




Upregulation of COX-2 and PGE₂ Induced by TNF- α Mediated Through TNFR1/MitoROS/PKC α /P38 MAPK, JNK1/2/FoxO1 Cascade in Human Cardiac Fibroblasts

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Purpose: Tumor necrosis factor- α (TNF- α) has been shown to exert as a pathogenic factor in cardiac fibrosis and heart failure which were associated with the up-regulation of cyclooxygenase (COX)-2/prostaglandin E₂ (PGE₂) axis. However, whether TNF- α -induced COX-2/PGE₂ upregulation mediated through ROS-dependent cascade remains elusive in human cardiac fibroblasts (HCFs). This study aims to address the underlying mechanisms of TNF- α -induced COX-2/PGE₂ expression.

Methods: Here, we used TNF receptor neutralizing antibody (TNFR nAb), pharmacologic inhibitors, and siRNAs to dissect the involvement of signaling components examined by Western blot and ELISA in TNF- α -mediated responses in HCFs. MitoSOX Red was used to measure mitoROS generation. Isolation of subcellular fractions was performed to determine membrane translocation of PKC α . Promoter luciferase assay and chromatin immunoprecipitation (ChIP) assay were used to determine the role of transcription factor.

Results: We found that TNF- α time- and concentration-dependently upregulated COX-2 protein and mRNA expression as well as PGE₂ synthesis which was attenuated by TNFR1 nAb, the inhibitor of mitochondrial ROS scavenger (MitoTEMPO), protein kinase C [(PKC α , Gö6976), p38 MAPK [p38 inhibitor VIII, (p38i VIII)], JNK1/2 (SP600125), or forkhead box protein O1 [(FoxO1), AS1842856], and transfection with their respective siRNAs in HCFs. TNF- α -stimulated PKC α phosphorylation was inhibited by TNFR1 nAb, MitoTEMPO, or Gö6976. TNF- α stimulated phosphorylation of p38 MAPK and JNK1/2 was attenuated by TNFR1 nAb, MitoTEMPO, Gö6976, and their inhibitors p38i VIII and SP600125. Moreover, TNF- α -triggered FoxO1 phosphorylation was abolished by AS1842856, TNFR1 nAb, and its upstream inhibitors MitoTEMPO, Gö6976, p38i VIII, and SP600125. Phosphorylation of FoxO1 could enhance its interaction with the COX-2 promoter element revealed by ChIP assay, which was attenuated by AS1842856.

Conclusion: Our results suggested that TNF- α -induced COX-2/PGE₂ upregulation is mediated through TNFR1-dependent MitoROS/PKC α /p38 MAPK and JNK1/2 cascade to activate FoxO1 binding with the COX-2 promoter in HCFs.

Keywords: TNF- α , COX-2, PGE₂, PKC- α , mitochondrial ROS, FoxO1

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Introduction

Cardiac fibrosis is an important pathological process of heart remodeling following cardiovascular diseases and cardiac injuries from the mechanical force, chemical signals, or other insults. Cardiac fibroblasts, one of the major cells in heart tissue,

contribute to the process of cardiac remodeling and fibrosis by producing a large amount of fibrotic extracellular matrix and depositing collagen to repair the cardiomyocyte damage, alter electric conduction, increase vessel resistance, and cause the failure of the left ventricle.^{1,2} Following a cardiac injury event, the innate immune system is activated which subsequently increases pro-inflammatory cytokines release including tumor necrosis factor- α (TNF- α).³ TNF- α causes cardiomyopathy, left ventricular dysfunction, heart failure, and pulmonary edema, when it is overexpressed in human subjects.⁴⁻⁷ However, the detailed mechanisms of TNF- α -induced responses in cardiac fibroblasts are still unclear. Thus, in the present study, we applied human cardiac fibroblasts (HCFs) to investigate the TNF- α -mediated inflammatory responses.

Cyclooxygenase-2 (COX)-2 is a well-known enzyme that can catalyze the generation of prostanoids such as prostaglandin E₂ (PGE₂). PGE₂ is involved in many types of diseases particularly those related to inflammation. Previous studies have demonstrated that the COX-2/PGE₂ axis is upregulated in various types of cells including fibroblasts upon exposure to TNF- α .⁸⁻¹⁰ In cardiac physiology and diseases, the exact role of COX-2 remains controversial due to either protective or detrimental in the hearts reported in human beings and experimental models. However, the elevation of PGE₂ level could increase proliferation and cardiac fibrosis characteristics mediated through EP1 receptor via stimulating calcium signal pathway but not EP3 and EP4 in cardiac fibroblasts.^{11,12} Nonetheless, the role of TNF- α in COX-2 expression and PGE₂ production remains elusive in HCFs. We hypothesize that TNF- α could induce COX-2 expression and PGE₂ production in HCFs.

Excessive mitochondrial reactive oxygen species (mitoROS) generation due to mitochondrial dysfunction is implicated in triggering inflammation and contributing to cardiovascular diseases.¹³ Since intervention targeting mitoROS could be beneficial for cardiovascular diseases.^{14,15} Previous studies have indicated that ROS are involved in COX-2 upregulation in various types of cells.^{16,17} Protein kinase C (PKC) has been suggested as a target in the strategy of heart failure and ischemic heart disorders.^{18,19} The previous studies have shown that PKC α is required for COX-2 expression induced by TNF- α .^{10,20} Our previous study also demonstrated that lysophosphatidylcholine-induced COX-2 expression via a PKC α dependent cascade.²¹ MAPKs were also noticed to play a role in COX-2 expression by various stimuli including TNF- α in various types of cells.²²⁻²⁵ Our previous study, in HCFs, has

revealed that COX-2 expression/PGE₂ production is mediated through forkhead box protein O1 (FoxO1) activity.²¹ Therefore, the present study aimed to investigate whether TNF- α induced COX-2/PGE₂ axis upregulation mediated through its own receptors, mitoROS, and protein kinases to activate transcription factor FoxO1 activity. Here, we demonstrated that TNF- α exposure increases COX-2 and PGE₂ levels, at least in part, mediated through TNFR1/MitoROS/PKC α /p38 MAPK and JNK1/2/FoxO1 cascade in HCFs.

Materials and Methods

Materials

DMEM/F-12 medium, fetal bovine serum (FBS), and TRIzol reagent were purchased from Invitrogen (Carlsbad, CA). Hybond C membrane and enhanced chemiluminescence (ECL) Western blotting detection system were purchased from GE Healthcare Biosciences (Buckinghamshire, England, UK). Antibodies against TNF receptor (TNFR) 1 and 2, phospho-p38 MAPK, and phospho-JNK1/2 were purchased from Cell Signaling (Danvers, MA). SP600125, p38 inhibitor (p38i) VIII, Gö6976, MitoTEMPO, and AS1842856 were purchased from Enzo Life Science (Farmingdale, NY). Monoclonal anti-COX-2 antibody was purchased from NeoMarkers (Fremont, CA). Anti-GAPDH (mouse monoclonal antibody, Cat# MCA-1D4) antibody was obtained from EnCor Biotechnology (Gainesville, FL). Enzymes and other chemicals were purchased from Sigma (St. Louis, MO).

Cell Culture and Treatment

HCFs were purchased from ScienCell Research Laboratories (San Diego, CA) and cultured in DMEM/F-12 medium supplemented with 10% FBS and antibiotics (100 U/mL penicillin G, 100 ng/mL streptomycin, and 250 ng/mL fungizone) at 37°C in a humidified 5% CO₂ atmosphere. The use of the cell lines had been approved by Chang Gung University Institutional Animal Care and Use Committee (Approval Document No. CGU 16-046). When the cells reached confluence, they were treated with 0.05% trypsin/0.53 mM EDTA for 1 min at 37°C. The cell suspension was diluted with DMEM/F-12 containing 10% FBS to a concentration of 2×10⁵ cells/mL. The cell suspension was seeded in (1 mL/well) 12-well culture plates, (2 mL/well) 6-well culture plates, and (10 mL/dish) 10-cm culture dishes, and made quiescent at confluence by incubation in serum-free DMEM/F-12 for

24 h and then treated with TNF- α . The following experiments were carried out using HCF passages from 5 to 7.

Preparation of Samples and Western Blotting Analysis

HCFs were seeded in 12-well culture plates and made quiescent at confluence by incubation in serum-free DMEM/F-12 for 24 h. The cells were incubated with or without different concentrations of TNF- α at 37°C for the indicated time intervals. When inhibitors were applied, they were added 1 h prior to the exposure to TNF- α . The cell lysates were prepared as previously described.²¹ Then the denatured proteins were centrifuged at 13,000 \times g for 30 sec. The mixed samples (15 μ L) were subjected to SDS-PAGE using a 10% running gel. Proteins were transferred to nitrocellulose membrane and the membrane was incubated successively at room temperature with 5% (w/v) BSA in TTBS [Tris-HCl 50 mM, NaCl 150 mM, 0.05% (w/v), Tween 20, pH 7.4] for 1 h. Membranes were incubated overnight at 4°C with an antibody used at a dilution of 1:500–1000 in TTBS. Membranes were washed with TTBS several times and incubated with 1:2000 dilution of an anti-mouse or anti-rabbit antibody for 1 h. Following incubation, the membranes were washed comprehensively with TTBS. The immunoreactive bands were visualized by ECL reagent. The images of the immunoblots were captured using a UVP BioSpectrum 500 imaging system (Upland, CA), and densitometry analysis was conducted using UN-SCAN-IT gel software (Orem, UT).

Total RNA Extraction and Real-Time PCR Analysis

Total RNA was extracted with TRIzol according to the manufacturer's instructions. First-strand cDNA synthesis was performed with 5 μ g of total RNA using Oligo(dT)₁₅ as primers in a final volume of 20 μ L [25 ng/ μ L Oligo(dT)₁₅, 0.5 mM dNTPs, 10 mM DTT, 2 units/ μ L RNase inhibitor, and 10 units/ μ L 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. Real-time PCR was performed with the TaqMan (GeneDireX®, San Diego, CA) gene expression assay system, using primer and probe mixes for COX-2 and endogenous GAPDH control genes. PCRs were performed using a 7500 Real-Time PCR System (Applied Biosystems,

Foster City, CA). The relative amount of the target gene was calculated using $2^{(Ct_{\text{test gene}} - Ct_{\text{GAPDH}})}$ (Ct = threshold cycle). All experiments were performed in triplicate.

The cDNA obtained from 0.5 μ g total RNA was used as a template for PCR amplification. Oligonucleotide primers were designed based on GenBank entries for human COX-2 and GAPDH. The following primers were used for amplification reaction:

COX-2

Forward primer: 5'-

CAAAGTCAAATTTGACCCAGAACTAC-3';

Reverse primer: 5'-ACTGTTGATAGTTGTATTTCTGGTCATGA-3';

Probe: 5'-

AACACCCTCTACTGTCATCCCCTTC-3'.

GAPDH

Forward primer: 5'-GCCAGCCGAGCCACAT-3';

Reverse primer: 5'-CTTTACCAGAGTTAAAAGCA GCCC-3';

Probe: 5'- CCAAATCCGTTGACTCCGACCTTCA-3'.

Transfection and Promoter Luciferase Assay

Gene expression is derived from gene activation through various transcription activators and co-activators. In order to investigate TNF- α -induced COX-2 gene activity, COX-2-luciferase plasmid (COX-2-luci-plasmid) was constructed. COX-2-luci-plasmid was cloned with -459 to +9 of human COX-2 promoter region into pGL3-basic vector, as previously described.²¹ COX-2-luci-plasmid was transfected into HCFs together with β -galactosidase plasmid using the Lipofectamine reagent according to the manufacturer's instructions. To assess promoter activity, after treatment with TNF- α , cells were collected and disrupted by sonication in lysis buffer (25 mM Tris, pH 7.8, 2 mM EDTA, 1% Triton X-100, and 10% glycerol). After centrifugation, aliquots of the supernatants were tested for luciferase activity using the luciferase assay system (Promega, Madison, WI) according to the manufacturer's instructions. Firefly luciferase activities were standardized for β -galactosidase activity.

Measurement of PGE₂ Release

The growth-arrested cells were treated with 15 ng/mL TNF- α for the indicated time intervals. The media were

collected to analyze PGE₂ levels by using an ELISA kit as specified by the manufacturer (Cayman Chemicals).

Determination of MitoROS

HCFs were seeded in 6-well culture plates with coverslips, shifted to serum-free DMEM/F-12, and then incubated with 15 ng/mL TNF- α for the indicated time intervals in the presence or the absence of pharmacological inhibitors for 1 h prior to TNF- α exposure. Then, cells were incubated in DMEM/F12 medium containing 5 μ M of MitoSOX Red at 37°C for 10 min. The fluorescence signals were recorded (excitation/emission: 510 nm/570 nm) using a fluorescence plate reader (Synergy HT1; BioTek, Winooski, VT). To visualize mitoROS generation, the cells were washed thrice with medium, and their fluorescence intensity was determined by a fluorescence microscopy with a rhodamine filter (Axiovert 200M; Carl Zeiss, Thornwood, NY) and quantified using ImageJ software (1.41v; US National Institutes of Health).

Transient Transfection with siRNAs

SMARTpool RNA duplexes corresponding to JNK2 (HSS108550) and p38 α (HSS102352, HSS102353, HSS175313) siRNA were purchased from Invitrogen Life Technologies (Carlsbad, CA), and PKC α (SASI_Hs01_00018816), JNK1 (SASI_Hs02_00319556), FoxO1/FoxO1 (SASI_Hs01_0076732), and scramble siRNA were obtained from Sigma-Aldrich (St. Louis, MO). HCFs cultured onto 12-well plates at 70–80% confluence, transient transfection of siRNAs was carried out using Lipofectamine 2000 transfection reagent. Briefly, siRNA (100 nM) was formulated with Lipofectamine 2000 transfection reagent according to the manufacturer's instruction. The transfection complex was diluted into 900 μ L of DMEM/F12 medium and added directly to the cells. The cells were washed with PBS and maintained in serum-free DMEM/F-12 medium for 24 h before treatment with TNF- α .

Isolation of Subcellular Fractions

HCFs were seeded in 10 cm dishes and reached 90% confluence, they were shifted to serum-free DMEM/F-12 medium for 24 h, and then incubated with 15 ng/mL TNF- α 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). The protein concentrations of samples were determined by BCA assay. Samples from these fractions (200 μ L protein) were denatured, subjected to SDS-

PAGE using a 12% (w/v) running gel, and determined as described above.

ChIP Assay

To detect the association of transcription factors with human COX-2 promoter, ChIP analysis was performed, as previously described.²¹ 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 lysed, immune-precipitated, washed, and eluted. 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'-ACAATTGGTCGCTAACCGAG-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).

Statistical Analysis of Data

Statistical analysis was performed by using GraphPad Prism Program 6.0 software (GraphPad, San Diego, CA). We used one-way ANOVA followed by Dunnett's post hoc test when comparing more than two groups of data or nonparametric Kruskal–Wallis test, followed by Dunn's post hoc test when comparing multiple independent groups and ANOVA normality assumptions not met. P values of 0.05 were considered to be statistically significant. Post hoc tests were run only if F achieved P < 0.05 and there was no significance in the homogeneity of variance. All the data were expressed as the mean \pm SEM, at least three individual experiments (n= number of independent cell culture preparations). Error bars were omitted when they fell within the dimensions of the symbols.

Results

TNF- α Induces COX-2 Expression and PGE₂ Production in HCFs

To determine the effects of TNF- α on expression of COX-2 protein and gene in HCFs, the cells were incubated with various concentrations of TNF- α for the indicated time intervals before being harvested for analyses. As shown in Figure 1A, TNF- α induced COX-2 protein expression in a time- and concentration-

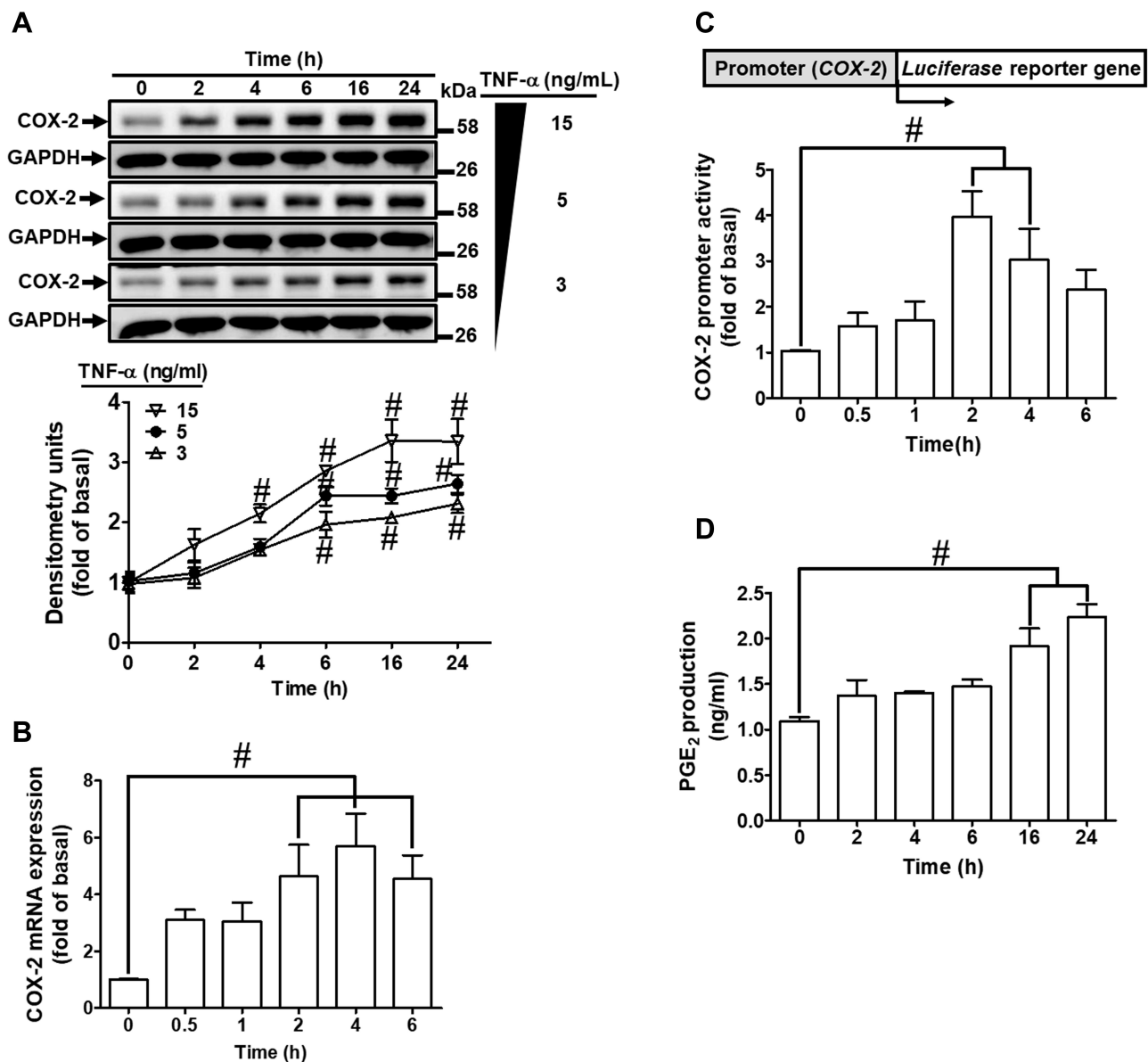


Figure 1 TNF- α -induced COX-2 expression and PGE₂ production in HCFs. **(A)** The cells were incubated with different concentrations of TNF- α (3, 5, and 15 ng/mL) for the indicated time intervals (2, 4, 6, 16, and 24 h). The levels of COX-2 and GAPDH protein were determined by Western blot. **(B)** The cells were incubated with 15 ng/mL TNF- α for the indicated time intervals (0.5, 1, 2, 4, and 6 h). The levels of *cox-2* and *gapdh* mRNA were analyzed by real-time PCR. **(C)** The cells were co-transfected with COX-2-luci-plasmid and β -galactosidase plasmid, and then incubated with 15 ng/mL TNF- α for the indicated time intervals (0.5, 1, 2, 4, and 6 h). The promoter activity was determined in the cell lysates using a promoter assay kit. **(D)** The media of 15 ng/mL TNF- α from **(A)** were saved to determine the levels of PGE₂ synthesis using a PGE₂ ELISA kit. Data are expressed as the mean \pm S.E.M. of three independent experiments. # $p < 0.05$, as compared with the control.

dependent manner. There was a significant increase response being observed within 6 h, when the cells were exposed to 15 ng/mL TNF- α . The maximal response was obtained within 16 h, and slightly declined within 24 h. GAPDH expression was served as an internal control to reveal equivalent loading amounts of protein. To further examine whether the effects of TNF- α on COX-2 expression were involved at the level of transcription, mRNA expression of COX-2

was determined by real-time PCR (Figure 1B). TNF- α -induced *cox-2* mRNA was significantly increased within 2 h, reached a maximum response within 4 h, and sustained for 6 h during the period of observation. To further investigate the effect of TNF- α on COX-2 expression at transcriptional level, COX-2 promoter activity was determined by using a firefly luciferase gene as a reporter of promoter activity (Figure 1C). TNF- α stimulated COX-2 promoter activity and reached

a maximum within 1 h. To observe whether TNF- α -increased the amount of COX-2 protein was accompanied by a corresponding production of PGE₂, the culture media were collected to measure PGE₂ levels using an ELISA kit (Figure 1D). Addition of TNF- α to HCFs induced a significant increase in PGE₂ production within 16 h and reached a maximum within 24 h. These results suggested that TNF- α -induced COX-2 expression accounts for increased PGE₂ production in HCFs.

TNF- α -Induced COX-2 Expression via TNFR1 in HCFs

To investigate which type of TNFRs is involved in TNF- α -induced COX-2 expression, HCFs were pretreated with either TNF receptor 1 or 2 neutralized antibody (TNFR1 or 2 nAb) for 1 h and then incubated with TNF- α (15 ng/mL) for 16 h. As shown in Figure 2A, pretreatment of HCFs with TNFR1 nAb significantly inhibited the TNF- α -induced COX-2 expression in a concentration-dependent manner, but not with TNFR2 nAb (Figure 2B). TNF- α -induced *cox-2* mRNA expression was also inhibited by pretreatment with TNFR1 nAb (Figure 2C), but not with TNFR2 nAb (Figure 2D) revealed by real-time PCR. These results suggested that TNF- α -induced COX-2 expression is mediated through TNFR1 in HCFs.

MitoROS Generation Induced by TNF- α mediates COX-2 and PGE₂ Upregulation

Previous studies have shown that TNF- α exposure alters mitochondrial integrity and causes mitochondrial DNA damage which is mediated by mitoROS elevation.^{26,27} To determine whether TNF- α increases mitoROS generation, HCFs were pretreated without or with MitoTEMPO for 1 h, and then treated with TNF- α for the indicated time intervals. As shown in Figure 3A, TNF- α (15 ng/mL) induced mitoROS generation which was attenuated by pretreatment with MitoTEMPO (100 nM) or TNFR1 nAb (1 μ g/mL) during the period of observation. To investigate whether mitoROS is involved in COX-2 upregulation, various concentrations of MitoTEMPO (10, 30, 100 nM) were applied before addition of TNF- α . As shown in Figure 3B, COX-2 protein expression induced by TNF- α was inhibited by MitoTEMPO in a concentration-dependent manner. The levels of *cox-2* mRNA expression and COX-2 promoter activity were also reduced by MitoTEMPO pretreatment (Figure 3C). In addition, we found that PGE₂ generation was returned to the basal

level following MitoTEMPO pretreatment in HCFs (Figure 3D). These results suggested that mitoROS generation induced by TNF- α mediates COX-2 and PGE₂ upregulation in HCFs.

Involvement of PKCs in COX-2 Expression Induced by TNF- α

Several studies have shown that PKC activation plays an important role in the cellular functions modulated by TNF- α .²⁸ To address the possible involvement of PKC activation in the TNF- α -induced COX-2 expression, the cells were pretreated with a pharmacological inhibitor of PKC α (Gö6976) for 1 h and then incubated with 15 ng/mL of TNF- α for 16 h. As shown in Figure 4A, pretreatment of HCFs with Gö6976 significantly attenuated the TNF- α -induced COX-2 expression in a concentration-dependent manner. TNF- α -induced *cox-2* mRNA expression and COX-2 promoter activity were also inhibited by pretreatment with Gö6976, revealed by real-time PCR and promoter activity assay, respectively (Figure 4B), suggesting that PKC α is involved in COX-2 expression induced by TNF- α in HCFs. To ensure the role of PKC α in COX-2 expression, as shown in Figure 4C, transfection with PKC α siRNA down-regulated the PKC α protein expression and also attenuated the TNF- α -induced COX-2 expression. To further investigate the role of PKC α activation in the TNF- α -induced COX-2 expression, we determined the effect of TNF- α on membrane translocation of PKC α in HCFs, revealed by Western blotting using an anti-PKC α antibody. We found that TNF- α stimulated translocation of PKC α from cytosol to membrane in a time-dependent manner (Figure 4D). To further observe whether PKC α phosphorylation participates in the TNF- α -induced COX-2 expression in HCFs, activation of this kinase was assayed by Western blotting using an antibody specific for the phosphorylated, active form of PKC α . We found that TNF- α stimulated a time-dependent phosphorylation of PKC α , which was attenuated by Gö6976 (Figure 4E). In addition, pretreatment with either TNFR1 nAb or MitoTEMPO also attenuated TNF- α -stimulated PKC α phosphorylation (Figure 4E), indicating that PKC α was a downstream component of TNFR1 and mitoROS in HCFs. We also found that TNF- α -induced PGE₂ production was inhibited by Gö6976 (Figure 4F). These results suggested that TNFR1/mitoROS-dependent activation of PKC α is involved in

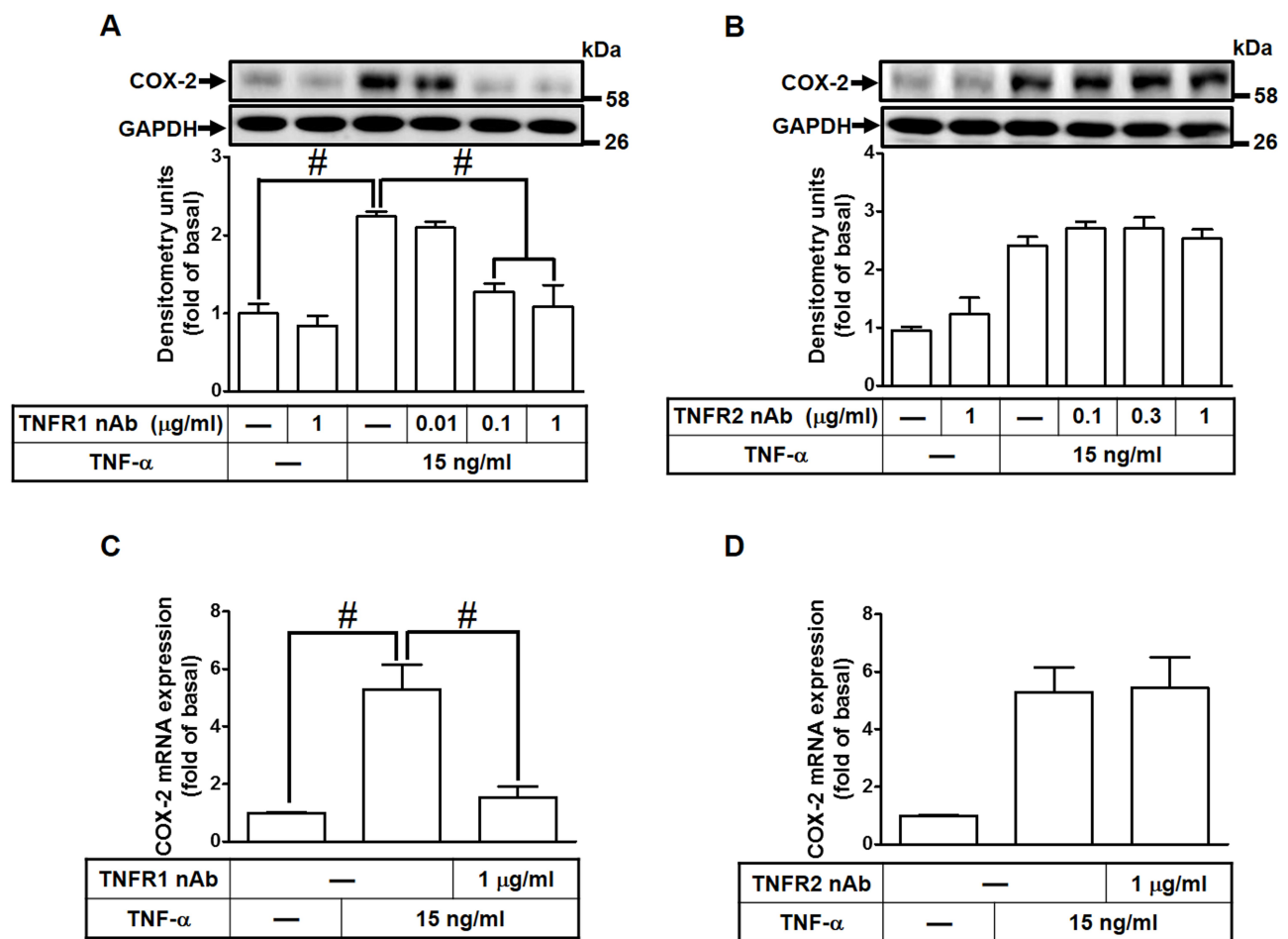


Figure 2 TNF- α -induced COX-2 expression and PGE₂ production mediated through TNFR1 in HCFs. **(A, B)** The cells were pretreated with various concentrations of either **(A)** TNFR1 or **(B)** TNFR2 neutralized antibody for 1 h and then incubated with 15 ng/mL TNF- α for 16 h. The levels of COX-2 and GAPDH protein were determined by Western blot. **(C, D)** The cells were pretreated with either **(C)** TNFR1 nAb or **(D)** TNFR2 nAb for 1 h and then incubated with TNF- α (15 ng/mL) for 4 h. The levels of *cox-2* and *gapdh* mRNA were analyzed by real-time PCR. Data are expressed as the mean \pm S.E.M. of three independent experiments. # p <0.05, as compared with the control.

COX-2 expression and PGE₂ production induced by TNF- α in HCFs.

TNF- α Induces COX-2 Expression via p38 MAPK Phosphorylation

To determine whether p38 MAPK is involved in the TNF- α -induced COX-2 expression in HCFs, a p38 MAPK inhibitor VIII (p38i VIII) was used. As shown in [Figure 5A](#), pretreatment with p38i VIII resulted in a significant attenuation of the TNF- α -induced COX-2 expression in a concentration-dependent manner revealed by Western blotting. Moreover, TNF- α -induced *cox-2* mRNA expression and COX-2 promoter activity were also inhibited by p38i VIII, revealed by real-time PCR and promoter activity, respectively ([Figure 5B](#)), suggesting that p38 MAPK is involved in

COX-2 expression induced by TNF- α in HCFs. To ensure the role of p38 MAPK in COX-2 expression, as shown in [Figure 5C](#), transfection with p38 MAPK siRNA down-regulated the p38 MAPK protein expression and also attenuated the TNF- α -induced COX-2 expression. To further investigate whether p38 MAPK phosphorylation participates in TNF- α -induced COX-2 expression in HCFs, activation of this kinase was assayed by Western blotting using an antibody specific for the phosphorylated, active form of p38 MAPK. We found that TNF- α stimulated a time-dependent phosphorylation of p38 MAPK which was attenuated by p38i VIII ([Figure 5D](#)). In addition, pretreatment with TNFR1 nAb, Gö6976, or MitoTEMPO also attenuated TNF- α -stimulated p38 MAPK phosphorylation ([Figure 5D](#)). TNF- α -stimulated PKC α phosphorylation

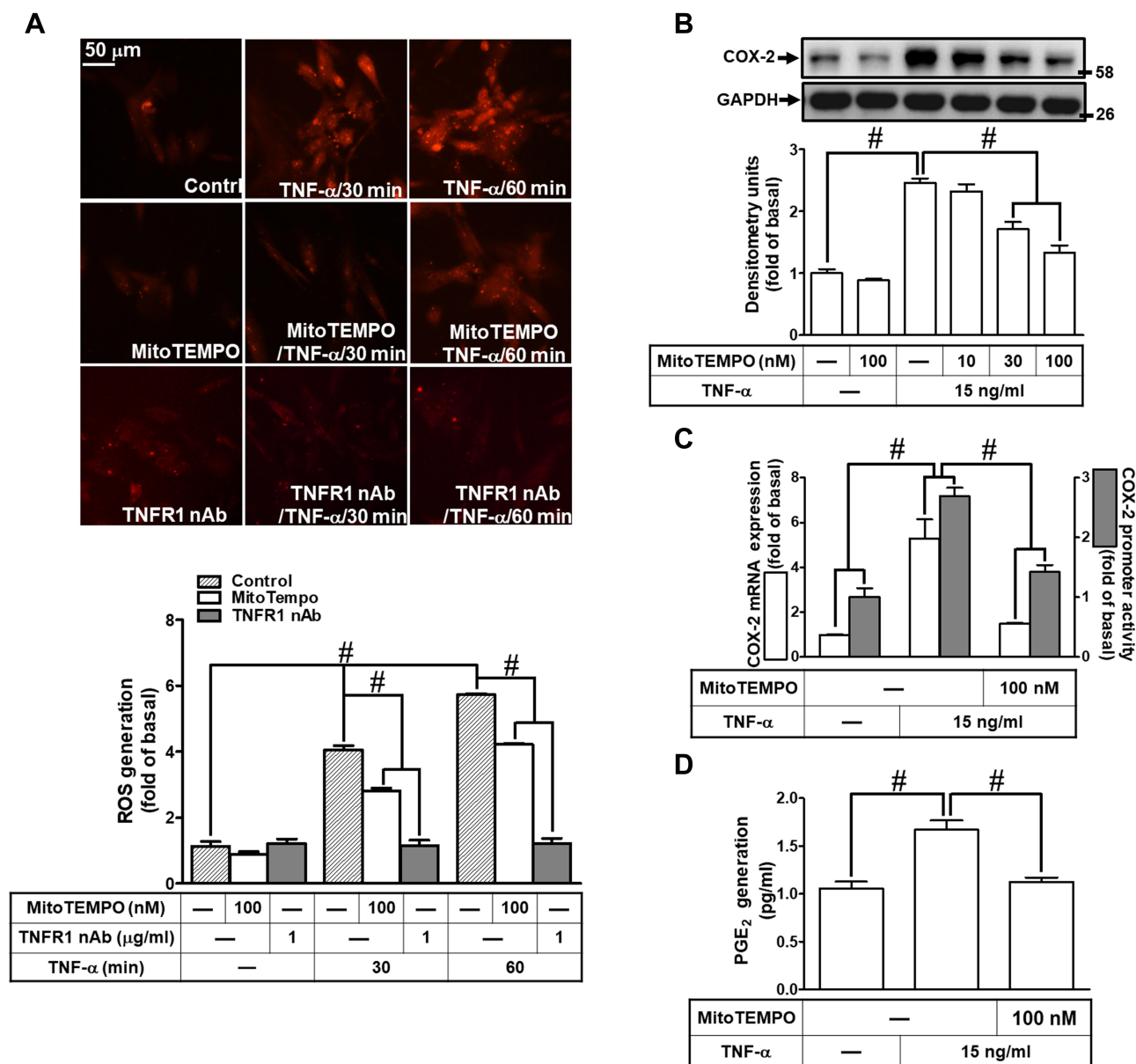


Figure 3 MitoROS generation induced by TNF- α mediates COX-2 and PGE₂ upregulation. **(A)** HCFs were pretreated without or with 100 nM MitoTEMPO or TNFR1 nAb (1 μ g/mL) for 1 h, and then incubated with 15 ng/mL TNF- α for the indicated time intervals (30 and 60 min). MitoROS production was determined by MitoSOX Red staining (scale bar = 50 μ m). The fluorescence unit of MitoSOX Red was measured using a fluorescent microplate reader. **(B)** HCFs were pretreated with various concentrations of MitoTEMPO (10, 30, and 100 nM) for 1 h and then incubated with 15 ng/mL TNF- α for 16 h. The levels of COX-2 and GAPDH protein were determined by Western blot. **(C)** The cells were pretreated with 100 nM MitoTEMPO for 1 h and then incubated with TNF- α (15 ng/mL) for 4 h. The levels of *cox-2* and *gapdh* mRNA were analyzed by real-time PCR (open bars). Cells were co-transfected with COX-2-luci-plasmid along with a β -galactosidase plasmid, sequentially pretreated with 100 nM MitoTEMPO for 1 h, and then incubated with 15 ng/mL TNF- α for 2 h. Promoter activity was determined in the cell lysates using a promoter assay kit (solid bars). **(D)** The media saved from **(A)** were used to determine the levels of PGE₂. Data are expressed as the mean \pm S.E.M. of three independent experiments. #*p*<0.05, as compared with the control.

was not significantly inhibited by p38i VIII (Figure 5D). Further, TNF- α -induced PGE₂ production was also inhibited by p38i VIII (Figure 5E). These results suggested that TNF- α -induced COX-2 expression and PGE₂ production is mediated through TNFR1/mitoROS/PKC α -dependent activation of p38 MAPK in HCFs.

TNF- α Induces COX-2 Expression via JNK1/2 Phosphorylation

To determine whether activation of JNK1/2 is involved in the TNF- α -induced COX-2 expression in HCFs, a pharmacological inhibitor of JNK1/2 (SP600125) was used. As shown in Figure 6A, pretreatment of HCFs with

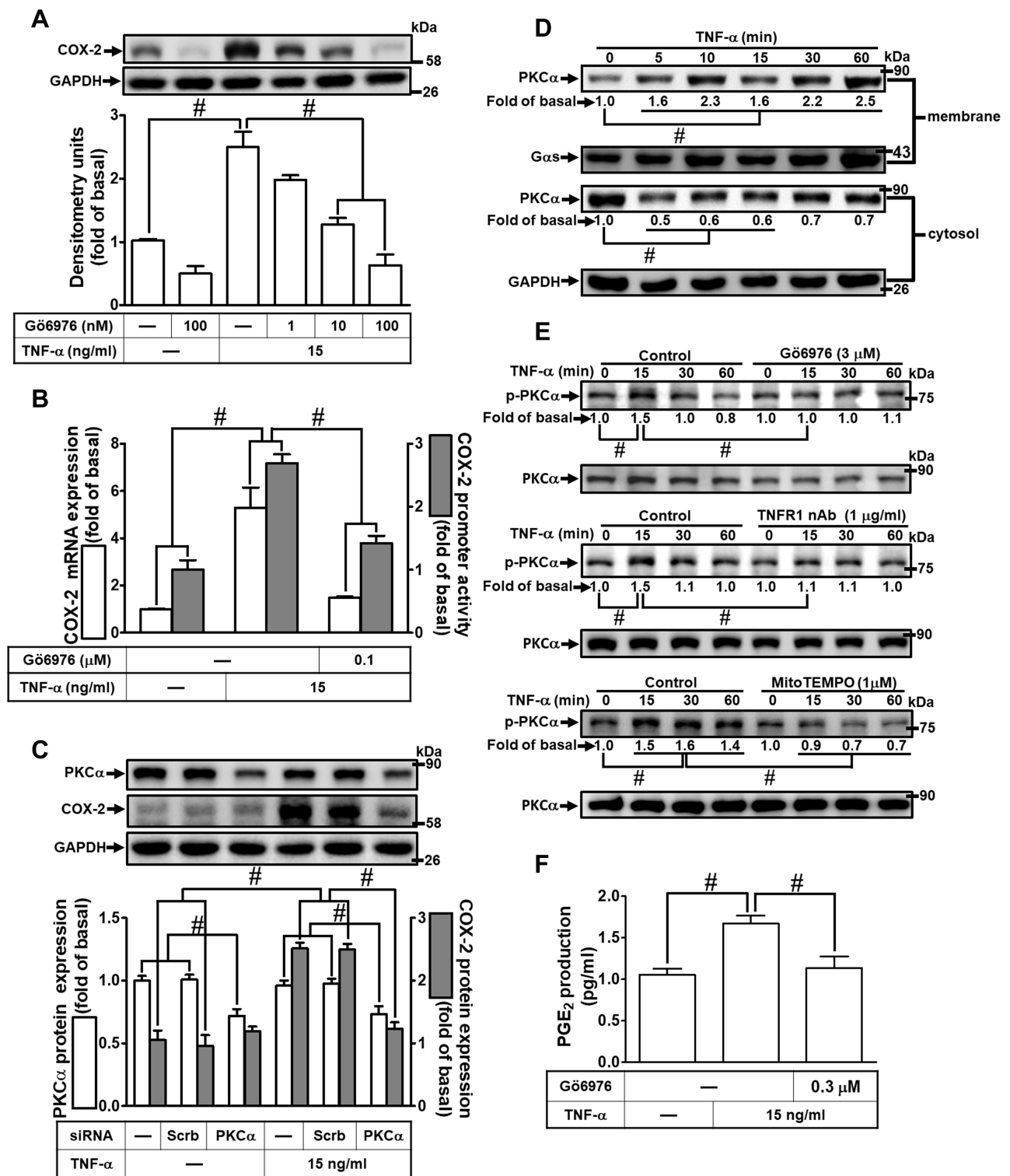


Figure 4 PKC α was involved in TNF- α -induced COX-2 expression and PGE $_2$ production in HCFs. **(A)** The cells were pretreated with various concentrations of Gö6976 (1, 10, and 100 nM) for 1 h and then incubated with 15 ng/mL TNF- α for 16 h. The levels of COX-2 and GAPDH protein were determined by Western blot. **(B)** The cells were pretreated with 100 nM Gö6976 for 1 h and then incubated with 15 ng/mL TNF- α for 4 h. The levels of *cox-2* and *gadh* mRNA were analyzed by real-time PCR (open bars). Cells were co-transfected with COX-2-luci-plasmid along with a β -galactosidase plasmid, pretreated with 100 nM Gö6976 for 1 h, and then incubated with 15 ng/mL TNF- α for 1 h. Promoter activity was determined in the cell lysates using a promoter assay kit (solid bars). **(C)** The cells were transfected with scrambled or PKC α siRNA and then incubated with 15 ng/mL TNF- α for 16 h. The levels of COX-2, PKC α , and GAPDH proteins were analyzed by Western blot. **(D)** The cells were incubated with 15 ng/mL TNF- α for the indicated time intervals (5, 10, 15, 30, and 60 min). The cytosolic and membrane fractions were analyzed by Western blot to determine the levels of PKC α with Gus and GAPDH as membrane and cytosolic internal control, respectively. **(E)** The cells were pretreated without or with 3 μ M Gö6976, 1 μ g/mL TNFR1 nAb, or 1 μ M MitoTEMPO for 1 h and then incubated with 15 ng/mL TNF- α for the indicated time intervals (15, 30, and 60 min). The levels of phospho-PKC α and total PKC α proteins were analyzed by Western blot. **(F)** The media saved from **(A)** were used to determine the levels of PGE $_2$. Data are expressed as the mean \pm S.E.M. of three independent experiments. # p <0.05, as compared with the control.

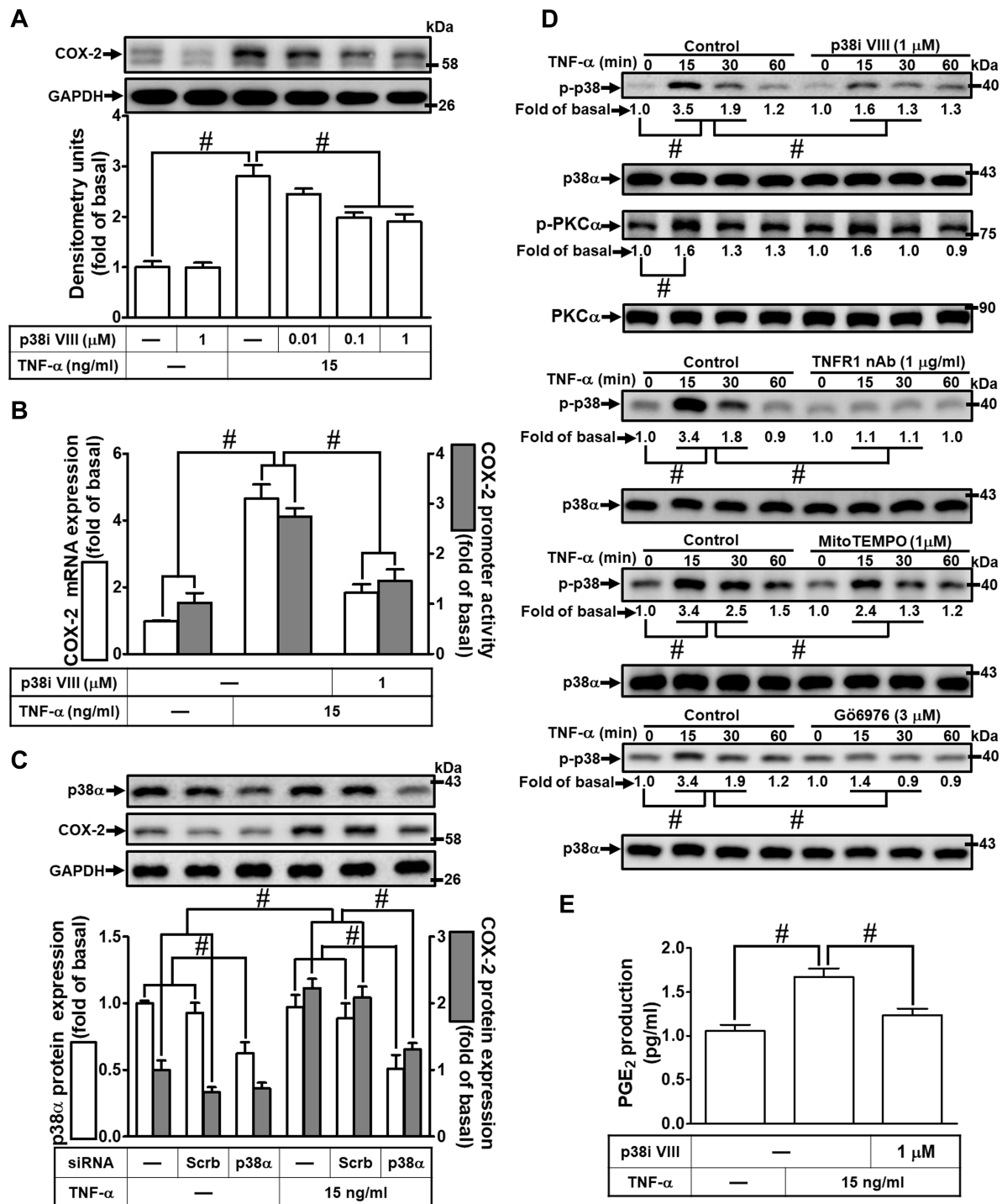


Figure 5 Involvement of p38 MAPK in TNF- α -induced COX-2 expression and PGE₂ production in HCFs. **(A)** The cells were pretreated with various concentrations of p38 inhibitor VIII (p38i VIII; 0.01, 0.1, and 1 μ M) for 1 h and then incubated with 15 ng/mL TNF- α for 16 h. The levels of COX-2 and GAPDH protein were determined by Western blot. **(B)** The cells were pretreated with 1 μ M p38i VIII for 1 h and then incubated with 15 ng/mL TNF- α for 4 h. The levels of *cox-2* and *gapdh* mRNA were analyzed by real-time PCR (open bars). Cells were co-transfected with COX-2-luci-plasmid along with a β -galactosidase plasmid, pretreated with 1 μ M p38i VIII for 1 h, and then incubated with 15 ng/mL TNF- α for 2 h. Promoter activity was determined in the cell lysates using a promoter assay kit (solid bars). **(C)** The cells were transfected with scrambled or p38 MAPK siRNA and then incubated with 15 ng/mL TNF- α for 16 h. The levels of COX-2, p38 MAPK, and GAPDH proteins were analyzed by Western blot. **(D)** The cells were pretreated with 1 μ M p38i VIII, 1 μ g/mL TNFR1 nAb, 3 μ M Gö6976, or 1 μ M MitoTEMPO for 1 h and then incubated with 15 ng/mL TNF- α for the indicated time intervals (15, 30, and 60 min). The levels of phospho-p38 MAPK, phospho-PKC α , total p38 MAPK, and total PKC α proteins were analyzed by Western blot. **(E)** The media saved from **(A)** were used to determine the levels of PGE₂. Data are expressed as the mean \pm S.E.M. of three independent experiments. #*p*<0.05, as compared with the control.

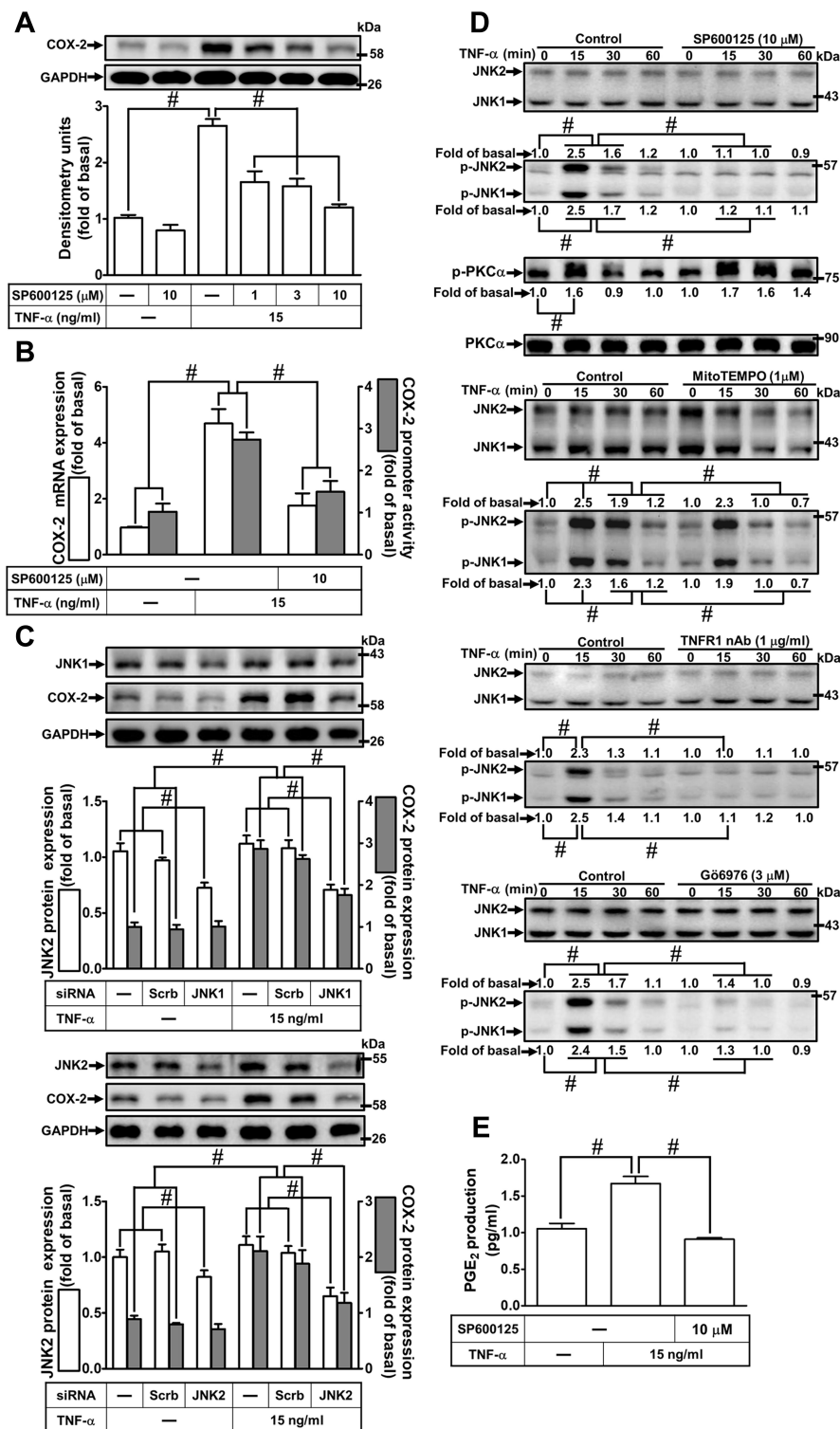


Figure 6 Involvement of JNK1/2 in TNF-α-induced COX-2 expression and PGE₂ production in HCFs. **(A)** The cells were pretreated with various concentrations of SP600125 (1, 3, and 10 μM) for 1 h and then incubated with 15 ng/mL TNF-α for 16 h. The levels of COX-2 and GAPDH protein were determined by Western blot. **(B)** The cells were pretreated with 10 μM SP600125 for 1 h and then incubated with 15 ng/mL TNF-α for 4 h. The levels of *cox-2* and *gapdh* mRNA were analyzed by real-time PCR (open bars). The cells were co-transfected with COX-2-luci-plasmid along with a β-galactosidase plasmid, pretreated with 10 μM SP600125 for 1 h, and then incubated with 15 ng/mL TNF-α for 2 h. Promoter activity was determined in the cell lysates using a promoter assay kit (solid bars). **(C)** The cells were transfected with scrambled, JNK1, or JNK2 siRNA, individually, and then incubated with 15 ng/mL TNF-α for 16 h. The levels of COX-2, JNK2, JNK1, and GAPDH proteins were analyzed by Western blot. **(D)** The cells were pretreated with 10 μM SP600125, 1 μg/mL TNFR1 nAb, 3 μM Gö6976, or 1 μM MitoTEMPO for 1 h and then incubated with 15 ng/mL TNF-α for the indicated time intervals (15, 30, and 60 min). The levels of phospho-JNK1/2, phospho-PKCα, total JNK1/2, and total PKCα proteins were analyzed by Western blot. **(E)** The media saved from **(A)** were used to determine the levels of PGE₂. Data are expressed as the mean ± S.E.M. of three independent experiments. #p<0.05, as compared with the control.

SP600125 effectively blocked the TNF- α -induced COX-2 expression in a concentration-dependent manner revealed by Western blotting. TNF- α -induced *cox-2* mRNA expression and COX-2 promoter activity were also inhibited by pretreatment with SP600125, revealed by real-time PCR and promoter activity assay, respectively (Figure 6B), suggesting that JNK1/2 is involved in COX-2 expression induced by TNF- α in HCFs. To ensure the role of JNK1/2 in COX-2 expression as shown in Figure 6C, transfection with JNK1/2 siRNA down-regulated the JNK1/2 protein expression and attenuated TNF- α -induced COX-2 expression. To further investigate whether JNK1/2 phosphorylation participates in TNF- α -induced COX-2 expression in HCFs, activation of this kinase was assayed by Western blotting using an antibody specific for the phosphorylated, active forms of JNK1/2. We found that TNF- α stimulated a time-dependent phosphorylation of JNK1/2 which was attenuated by SP600125 (Figure 6D). In addition, pretreatment with TNFR1 nAb, Gö6976, or MitoTEMPO also attenuated TNF- α -stimulated JNK1/2 phosphorylation (Figure 6D). TNF- α -stimulated PKC α phosphorylation was not significantly inhibited by SP600125 (Figure 6D). Further, TNF- α -induced PGE₂ production was also inhibited by SP600125 (Figure 6E). These results suggested that TNFR1/mitoROS/PKC α -dependent activation of JNK1/2 is involved in COX-2 expression and PGE₂ production induced by TNF- α .

FoxO1 is Involved in TNF- α -Induced COX-2 Expression in HCFs

The promoter region of COX-2 possesses serial binding elements for recognition of transcription factors including FoxO1. Thus, we examined whether activation of FoxO1 is required for COX-2 expression induced by TNF- α in HCFs, a pharmacological inhibitor of FoxO1 (AS1842856) was used. As shown in Figure 7A, pretreatment of HCFs with AS1842856 significantly attenuated the TNF- α -induced COX-2 protein expression. Moreover, TNF- α -induced *cox-2* mRNA and COX-2 promoter activity were also inhibited by pretreatment with AS1842856, revealed by real-time PCR and promoter activity assay, respectively (Figure 7B), suggesting that FoxO1 is involved in COX-2 expression induced by TNF- α in HCFs. To ensure the role of FoxO1 in COX-2 expression, as shown in Figure 7C, transfection with FoxO1 siRNA down-regulated the FoxO1 protein expression and attenuated the TNF- α -induced COX-2 expression. To further

investigate whether FoxO1 phosphorylation participates in TNF- α -induced COX-2 expression in HCFs, activation of this kinase was assayed by Western blotting using an antibody specific for the phosphorylated, active form of FoxO1. We found that TNF- α induced a time-dependent phosphorylation of FoxO1 which was attenuated by AS1842856 (Figure 7D). In addition, pretreatment with TNFR1 nAb, Gö6976, p38i VIII, SP600125, or MitoTEMPO also attenuated the TNF- α -stimulated FoxO1 phosphorylation (Figure 7D), indicating that FoxO1 was a downstream component of TNFR1, mitoROS, PKC α , p38 MAPK and JNK1/2 in HCFs. We further investigate the binding activity of FoxO1 with the COX-2 promoter following TNF- α exposure. We revealed that TNF- α exposure enhanced FoxO1 binding activity with the COX-2 promoter which was attenuated by AS1842856 pretreatment (Figure 7E). In addition, TNF- α -induced PGE₂ production was also inhibited by AS1842856 (Figure 7F). These results suggested that TNFR1/mitoROS/PKC α /p38 MAPK and JNK1/2-dependent activation of FoxO1 is involved in COX-2 expression and PGE₂ production induced by TNF- α in HCFs.

Discussion

Cardiac fibrosis occurs in association with coronary heart diseases and myocardial infarction which ultimately lead to heart failure. Elevated PGE₂ level is one of the key factors that contribute to cardiac fibrosis initiation by stimulating cardiac fibroblast migration and proliferation.^{11,12,29} The increase in PGE₂ level is closely associated with COX-2 upregulation as it catalyzes the formation of PGE₂. The signaling of several pro-inflammatory cytokines such as TNF- α has been known to induce COX-2-mediated PGE₂ elevation in human gingival fibroblasts, human lung epithelial cells, and human tracheal smooth muscle cells.⁸⁻¹⁰ Moreover, TNF- α has been involved in cardiovascular diseases where its activation has resulted in vascular dysfunction, cardiac remodeling, and heart failure.⁴ In the present study, we elucidated that the mechanisms by which TNF- α induces COX-2 overexpression and PGE₂ elevation are mediated through TNFR1-dependent MitoROS/PKC α /p38 MAPK and JNK1/2 cascade to activate FoxO1 binding with the COX-2 promoter in HCFs (Figure 8).

TNF- α is a key regulator in the initiation and potentiation of inflammation due to its role in the immune system that contributes to innate and adaptive immunity.³⁰ Deregulation of TNF- α expression and related-signalings

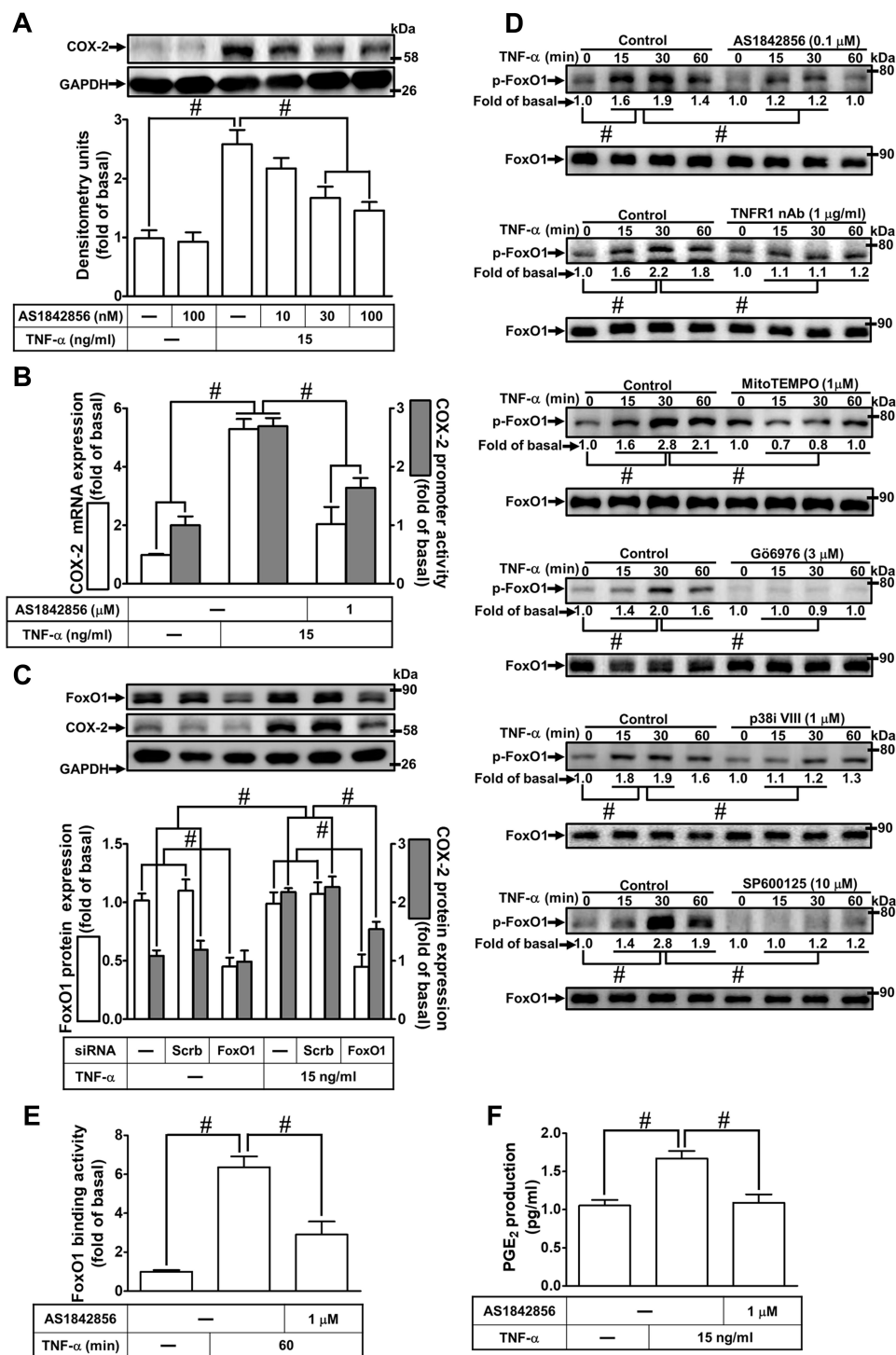


Figure 7 Involvement of FoxO1 in TNF- α -induced COX-2 expression and PGE₂ production in HCFs. **(A)** The cells were pretreated with various concentrations of AS1842856 (10, 30, and 100 nM) for 1 h and then incubated with 15 ng/mL TNF- α for 16 h. The levels of COX-2 and GAPDH protein were determined by Western blot. **(B)** The cells were pretreated with 1 μ M AS1842856 for 1 h and then incubated with 15 ng/mL TNF- α for 4 h. The levels of *cox-2* and *gapdh* mRNA were analyzed by real-time PCR (open bars). Cells were co-transfected with COX-2-luci-plasmid along with a β -galactosidase plasmid, sequentially pretreated with 1 μ M AS1842856 for 1 h, and then incubated with 15 ng/mL TNF- α for 2 h. Promoter activity was determined in the cell lysates using a promoter assay kit (solid bars). **(C)** The cells were transfected with scrambled or FoxO1 siRNA and then incubated with 15 ng/mL TNF- α for 16 h. The levels of COX-2, FoxO1, and GAPDH proteins were analyzed by Western blot. **(D)** The cells were pretreated with 0.1 μ M AS1842856, 1 μ g/mL TNFR1 nAb, 3 μ M Gö6976, 1 μ M MitoTEMPO, 1 μ M p38i VIII, or 10 μ M SP600125 for 1 h and then incubated with 15 ng/mL TNF- α for the indicated time intervals (15, 30, and 60 min). The levels of phospho-FoxO1 and total FoxO1 proteins were analyzed by Western blot. **(E)** The cells were pretreated with 0.1 μ M AS1842856 and then incubated with 15 ng/mL TNF- α for 60 min. The DNA binding ability of FoxO1 was detected by chromatin immunoprecipitation assay. **(F)** The media saved from **(A)** were used to determine the levels of PGE₂. Data are expressed as the mean \pm S.E.M. of three independent experiments. #p<0.05, as compared with the control.

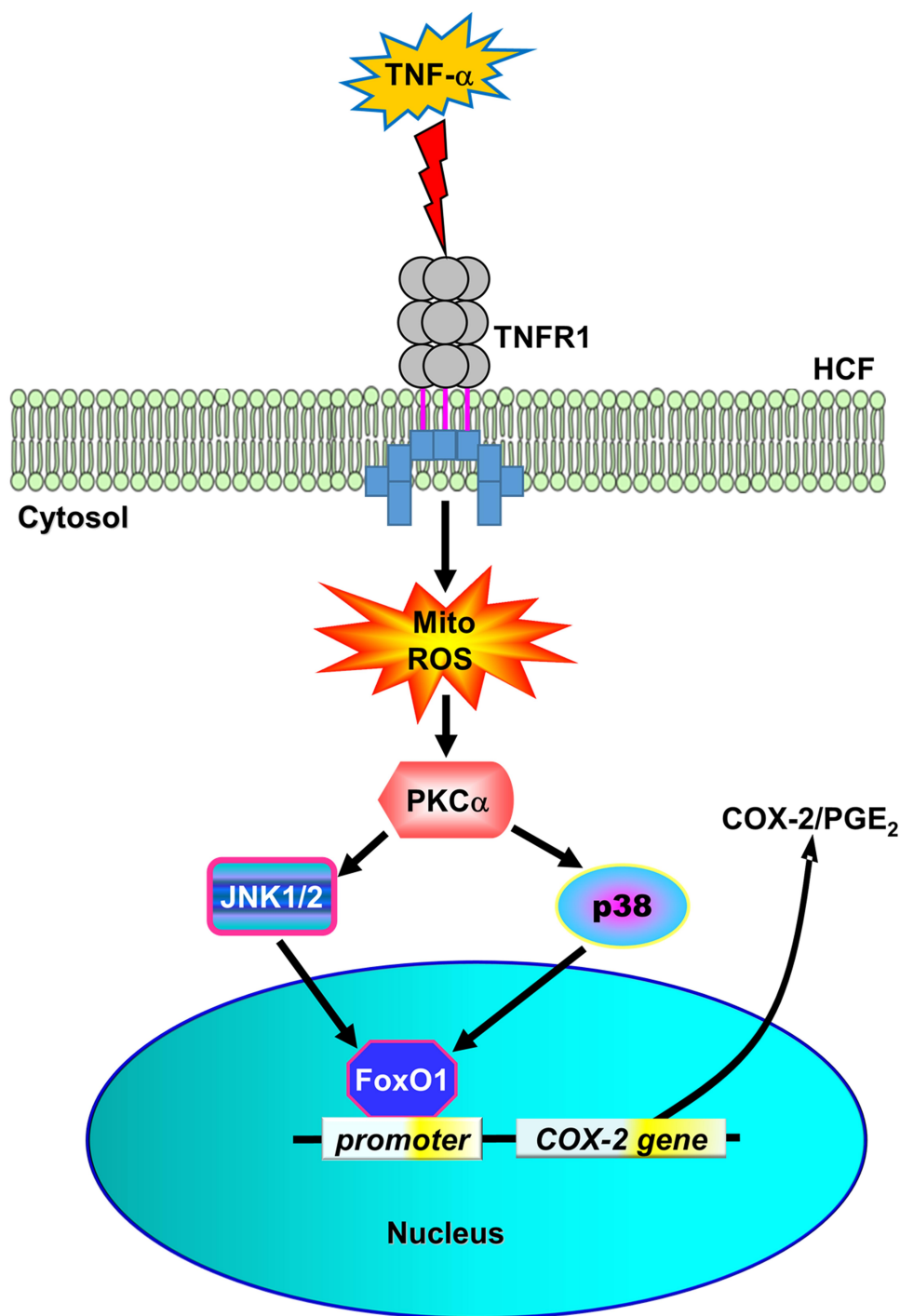


Figure 8 The schematic signaling pathways involved in TNF- α -induced COX-2 expression and PGE₂ production in HCFs. TNF- α activated TNFR1 to stimulate mitoROS generation, in turn, activating PKC α /p38 MAPK and JNK1/2 cascade-dependent FoxO1 activity and binding with COX-2 promoter leading to COX-2 expression and PGE₂ production in HCFs.

may trigger pathogenesis associated with tissue damage and chronic inflammation. Indeed, increased levels of TNF- α were identified in patients with autoimmune and degenerative diseases. An early study indicated that circulating levels of TNF- α are increased in patients with end-

stage cardiac disease.⁶ These findings have evoked a large effort to investigate the effects of TNF- α in the failing heart. Thus, several lines of evidence have shown that increased expression of TNF- α is involved in injured and failing hearts including animal experiments and human

patients.^{7,31} In fibroblasts, TNF- α may lead to extracellular matrix (ECM) degradation mediated through disrupting the balance between matrix metalloproteinases (MMPs) and their inhibitors.³² TNF- α binds to the two kinds of type I transmembrane receptors TNFR1 and TNFR2 that in their extracellular domains, contain four cysteine-rich domains (CRD). These cause TNF- α to induce opposing action in the immune system. TNF- α -mediated proinflammatory functions are most predominantly mediated through TNFR1 signaling. In contrast, in immunity, TNFR2 expression is mostly to suppress the development of autoimmune responses via regulation of immune response signaling in regulatory T cells (Tregs).³³ In the present study, we demonstrated that TNF- α -induced COX-2 upregulation is most predominantly mediated through a TNFR1-related pathway, because pretreatment with TNFR1 nAb attenuated COX-2 induction but not with TNFR2 nAb in HCFs. This finding is consistent with previous studies suggesting that the effect of TNF- α on COX-2 expression is mediated via TNFR1.^{34,35} These results may result from different binding affinity between TNF- α and two types of TNFR. A study has indicated that TNFR1 with a higher affinity ($K_d = 1.9 \times 10^{-11}$ M) than TNFR2 ($K_d = 4.2 \times 10^{-10}$ M) binds with TNF- α .³⁶ These results suggest that TNF- α -induced COX-2/PGE₂ elevation in cardiac fibroblasts is initiated via TNFR1 activity and its downstream signaling cascades and possibly involved in the development of cardiovascular disorders.³⁷

ROS are involved in the pathogenesis of disease development, which induce modifications in subcellular organelles such as mitochondria, nucleus, sarcoplasmic reticulum, and sarcolemma through oxidative modification of most intracellular macromolecules (such as DNA, proteins, or lipids). Thus, an increased production of ROS has already been found to participate in several cardiac diseases such as myocardial fibrosis,^{38,39} heart failure,⁴⁰ and myocardial infarction.⁴¹ Although global oxidative stress comes from different resources, mitochondrial compartment contributes to the majority of intracellular ROS about 90%. Mitochondrial dysfunction triggers excessive ROS generation and simultaneously induces irreversible damage to mitochondria due to mitochondria are also a vulnerable target of ROS, which occurs during the development of cardiovascular diseases.⁴² Growing evidence suggests that mitochondrial ROS production plays a crucial role in many diabetes-related cardiovascular complications and cardiac hypertrophy.^{43,44} It has been noted that TNFR1 activation positively affects ROS and

reactive nitrogen species generation.^{17,45} By utilizing MitoTEMPO, a mitochondria-targeted ROS scavenger, we demonstrated that TNF- α -induced mitoROS was decreased along with COX-2 expression and PGE₂ production. These results revealed the role of mitoROS in the TNF- α -induced COX-2 expression and PGE₂ production. These results are consistent with previous studies demonstrating that ROS are an important factor for COX-2 upregulation in various types of cells.^{16,17}

PKC plays an important role in many human cardiovascular diseases, including ischaemic heart disease¹⁸ and heart failure.⁴⁶ In particular, PKC α , one of the PKC isozymes has emerged as an important protein kinase contributing to cardiac dysfunction, cardiac fibrosis, and even heart failure.^{47,48} PKC α can cause decreased cardiac contractility, the force of myofilaments, and uncoupling of β -adrenergic receptors in the pathology of heart failure.^{19,49} Although there are not consistent results to be concluded in PKC as a therapeutic target for cardiovascular diseases, PKC inhibitors have been considered as a potential strategy for cardiovascular diseases.^{50,51} Several lines of evidence indicated that various stimuli including TNF- α might activate PKC α leading to upregulation of COX-2 expression and PGE₂ release in various types of cells.^{20,52} Our results also consistently showed that pretreatment with Gö6976 (PKC α inhibitor) or transfection with PKC α siRNA attenuated COX-2 expression induced by TNF- α . Under normal status, inactive PKC α is distributed in the cytosol, upon stimulation, PKC α can be activated and translocated to the cell membrane and subsequently activates its downstream signaling components.⁵³ As expected, following TNF- α exposure, we observed the elevated protein expression of PKC α in the cell membrane as early as 5 min until the end of time intervals observed. In contrast, PKC α expression in the cytosol was simultaneously decreased during the first 15 min of TNF- α exposure. Our data demonstrated that PKC α activation and translocation following TNF- α exposure occurred at a relatively early event. Our results revealed that pretreatment with TNFR1 nAb or MitoTEMPO decreased PKC α phosphorylation, indicating that TNFR1/mitoROS is required for PKC α phosphorylation in HCFs.

Previous studies have shown that p38 MAPK and JNK1/2 signaling cascades mediate TNFR1 responses elicited by TNF- α in heart diseases.³⁷ TNF- α has been demonstrated to induce COX-2 expression mediated through p38 MAPK activity in various types of cells.^{24,25} Consistently, we revealed that TNF- α -induced COX-2 and PGE₂ upregulation were mediated by p38 MAPK signaling pathway, which was attenuated by pretreatment with p38i VIII or transfection with p38 siRNA.

JNK1/2 was also involved in COX-2 upregulation induced by various mediators in different types of cells.^{22,23} Our results also suggested that JNK1/2 plays a crucial role in COX-2 expression and PGE₂ production induced by TNF- α in HCFs, which was inhibited by pretreatment with JNK1/2 inhibitor SP600125 or transfection with JNK1/2 siRNA. In addition, our data suggested that PKC α is required for p38 MAPK and JNK1/2 activation, verified by that the levels of both p38 MAPK and JNK1/2 phosphorylation were attenuated by pretreatment with TNFR1 nAb, mitoROS inhibitor MitoTEMP, or PKC α inhibitor Gö6976. In contrast, pretreatment with either p38i VIII or SP600125 had no significant effect on PKC α phosphorylation stimulated by TNF- α . Thus, our results further revealed that in HCFs, both p38 MAPK and JNK1/2 are activated by TNF- α through a TNFR1-dependent mitoROS/PKC α signaling pathway leading to COX-2 expression and PGE₂ production.

The FoxO1 transcription factor plays an important role in the widely physiological processes, including metabolism, proliferation, apoptosis, stress, and inflammation by regulating its target genes.⁵⁴ FoxO1 has been shown to play a crucial role in the development of cardiac hypertrophy due to regulating the expression of target genes in the myocardium that is involved in calcium homeostasis, autophagy, cardiac growth/protein synthesis, and cell apoptosis.⁵⁴ FoxO1 is activated upon exposure to various inflammatory mediators such as sphingosine 1-phosphate,⁵⁵ lysophosphatidylcholine,⁵⁶ and LPS.⁵⁷ Besides, TNF- α has been demonstrated to activate FoxO1 in both in vitro and in vivo studies.⁵⁸ In our previous studies, FoxO1 has been demonstrated to be involved in COX-2 expression.^{55,56} In the present study, we clarified that TNF- α -induced COX-2 and PGE₂ upregulation is, at least in part, dependent on FoxO1 activity. To our best knowledge, this is the first time to reveal that FoxO1 is involved in the TNF- α -induced COX-2 expression in HCFs. In addition, this result was verified by ChIP assay showing that the TNF- α -triggered binding activity of FoxO1 to COX-2 promoter was blocked by pretreatment with FoxO1 inhibitor AS1842856. Moreover, our results showed that FoxO1 activity was inhibited by pretreatment with TNFR1 nAb, MitoTEMPO, Gö6976, p38i VIII, or SP600125 in HCFs challenged with TNF- α .

Conclusions

In this study, we depicted the mechanisms by which TNF- α induced COX-2 upregulation and PGE₂ release in HCFs are, at least partially, mediated through TNFR1/MitoROS/

PKC α /JNK1/2 and p38 MAPK-dependent FoxO1 activity. The elucidation of the mechanism of TNF- α -induced COX-2/PGE₂ production in HCFs provides us with a better understanding of the pathogenesis of TNF- α in heart disorders and potential strategies in therapies for cardiovascular diseases.

Abbreviations

BSA, Bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; ECL, Enhanced Chemiluminescence; ELISA, Enzyme-linked immunosorbent assay; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; HO-1, Heme oxygenase-1; HCFs, Heart cardiac fibroblasts; JNK, c-Jun N-terminal kinase; MAPK, Mitogen-activated protein kinase; PBS, Phosphate-buffered saline; PKC, Protein kinase C; ROS, Reactive oxygen species; SDS-PAGE, Sodium dodecyl sulfate polyacrylamide gel electrophoresis; siRNA, Small interfering RNA; Tris-HCl, Tris[hydroxymethyl]aminomethane; TTBS, Tween-Tris-buffered saline.

Data Sharing Statement

The data sets used and analyzed during the current study are available from the corresponding author on reasonable request.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors report no conflicts of interest in this work.

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