated with LPC (40 μM) for the indicated time points. E: mitoROS production was determined by MitoSOX Red staining. The fluorescence unit of MitoSOX Red was measured using a fluorescent microplate reader. Results are relative to control, n = 7. F: The protein levels were determined by Western blotting. The densitometry measurements are presented in supplemental Fig. S2A, n = 7. Data are expressed as the mean ± SEM and were analyzed by one-way ANOVA with Tukey’s post hoc tests. *P < 0.05; **P < 0.01. DM, 0.5% DMSO; EtOH, 0.5% EtOH.
confirmed that overexpression of WT FoxO1 or S256D FoxO1 increased COX-2 expression in HCFs (supplemental Fig. S5A). Moreover, overexpression of either WT FoxO1 or S256D FoxO1 significantly enhanced collagen production (Fig. 7C), which was attenuated by transfection with celecoxib (supplemental Fig. S5B). These results suggested that phosphorylation of FoxO1 at Ser256 residue contributes to collagen production in a COX-2-dependent manner in HCFs.

**LPC-induced COX-2-dependent collagen secretion is mediated via EP4 receptors**

The COX-2/PGE2 axis has been shown to mediate biological events, including ECM synthesis, via prostanoid EP receptors (22–26). While investigating whether production...
of PGE$_2$ was involved in the LPC-triggered responses in HCFs, we found that LPC-induced PGE$_2$ production was inhibited by pretreatment with either MitoTEMPO, Gö 6976, mdivi-1, SP600125, or AS1842856 (Fig. 8A). Next, we characterized the expression of EP receptors in HCFs. The prostanooid EP receptors, including EP$_2$–EP$_4$ were expressed on HCFs (supplemental Fig. S6A). Further, we determined which of the EP receptors mediated the COX-2/PGE$_2$ axis on LPC-induced collagen production. Transfection with EP$_2$–EP$_4$ siRNA knocked down the level of respective EP mRNA (supplemental Fig. S6B). We found that knockdown of EP$_2$ but not EP$_2$ and EP$_3$ receptors significantly attenuated the LPC-induced collagen secretion (Fig. 8B). Taken together, our results suggested that LPC-induced collagen secretion via a COX-2/PGE$_2$ axis is predominantly mediated through EP$_4$ receptors in HCFs.

**DISCUSSION**

The excessive production and deposition of scar tissues are well-known characteristics of cardiac fibrosis. This
pathological event is characterized by irreversible injury in cardiomyocytes, while noncardiomyocytes, such as CFs, are less susceptible to injury (32). Although CFs are quiescent, they are responsible for maintaining heart functions during injury and remodeling. We attempted to explore the mechanisms underlying LPC-induced COX-2/PGE2-dependent collagen expression in HCFs. First, we found that the increased COX-2 induction was accompanied by PGE2 accumulation upon LPC treatment. Subsequently, the COX-2/PGE2 axis contributed to extracellular collagen induction via EP4 receptors. Second, LPC-induced COX-2 expression was mediated through mitoROS production. Furthermore, the increase in mitoROS altered the mitochondrial morphology via the translocation of Drp1 from the cytoplasm into the mitochondria, leading to mitochondrial fission and depolarization. Third, PKCα was an upstream regulator of Drp1 and interacted with Drp1 in response to LPC-mediated mitoROS generation in HCFs. Activation of signaling components involved in COX-2 expression by LPC was mediated through a mitoROS-dependent PKCα-Drp1-JNK1/2 cascade. Fourth, phosphorylation of FoxO1 at Ser256 was regulated by a mitoROS-mediated PKCα-Drp1-JNK1/2 pathway and resulted in the accumulation of FoxO1 in the nucleus, which led to COX-2/PGE2 expression. Fifth, inhibition of COX-2 expression by pharmacological inhibitors or silencing of COX-2 attenuated WT FoxO1 and S256D FoxO1-mediated collagen induction. We concluded that LPC promoted mitoROS generation and, consequently, activated a PKCα-Drp1-JNK1/2 cascade, which contributed to COX-2/PGE2/EP4-mediated collagen induction by enhancing FoxO1 transcriptional activity in HCFs. Moreover, mitoROS

![Graph A](image1.png)

![Graph B](image2.png)

![Graph C](image3.png)

![Graph D](image4.png)
LPC-induced mitochondrial fission and collagen production

LPC is known to be accumulated in ischemic heart (4, 38), and the concentration of LPC in the ischemic heart is approximately 100 to 200 μM (39–41), which is dependent on the species and tissue compartments. In vivo conditions, LPC may be nonspecifically bound to plasma proteins in the myocardium, while the free form of LPC has a lower concentration than that of protein bound LPC by about five to ten times (42, 43). Under cell-cultured experiments, LPC could affect cell viability ranging from 10 to 80 μM in rat cardiomyocytes (3, 44). Therefore, we determined cell viability in HCFs exposed to LPC: the concentrations of LPC less than 50 μM failed to affect cell viability (data not shown). Fibrotic events have been reported to be regulated by various events that promote fibroblast activation and ECM expression (2). The LPC content is associated with fibrotic responses in the animal model of ischemic heart (Fig. 7C).

Fig. 8. LPC-induced COX-2-dependent collagen secretion is mediated via EP4 receptors. A: HCFs were pretreated with MitoTEMPO (1 μM), G6 6976 (100 nM), mdivi-1 (50 nM), dynasore (300 nM), SP600125 (1 μM), AS1842856 (100 nM), celecoxib (10 μM), or NS-398 (10 μM) for 1 h and then incubated with LPC (40 μM) for 6 h. The conditioned media were subjected to determination of PGE2 production, n = 7. B: HCFs were transfected with siRNA of EP2, EP3, or EP4 and scrambled sequences, and then incubated with LPC for 48 h. RT-qPCR was performed to determine the EPs gene expression as shown in supplemental Fig. S6B. A Sircol collagen assay was conducted to determine levels of collagen, n = 6. Results are relative to control (0.5% EtOH). Values are expressed as the mean ± SEM and were analyzed using one-way ANOVA with Tukey’s post hoc tests. *P < 0.05; †P < 0.01. DM, 0.5% DMSO; EtOH, 0.5% EtOH. C: Schematic diagram illustrates that LPC-induced COX-2/PGE2/EP4 expression was regulated by PKCα/Drp1/JNK/FoxO1 cascades via regulation of FoxO1 transcriptional activity, linking to LPC-induced collagen production in HCFs. LPC-induced mitoROS enhance PKCα and Drp1 interaction; thereby, PKCα regulates phosphorylation of Drp1 at Ser616, leading to Drp1 translocation into the mitochondrial outer membrane. Activation of Drp1 mediates mitochondrial fission, which causes mitochondrial depolarization and excessive ROS generation. In consequence, activation of these signaling components promotes JNK1/2 activation, resulting in FoxO1 regulating COX-2 promoter activation and contributing to COX-2 gene expression. Moreover, induction of COX-2 increases PGE2 production and auto-regulates collagen secretion by mediating EP4 activation.
cardiac fibrosis (3, 4, 38); however, the role of LPC in the progression of fibrotic responses has not been well defined. Previous studies have demonstrated that LPC is involved in valvular sclerosis in aortic valve interstitial cells via expression and deposition of collagen I (45). Here, we suggested that LPC increases extracellular collagen levels, which potentially enhances collagen deposition in the heart. In fact, LPC is produced from the hydrolysis of phospholipids by PLAS. In in vivo studies, myocardial I/R injury increases cPLA2 activity and cardiomyocyte apoptosis, which are reduced with group V sPLA2 knockout (46). In addition, circulating sPLA2 is associated with LPC generation in the animal model of cardiac fibrosis. Therefore, we speculated that expression of PLA2 protein may be upregulated and hydrolyze phospholipids to LPC in the setting of injury. There are two possible mechanisms for LPC production: i) LPC is released from apoptotic myocytes, which are generated by cPLA2; or ii) elevation of sPLA2 hydrolytic activity increases LPC production.

The involvement of COX-2/PGEs in different diseases via EP1receptors has been demonstrated in several cell culture and animal models. Treatment with both celecoxib and the EP1 receptor antagonist, SC 19220, and knockdown of the EP1 receptor have been demonstrated to attenuate the deposition of vascular collagen in hypertensive animals (22). COX-2 expression, associated with PGE2 secretion, has been shown to promote profibrotic activities via the EP1 receptor in human pancreatic stellate cells (23). In contrast, knockout of the EP2 receptors promotes eccentric cardiac hypertrophy and fibrosis in mice (24). COX-2-mediated PGE2 production suppresses the synthesis of collagen via the EP2 receptors in pancreatic stellate cells (25) and acts as an autocrine inhibitor of fibrogenesis in lung fibroblasts in vitro (47). Activation of the EP2 receptors by PGE2 inhibits TGF-β1-induced collagen synthesis in dermal fibroblasts (26). Our previous study has also shown that LPC induces COX-2 and IL-6 expression, which is associated with cardiac fibrosis in HCFs (5). In this study, we further revealed that LPC-induced COX-2/PGE2 expression was accompanied by secretion of collagen in HCFs, which was predominantly mediated via the EP2 receptors in HCFs.

Using antioxidant with nitrate is a potentially beneficial combinational therapy to improve myocardial infarct size for heart patients (48). Pretreatment with MitoTEMPO could reduce mitoROS levels and prevent chronic heart remodeling and myocyte viability and fibrotic area in experimental animals (49). Our data confirmed that MitoTEMPO improved LPC-induced collagen production in HCFs. Previous mechanistic evidence demonstrated that pretreatment with MitoTEMPO restores vascular function via suppression of mitoROS-mediated COX-2 expression (29). Consistently, our data demonstrated that LPC-induced mitoROS initially promoted signaling cascades and, consequently, activated COX-2/EP2-dependent collagen induction in HCFs. Further, our ex vivo data suggested that scavenging of mitoROS reduced oxidative stress and COX-2 expression in HCFs.

Abnormal activation of PKα is associated with the occurrence of heart failure via excessive collagen synthesis (50). Our results indicated that inhibition of PKα activation attenuated LPC-induced collagen production. In addition, LPC-activated PKα was necessary for the COX-2 expression in HCFs, similar to the other cell types (51). Our previous data demonstrated that PKα is an upstream component that activates NADPH oxidase activity leading to ROS generation (27); whereas, ROS are considered as an activator of PKC (35). Our present data revealed that scavenging of mitoROS attenuated PKα activation and suppressed the interaction between PKα and Drp1. Previous data have also demonstrated that PKCα enhances interaction with Drp1 and translocation into mitochondria and causes mitochondrial fragmentation in neuronal cell lines under oxidative stress or in hypertensive rats (11). On the other hand, sevoflurane-induced cardioprotection depends on PKCα activation via production of ROS (52). Although we have not yet demonstrated that activation of PKα may enhance mitochondrial translocation of Drp1, our present data revealed that activation of PKα enhanced its interaction with Drp1, which, consequently, led to mitochondrial fission and depolarization.

Mitochondrial membrane dynamics are regulated by Drp1, which is phosphorylated at different sites and, accordingly, exerts opposite effects in LPC-mediated responses. Phosphorylation of Drp1 at Ser616 and dephosphorylation of Drp1 at Ser637 enhances its activity and favors fission that results in mitochondrial dysfunction (13, 53). Drp1, which is phosphorylated at Ser616, is a substrate of CaMKII or PKCα (11, 54). The Ser637 of Drp1 is phosphorylated by PKA, which suppresses its GTPase activity and mitochondrial translocation (55, 56). In addition, dephosphorylation of Drp1 at Ser616 promotes mitochondrial fragmentation (57). Our data demonstrated PKα interacted with Drp1 and phosphorylated Drp1 at Ser616. In addition, pretreatment with an inhibitor of CaMKII failed to inhibit Drp1 phosphorylation at Ser616 (data not shown). Mitochondrial fission has been reported to cause mitoROS generation, while suppression of Drp1 activation by mdivi-1 attenuates mitochondrial fission and ROS production that is a promising target for treatment of cardiomyopathy and IR injury (9). Recent studies have reported that mitoROS initiates signaling cascades to mediate phosphorylation of Drp1 at Ser616 (13). Our data revealed that scavenging of mitoROS or inhibition of PKα attenuated the formation of a PKα-Drp1 complex and led to mitochondrial fission. Surprisingly, pretreatment with Gö 6976 or mdivi-1 could reduce mitoROS generation upon LPC stimulation for 60 min, whereas pretreatment with these inhibitors had no effect on mitoROS generation upon LPC stimulation for 30 min. Therefore, we speculated that LPC stimulated mitoROS generation, which regulated the formation of a PKα-Drp1 complex and, consequently, contributed to mitochondrial fission.

Drp1 mediates the changes in mitochondrial morphology and regulates gene expression. Previously, suppression of Drp1 activation by mdivi-1 reduced NF-κB-dependent gene expression by inhibiting ROS generation (33). Our previous data demonstrated that NADPH oxidase-derived ROS-mediated COX-2 expression by regulating JNK1/2, FoxO1 and -NF-κB transcriptional activity (5). We further
demonstrated that inhibition of Drp1 activation attenuated nuclear levels of FoxO1, phosphorylated at Ser256, and FoxO1-mediated COX-2 expression by downregulating ROS-mediated JNK1/2 activation. Furthermore, pretreatment with mdivi-1 reduced collagen expression as observed in ventricular fibroblasts (58). Our data suggested that Drp1 is associated with COX-2/PGE2-dependent collagen production in HCFs.

It has been demonstrated that knockout of Drp1 in a mouse model suppressed JNK1/2 phosphorylation in drug-induced liver toxicity (59). It is considered that activation of JNK 1/2 was regulated by mitoROS (59). Here, we demonstrated that pretreatment with a scavenger of mitoROS or an inhibitor of Drp1 phosphorylation at Ser210 attenuated JNK1/2 phosphorylation, suggesting that Drp1 could indirectly regulate JNK1/2 activation via ROS signaling of mitochondria. In addition, PKCa signaling has been observed to cross-talk with the JNK 1/2 pathway via activation of MEKK-SEK-MKK cascade. (60, 61). Our data demonstrated that both Gö6976 and mdivi-1 attenuated JNK1/2 phosphorylation. These observations suggested that PKCa and Drp1 are the regulators upstream of JNK1/2 activation. In particular, we have reported the involvement of the ROS-JNK1/2-FoxO1 axis in LPC-induced COX-2 expression (5). Moreover, LPC-induced collagen production via FoxO regulation was demonstrated in HCFs. Therefore, we further confirmed that the increase of collagen production induced by LPC was inhibited by respective pharmacological inhibitors.

The transcriptional activity of FoxO1 is related to its phosphorylation status and nuclear localization. FoxO1 accumulates in the nuclear fraction and contributes to differentiation and pro-collagen expression under TGF-β stimulation in CFs (37). Our data indicated that inhibition of FoxO1 attenuated extracellular collagen induction. Although TGF-β has been shown to inhibit FoxO1 phosphorylation, leading to its nuclear retention, our previous data demonstrated that accumulation of nuclear FoxO1 phosphorylated at Ser256 increased COX-2 expression via ROS induced by LPC (5). Furthermore, we demonstrated that induction of the COX-2/PGE2/EP4 axis by WT FoxO1 and S256D FoxO1 was necessary to induce collagen production, which was attenuated by silencing COX-2 or by celecoxib treatment in HCFs.

There are several limitations of this study. First, although the roles of these signaling molecules in the present study were examined using different pharmacological inhibitors, these may not be specific for these components. Second, the mechanisms of ROS generation from mitochondria are not clear in the present study. Third, although we investigate the cell models of HCFs that are derived from the human heart and in the setting of injury ex vivo, the present study cannot completely dissect the effects of LPC in vivo. Therefore, further studies are necessary to substantiate these findings and investigate the potential contribution of different cells in the heart, including cardiomyocytes and immune cells.

In conclusion, our findings demonstrated that the molecular mechanisms underlying LPC-induced mitoROS mediated the PKCa-Drp1-FoxO1 cascade and mitochondrial fission, which contributed to COX-2/PGE2-dependent collagen production via regulation of the EP4 receptor in HCFs, suggesting that pharmacological interventions that target EP4 receptors, scavenging of mitoROS, and attenuation of mitochondrial fission present therapeutic targets for strategies aimed at preventing cardiac fibrosis.

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