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RESEARCH PAPER

PAR1-dependent COX-2/PGE_2 production contributes to cell proliferation via EP_2 receptors in primary human cardiomyocytes

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BACKGROUND AND PURPOSE

Different protease-activated receptors (PARs) activated by thrombin are involved in cardiovascular disease, via up-regulation of inflammatory proteins including COX-2. However, the mechanisms underlying thrombin-regulated COX-2 expression in human cardiomyocytes remain unclear.

EXPERIMENTAL APPROACH

Human cardiomyocytes were used in the study. Thrombin-induced COX-2 protein and mRNA expression, and signalling pathways were determined by Western blot, real-time PCR and COX-2 promoter luciferase reporter assays, and pharmacological inhibitors or siRNAs. PGE_2 generation and cell proliferation were also determined.

KEY RESULTS

Thrombin-induced COX-2 protein and mRNA expression, promoter activity and PGE_2 release was attenuated by the PAR1 antagonist (SCH79797) or the inhibitors of proteinase activity (PPACK), MEK1/2 (U0126), p38 MAPK (SB202190) or JNK1/2 (SP600125), and transfection with small interfering RNA (siRNA) of PAR1, p38, p42 or JNK2. These results suggested that PAR1-dependent MAPKs participate in thrombin-induced COX-2 expression in human cardiomyocytes. Moreover, thrombin stimulated phosphorylation of MAPKs, which was attenuated by PPACK and SCH79797. Furthermore, thrombin-induced COX-2 expression was blocked by the inhibitors of AP-1 (tanshinone IIA) and NF-κB (helenalin). Moreover, thrombin-stimulated phosphorylation of c-Jun/AP-1 and p65/NF-κB was attenuated by tanshinone IIA and helenalin, respectively, suggesting that thrombin induces COX-2 expression via PAR1/MAPKs/AP-1 or the NF-κB pathway. Functionally, thrombin increased human cardiomyocyte proliferation through the COX-2/PGE_2 system linking to EP_2 receptors, as determined by proliferating cell nuclear antigen and cyclin D1 expression.

CONCLUSIONS AND IMPLICATIONS

These findings demonstrate that MAPKs-mediated activation of AP-1/NF-κB pathways is, at least in part, required for COX-2/PGE_2/EP_2-triggered cell proliferation in human cardiomyocytes.

Abbreviations

ANF, atrial natriuretic factor; PAR, protease-activated receptor; PCNA, proliferating cell nuclear antigen

Table of Links

TARGETS	LIGANDS	
COX-2	AH6809	Thrombin
EP_2 prostanoid receptor	Celecoxib	U0126
Erk1/2	PGE_2	
JNK1/2	SB202190	
p38 MAPK	SP600125	
PAR1	TFLLR-NH_2	

This Table lists the protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and the Concise Guide to PHARMACOLOGY 2013/14 (Alexander *et al.*, 2013a, Alexander *et al.*, 2013b).

Introduction

Heart failure, one of the cardiovascular conditions with high morbidity and mortality, describes a situation where the heart is incapable of supplying sufficient blood for circulation (Heineke and Molkentin, 2006). The characteristic response of heart failure occurs in the ventricular chambers. In response to cytokines, neurohormones, growth factors and cardiac injury, ventricular cardiomyocytes increase in size and thickness of walls and reorganize the sarcomeres but reduce the internal dimensions of the ventricular chamber in an attempt to provide sufficient blood for peripheral tissues and organs (Ritter and Neyses, 2003).

The major action of thrombin is to prevent blood loss at the sites of injury through converting fibrinogen to fibrin by forming rigid blood clots (Ariens, 2013) and most studies concerning thrombin have focused on vascular endothelium, platelets and other cardiovascular components, but little is known about its role in the heart. Thrombin exerts its physiological and pathological processes via cellular surface receptors, known as protease-activated receptors (PARs), a class of the GPCR family (Coughlin, 2000). The PARs are divided into four subtypes, PAR1, PAR-2, PAR-3 and PAR-4, in cardiovascular systems. PAR1 is widespread in cells and tissues and its activation regarding platelet activation and vasodilatation (Coughlin, 1999). PAR1, PAR-2 and PAR-4 are expressed in myocardium where activation of PAR1 activation leads to a broad range of signalling events in cardiomyocytes (Sabri *et al.*, 2003). PAR1 is functionally linked to G-protein, PLC, MAPKs and Akt (Barnes *et al.*, 2004). Moreover, PAR1 and PAR-2 may share the common signalling pathways and contribute to dilated hypertrophy in neonatal rat cardiomyocytes (Sabri *et al.*, 2000; Moshal *et al.*, 2005a). Activation of PAR1 by thrombin increases atrial natriuretic peptide mRNA levels in neonatal rat ventricular cardiomyocyte (Glembotski *et al.*, 1993). Furthermore, up-regulation of PAR1 has been demonstrated to contribute to cardiac hypertrophy and remodelling, suggesting that PAR1 may be a novel therapeutic target in heart failure (Moshal *et al.*, 2005b; Pawlinski *et al.*, 2007).

Two COX isoforms have been identified as the enzymes responsible for PG synthesis in most tissues (Duvivier *et al.*, 1975). The COXs convert arachidonic acid into its hydroperoxy-endoperoxide, PGG_2, which is quickly reduced to PGH_2 and thence to various PG end-products (Streicher and Wang, 2008). Among COXs, COX-1 is ubiquitously expressed in mammals mediating physiological homeostasis (Rolin *et al.*, 2007). COX-2 can be rapidly induced in response to many proinflammatory mediators, suggesting that COX-2 may participate in inflammation (Smith *et al.*, 2000). Moreover, up-regulation of COX-2 has been found in cardiomyocytes from heart failure patients whereas it was undetectable in healthy controls (Norman *et al.*, 1998; Saito and Giaid, 1999). In addition to COX-2, high levels of PGs also have been found in patients with heart failure (Kotlyar *et al.*, 2006). However, the relationship between thrombin and COX-2 in cardiomyocytes remains to be investigated.

In the present study, we sought to elucidate the mechanisms underlying thrombin-induced COX-2 expression in human cardiomyocytes. The results demonstrated that thrombin-induced COX-2 expression and PGE_2 generation is mediated through a PAR1-dependent signalling pathway, including p38 MAPK, Erk1/2, JNK1/2, AP-1 and NF-κB. Moreover, we confirmed that thrombin could induce cell proliferation via PAR1-dependent COX-2/PGE_2/EP_2 recptor signalling pathway. These results provided new insight into therapeutic targets on the mechanisms of thrombin acts that could potentially ameliorate heart failure.

Methods

Primary human cardiomyocyte culture

Primary human neonatal cardiomyocytes were purchased from ScienCell Research Laboratories (San Diego, CA, USA). The origin of cells was isolated and cultured from one neonatal donor, and confirmed by immunostaining for sarcomeric α-actinin myosin staining. The cell population consisted of 90~95% cardiomyocytes. The cells were cultured in a commercial growth medium supplemented with 5% FBS, growth supplements (10 μg·mL^{-1} BSA, 10 μg·mL^{-1} apo-transferrin, 5 μg·mL^{-1} insulin, 2 ng·mL^{-1} EGF, 2 ng·mL^{-1} FGF-2, 2 ng·mL^{-1} IGF-1, 1 μg·mL^{-1} hydrocortisone and 100 nM retinoic acid), and antibiotics (100 U·mL^{-1} penicillin G, 100 μg·mL^{-1} streptomycin and 250 ng·mL^{-1} fungizone) at 37°C in a humidified

5% CO_2 atmosphere. When the cultures reached confluence (4 days), cells were treated with 0.05% trypsin/1 mM EDTA for 3 min at 37°C. The cell suspension was diluted with commercial growth medium to a concentration of 2×10^5 cells·mL^{-1}. The cell suspension was plated onto (2 mL per well) 6-well culture plates and (10 mL per dish) 10 cm culture dishes for the measurement of protein expression and mRNA accumulation respectively. Culture medium was changed after 48 h and then every 3 days.

Sample preparation and Western blot analysis

Human cardiomyocytes were plated onto 6-well culture plates and made quiescent at confluence by incubation in DMEM/F-12 with 0.05% BSA and 2 mM glutamine for 24 h. Growth-arrested cells were incubated with or without different concentrations of thrombin at 37°C for the indicated time intervals. When inhibitors were used, they were added 1 h prior to the application of thrombin. After incubation, the cells were then rapidly washed with ice-cold PBS, scraped and collected by centrifugation at 1000× *g* for 10 min. The collected whole cells were lysed with ice-cold lysis buffer containing: 25 mM Tris-HCl, pH 7.4, 25 mM NaCl, 25 mM NaF, 25 mM sodium pyrophosphate, 1 mM sodium vanadate, 2.5 mM EDTA, 2.5 mM EGTA, 0.05% Triton X-100, 0.5% SDS, 0.5% deoxycholate, 0.5% NP-40, 5 μg·mL^{-1} leupeptin, 5 μg·mL^{-1} aprotinin, and 1 mM phenylmethylsulfonyl fluoride. The lysates were centrifuged at 45 000× *g* for 1 h at 4°C to yield the whole cell extract. The protein concentration was determined by using BCA reagents according to the instructions of the manufacturer. Samples from these supernatant fractions (30 μg protein) were denatured and subjected to SDS-PAGE using a 10% running gel. Proteins were transferred to nitrocellulose membrane and incubated successively at room temperature with 5% BSA in Tween-Tris buffered saline (50 mM Tris-HCl, 150 mM NaCl, 0.05% Tween 20, pH 7.4) for 1 h. Membranes were incubated overnight at 4°C with their respective component antibody or anti-GAPDH antibody used at a dilution of 1:2000 in Tween-Tris buffered saline. Membranes were washed with Tween-Tris buffered saline four times for 5 min each, incubated with a 1:1500 dilution of anti-mouse horseradish peroxidase antibody for 1 h. Following each incubation, the membrane was washed extensively with Tween-Tris buffered saline. The immunoreactive bands were detected by ECL reagents and captured by a UVP BioSpectrum 500 Imaging System (Upland, CA, USA). The image densitometry analysis was quantified by an UN-SCAN-IT gel software (Orem, UT, USA).

Total RNA extraction and RT-PCR analysis

Total RNA was isolated from human cardiomyocytes (10 cm culture dishes) incubated with thrombin for the indicated time intervals, using TRIzol according to the protocol of the manufacturer. RNA concentration was spectrophotometrically determined at 260 nm. First strand cDNA synthesis was performed with 2 μg of total RNA using random hexamers as primers in a final volume of 20 μL (5 μg·μL^{-1} random hexamers, 1 mM dNTPs, 2 units·μL^{-1} RNasin and 10 units·μL^{-1} Moloney murine leukaemia virus reverse transcriptase). The reaction was carried out at 37°C for 60 min. cDNAs encoding COX-2, β-actin, PAR1-4 and EP1-4 were amplified from 3–5 μL of the cDNA reaction mixture using specific gene primers. The primers, as previously described (Kunisch *et al.*, 2009; Seminario-Vidal *et al.*, 2009), were used for amplification reaction.

Real-time RT-PCR analysis

Real-time PCR was performed with the TaqMan gene expression assay system, using primers and probe mixes for COX-2 and endogenous GAPDH control genes. PCRs were performed using SYBR Green PCR reagents (Applied Biosystems, Branchburg, NJ, USA). Relative gene expression was determined by the ΔΔCt method, where Ct meant threshold cycle. All experiments were performed in triplicate (n = 3).

Human COX-2, AP-1 and NF-κB promoter cloning, transient transfection and promoter activity assays

The upstream region (–1280 to +19) of the human COX-2 promoter was cloned into the pGL3-basic vector containing the luciferase reporter system. Briefly, a 1.3 kb segment at the 5′-flanking region of the human COX-2 gene was amplified by PCR using specific primers for the human COX-2 gene (accession no. U36476): 5′-ccccggtaccGAAGGCGAAATGCT TTGCCC (forward/Kpn1) and 5′-ccccctcgaGGGTGAGAAC CGAAGCTTCTG (reverse/Xho1). The pGL3-Basic vector, containing a polyadenylation signal upstream from the luciferase gene, was used to construct the expression vectors by subcloning PCR-amplified DNA of the COX-2 promoter into the Kpn1/Xho1 site of this vector. The PCR products (pGL3-COX-2WT) were confirmed by their sizes, as determined by electrophoresis and by DNA sequencing. Additionally, the introduction of a mismatched primer mutation into the AP-1 and NF-κB to generate pGL3-COX-2ΔAP-1 and pGL3-COX-2ΔNF-κB was performed, using the following (forward) primer: ΔAP-1: 5′-ACACACACCCTGAGTTGGCG-3′. ΔNF-κB: 5′-GGTAGGCTTACTGGGCCCCCAC-3′ respectively. All plasmids were prepared by using QIAGEN plasmid DNA preparation kits. The COX-2 promoter reporter construct was transfected into HCM cells using the Lipofectamine reagent according to the instructions of manufacture. To assess promoter activity, cells were collected and disrupted by sonication in lysis buffer (25 mM Tris-phosphate, 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, USA). The chemiluminescence was determined by using a Synergy H1 Hybrid reader (BioTek, Winooski, VT, USA) and processed with Gene 5 software (BioTek) (version 2.0). Firefly luciferase activities were standardized to those of β-galactosidase activity. The data presented are summarized from three independent assays.

Measurement of PGE_2 release

To determine the level of PGE_2, human cardiomyocytes were treated with thrombin for indicated time intervals. Culture supernatants were analysed for secreted PGE_2, using a PGE_2 Enzyme Immunoassay Kit (Cayman Chem., Ann Arbor, MI, USA) according to the manufacturer's instructions.

Transient transfection with siRNAs

Human siRNAs of scrambled, PAR1, p38, Erk1, JNK2 c-Jun and p65 were from Sigma. Transient transfection of siRNAs

(100 nM) was performed using a Lipofectamine 2000 reagent according to the manufacturer's instructions.

Cell proliferation assay

Human cardiomyocytes (2×10^4 cells per well) were seeded in 24-well culture plates and incubated at 37°C in commercial growth medium. After reaching 70% confluence, human cardiomyocytes were serum-starved for 24 h and incubated with thrombin for the indicated time intervals. Proliferation of human cardiomyocytes was assessed by a cell counting kit (CCK-8), by measuring mitochondria-metabolized formazan generation, directly proportional to live cells. The absorbance of samples was measured with a wavelength of 450 nm on an ELISA reader according to the manufacturer's instructions. Cell counting assay was also performed to assess the cell proliferation. Trypsin-digested human cardiomyocytes were collected, centrifuged and resuspended in PBS. Subsequently, Trypan blue solution was mixed with cell suspension in a 1:1 ratio, and the cells were counted in a haemocytometer via microscope.

Data analysis.

Data are expressed as mean ± SEM and analysed by one-way ANOVA followed with Tukey's *post hoc* test. Analysis was carried out with the GraphPad Prism program (Graph Pad, San Diego, CA, USA). A value of $P < 0.05$ was considered significant.

Materials

Anti-COX-2 antibody (#2169-1) was from Epitomics (Burlingame, CA, USA). Anti-phospho-c-Jun (#2361), anti-phospho-p42/p44 MAPK (#9101), anti-phospho-p38 MAPK (#9211) and anti-phospho-JNK1/2 (#4668) antibodies were from Cell Signaling (Danver, MA, USA). Anti-PAR1(sc-154), anti-Erk2 (sc-154), anti-JNK2 (sc-827), anti-p38 MAPK (sc-535), anti-p65 (sc-7151), anti-c-Jun (sc-822), anti-PCNA (sc-56) and anti-cyclin D1 (sc-8396) antibodies were from Santa Cruz (Santa Cruz, CA, USA). Anti-PAR1 antibody (#659102) was from BioLegend (San Diego, CA, USA). Anti-GAPDH (#MCA-1D4) was from Encor (Gainesville, FL, USA). PPACK, SCH79797, U0126, SP600125, SB202190, tanshinone IIA and helenalin were from Biomol (Plymouth Meeting, PA, USA). Thrombin (T4648), AH6809, Enzymes and Cell Counting Kit-8 (CCK-8) were from Sigma (St. Louis, MO, USA). TFLLR-NH_2 was from R&D systems (Minneapolis, MN, USA). SDS-PAGE supplies were from MDBio Inc (Taipei, Taiwan).

Results

Thrombin up-regulates COX-2 expression and PGE_2 generation

Thrombin-induced COX-2 expression has been reported in rat vascular smooth muscle cells (Hsieh *et al.*, 2008). In our experiments, as shown in Figure 1A, thrombin induced COX-2 protein expression in a time- and concentration-dependent manner and a significant increase within 8 h and at 3–10 U·mL^{-1} of thrombin. Thrombin also time-dependently induced COX-2 mRNA expression, reaching a peak within 4 h and slightly declining after 6 h (Figure 1B) and increased COX-2 promoter activity within 2 h. The induction of COX-2 protein by thrombin was accompanied by increased PGE_2 biosynthesis (Figure 1C). Thrombin time dependently induced PGE_2 generation with a maximum within 16–24 h (the basal level of PGE_2 ranged from 2 to –4 ng·mL^{-1}). To further determine whether thrombin induced COX-2 expression via transcription and translation processes, an inhibitor of transcription, actinomycin D, or of translation, cycloheximide were used. Figure 1D showed that pretreatment with actinomycin D or CHI concentration-dependently attenuated thrombin-induced COX-2 expression. Moreover, pretreatment with actinomycin D, but not cycloheximide, attenuated thrombin-induced COX-2 mRNA expression (Figure 1E). These results indicated that thrombin-induced COX-2 expression is mediated through *de novo* mRNA and protein synthesis, resulting in PGE_2 production.

PAR1 mediates thrombin-induced COX-2 expression

PARs mediate biological effects of thrombin via relaying downstream signalling cascades in hearts (Shah, 2009). Therefore, we identified which subtype of PARs is present in human cardiomyocytes. As shown in Figure 2A, PAR1, -2 and -3 were expressed in human cardiomyocytes. Pretreatment with a proteolytic activity inhibitor PPACK or a PAR1 antagonist SCH79797 both concentration dependently blocked thrombin-induced COX-2 expression (Figure 2B and 2C). Moreover, thrombin-induced COX-2 mRNA expression and promoter activity was also attenuated by pretreatment with PPACK or SCH79797 (Figure 2D). To further ensure the role of PAR1 in these responses, as shown in Figure 2E, transfection with PAR1 siRNA attenuated thrombin-induced COX-2 expression. Pretreatment with PPACK or SCH79797 also attenuated thrombin-induced PGE_2 production (Figure 2F). To further confirm the role of PAR1 in these responses, a PAR1 activation peptide, TFLLR-NH_2, was used. The results showed that TFLLR-NH_2 also induced COX-2 expression, including protein, mRNA and promoter activity levels, which were inhibited by pretreatment with SCH79797 (Supporting Information Fig. S1A and S1B), suggesting that activation of PAR1 is critical for COX-2 expression in human cardiomyocytes. These results suggested that thrombin-induced COX-2 expression and PGE_2 production is mediated through its proteolytic activity and PAR1 in human cardiomyocytes.

Involvement of MAPKs in thrombin-induced COX-2 expression

MAPKs are activated through PARs and contribute to cardiac hypertrophy (Wang, 2007). We explored the roles of p38 MAPK, Erk1/2 and JNK1/2 in thrombin-induced COX-2 expression. Human cardiomyocytes were pretreated with the corresponding MAPK inhibitors before thrombin challenge. As shown in Figure 3A and 3B, thrombin-induced COX-2 protein and mRNA expression, and COX-2 promoter activity was attenuated by pretreatment with the inhibitor of p38 MAPK (SB202190), MEK1/2 (U0126) or JNK1/2 (SP600125). Thrombin also time-dependently stimulated phosphorylation of p38 MAPK, Erk1/2 and JNK1/2, which were attenuated by their respective inhibitors (i.e. SB202190, U0126 and

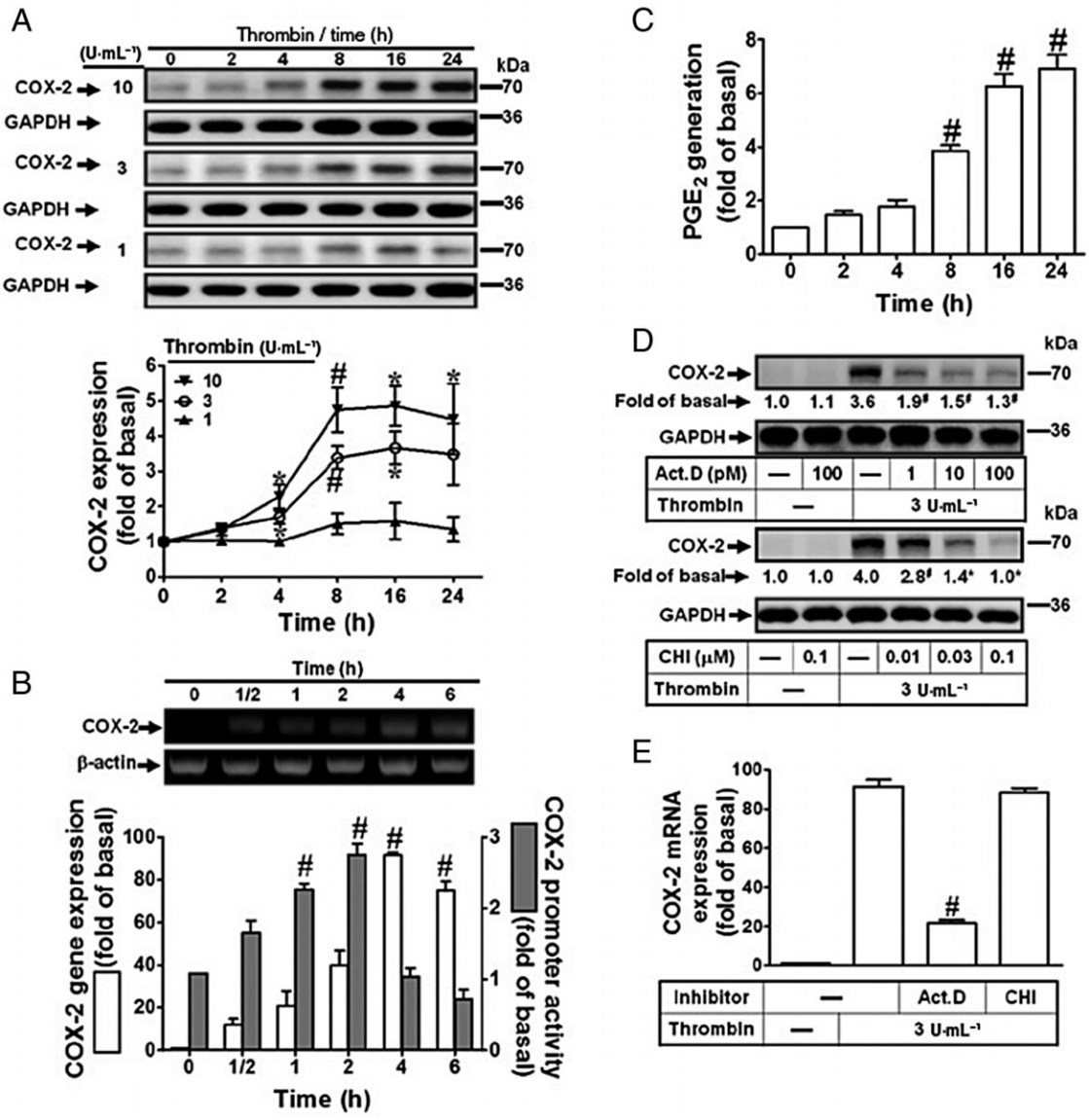

Figure 1

Thrombin induces COX-2 expression and PGE_2 generation in human cardiomyocytes. (A) Human cardiomyocytes were incubated with various concentrations of thrombin for the indicated time intervals. The cell lysates were subjected to Western blot analysis. (B) The cells were incubated with thrombin (3 U·mL^{-1}) for the indicated time intervals. The levels of COX-2 mRNA were analysed by RT-PCR and real-time PCR. Human cardiomyocytes were co-transfected with a COX-2 promoter luciferase reporter gene with a β-galactosidase plasmid and then incubated with thrombin (3 U·mL^{-1}) for indicated time intervals (black bar). The COX-2 luciferase activity was detected and normalized to β-galactosidase activity. (C) The conditioned media were collected for PGE_2 generation analysis by an EIA kit. The basal value of PGE_2 is ranged from 2–4 ng·mL^{-1}. (D–E) Human cardiomyocytes were pretreated with actinomycin D (Act. D) or cycloheximide CHI for 1 h and then incubated with thrombin (3 U·mL^{-1}) for (D) 16 h or (E) 4 h. The COX-2 protein and mRNA levels was determined by Western blot (D) and real-time PCR (E). Data are expressed as mean ± SEM (n = 3). *P < 0.05; #P < 0.01, significantly different from cells incubated with vehicle alone (A–C) or thrombin alone (D–E).

SP600125), during the period of observation (Figure 3C). The protein levels of MAPKs were not changed by incubation with thrombin or inhibitor. To further confirm the roles of these three MAPKs in thrombin-mediated responses, as shown in Figure 3D, transfection with p38 MAPK, p42 or JNK2 siRNA knocked down their respective proteins and subsequently attenuated thrombin-induced COX-2 expression. Moreover, pretreatment with SB202190, U0126 and SP600125 also inhibited thrombin-induced PGE_2 production (Figure 3E). The data demonstrated that thrombin-induced COX-2 expression and PGE_2 production is mediated through these MAPKs (i.e. p38 MAPK, Erk1/2, and JNK1/2) in human cardiomyocytes.

AP-1 and NF-κB are required for thrombin-induced COX-2 expression

AP-1 and NF-κB are two important transcription factors for regulating COX-2 gene expression in various cells upon

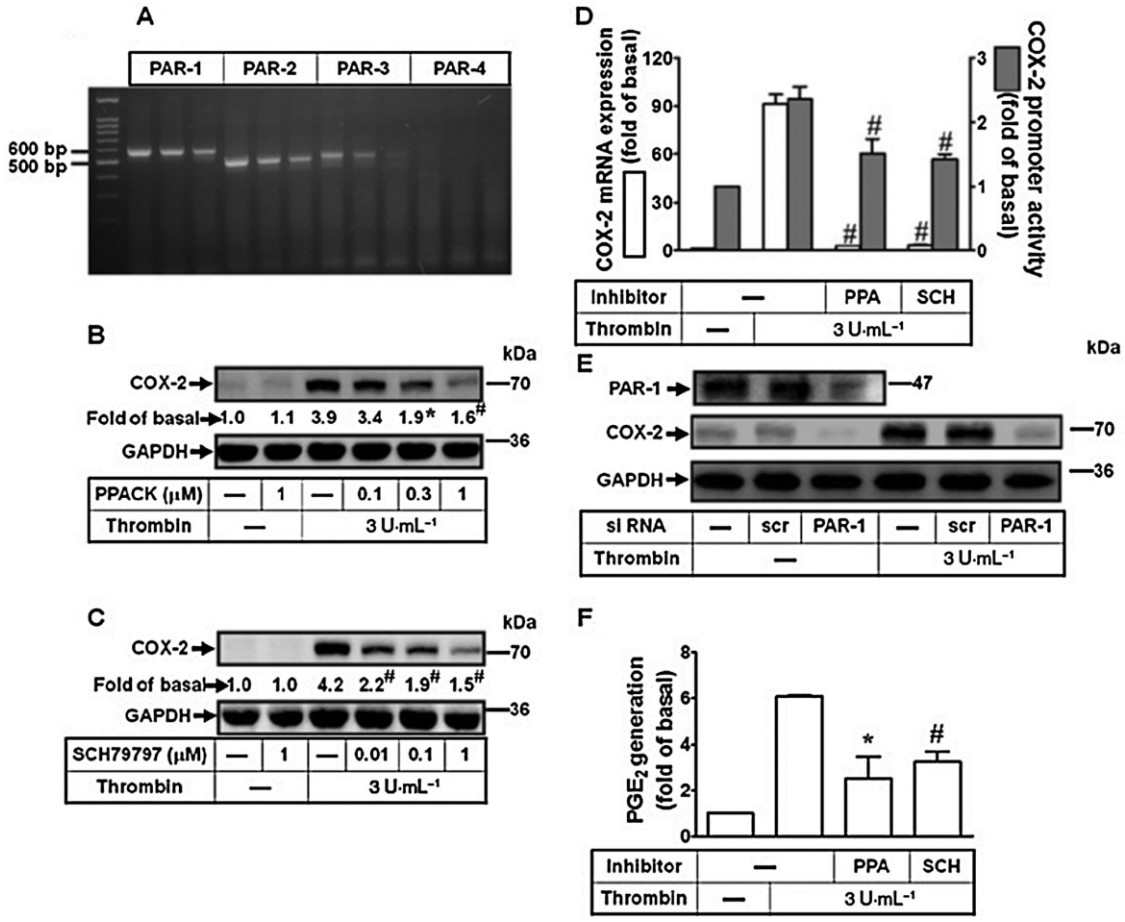

Figure 2

Thrombin induces COX-2 expression via a PAR1-dependent manner. (A) Expression of PAR subtypes in human cardiomyocytes was analysed by RT-PCR. (B–C) Cells were pretreated with PPACK (B) or SCH79797 (C) for 1 h and then incubated with thrombin for 16 h. The COX-2 protein was determined by Western blot. (D) Cells were pretreated with PPACK (PPA, 1 μM) or SCH79797 (SCH, 10 μM) for 1 h and then incubated with thrombin for 4 h. The COX-2 mRNA and promoter luciferase activity were detected by real-time PCR and promoter assay. (E) Cells were transfected with siRNA for scramble (scr) or PAR1 and then incubated with thrombin for 16 h. The COX-2 protein expression was determined by Western blot. (F) Cells were pretreated with PPACK (PPA, 1 μM) or SCH79797 (SCH, 1 μM) for 1 h and then incubated with thrombin for 16 h. The conditioned media were collected for PGE_2 generation analysed by an EIA kit. Data are expressed as mean ± SEM ($n = 3$). $^*P < 0.05$; $^\#P < 0.01$, significantly different from cells incubated with thrombin alone.

diverse stimuli (Kang *et al.*, 2007). Here, to investigate the roles of AP-1 and NF-κB in thrombin-induced COX-2 expression, the inhibitors of AP-1 (tanshinone IIA) and NF-κB (helenalin) were used. As shown in Figure 4A and 4B, pretreatment with either inhibitor attenuated thrombin-induced COX-2 protein, mRNA expression and promoter activity. Furthermore, the phosphorylation of c-Jun/AP-1 and p65 NF-κB stimulated by thrombin was blocked by tanshinone IIA or helenalin respectively (Figure 4C). To investigate whether thrombin can regulate AP-1 and NF-κB transcriptional activity, the AP-1 and NF-κB promoter plasmids were used. As shown in Figure 4D, thrombin enhanced the transcriptional activities of AP-1 and NF-κB, which were attenuated by PPACK, SCH79797, SB202190, U0126 and SP600125. We further confirmed that AP-1 and NF-κB are required for thrombin-induced COX-2 promoter luciferase activity by transfection of human cardiomyocytes with the wild-type (WT), mutated AP-1 (mtAP-1) or mutated NF-κB (mtNF-κB) of COX-2 promoter plasmid. As shown in Figure 4E, transfection with mt-AP-1 or mt-NF-κB COX-2 promoter plasmid significantly blocked thrombin-stimulated COX-2 promoter activity in human cardiomyocytes. To confirm the roles of c-Jun/AP-1 and p65 NF-κB in thrombin-induced COX-2 expression, as shown in Figure 4F, transfection with c-Jun or p65 siRNA knocked down c-Jun or p65 protein and attenuated thrombin-induced COX-2 expression. Finally, we also found that pretreatment with tanshinone IIA or helenalin significantly inhibited thrombin-induced PGE_2 generation (Figure 4G). These results indicated that both AP-1 and NF-κB are essential for thrombin-induced COX-2 expression in human cardiomyocytes.

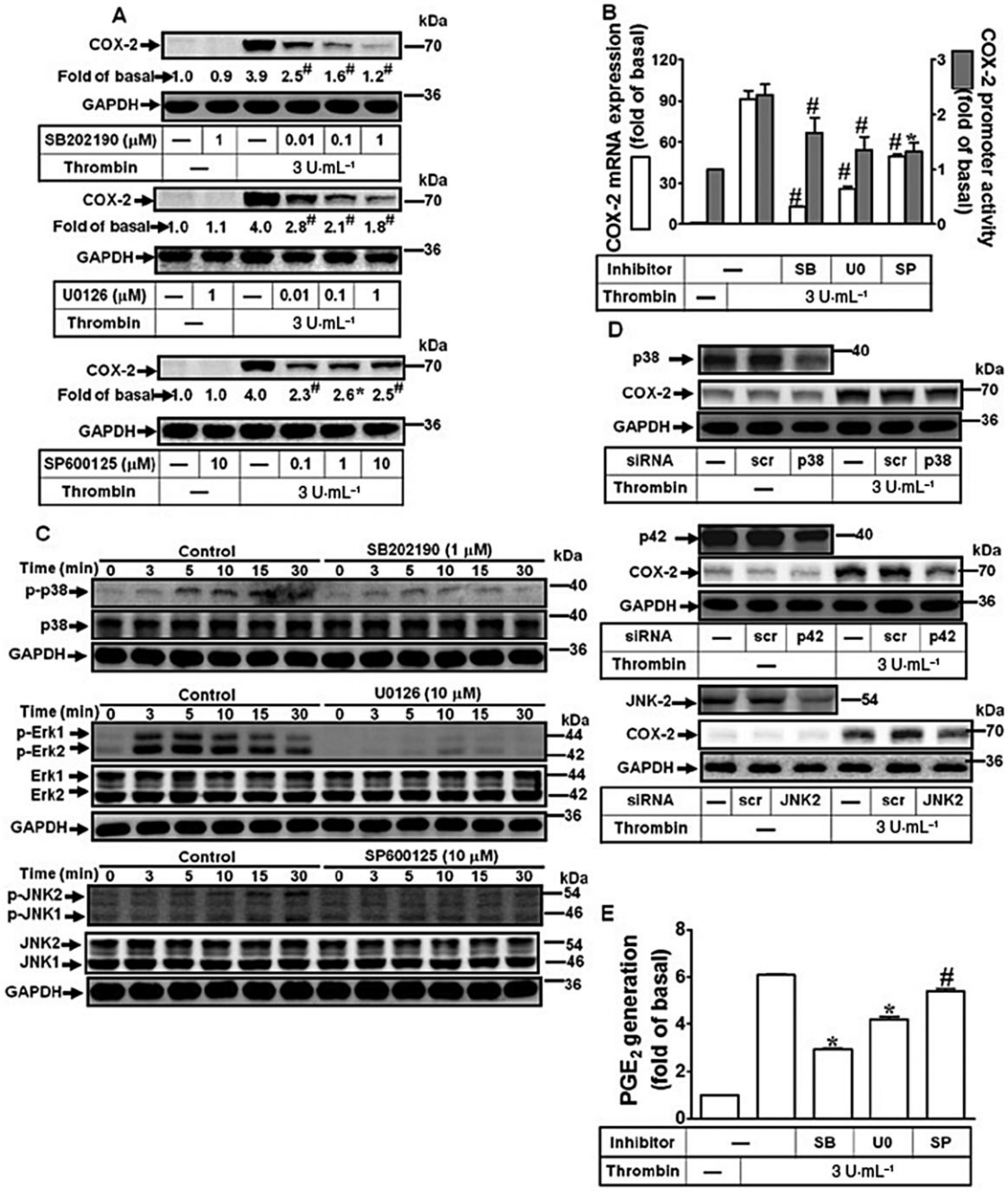

Figure 3

Effects of MAPKs in thrombin-induced COX-2 expression. (A) Cells were pretreated with SB202190, U0126, or SP600125 for 1 h and then incubated with thrombin for 16 h. The COX-2 protein expression was determined by Western blot. (B) Cells were pretreated with SB202190 (1 μM), U0126 (10 μM) or SP600125 (10 μM) for 1 h and then incubated with thrombin for 4 h. The COX-2 mRNA and promoter luciferase activity were detected. (C) Cells were pretreated with SB202190 (1 μM), U0126 (10 μM) or SP600125 (10 μM) for 1 h and then challenged with thrombin (3 U·mL^{-1}) for the indicated time intervals. The cell lysates were analysed by Western blot using an anti-phospho-p38, anti-phospho-Erk1/2, anti-phospho-JNK1/2 or anti-GAPDH (as an internal control) antibody. (D) Cells were transfected with siRNA for scramble (scr), p38, p42 (Erk2) or JNK2 and then incubated with thrombin for 16 h. The COX-2 protein expression was determined by Western blot. (E) Cells were pretreated with SB202190 (1 μM), U0126 (1 μM) or SP600125 (10 μM) for 1 h and then incubated with thrombin for 16 h. The conditioned media were collected for PGE_2 generation analysed by an EIA kit. Data were expressed as mean ± SEM (n = 3). *P < 0.05; #P < 0.01, significantly different from thrombin alone.

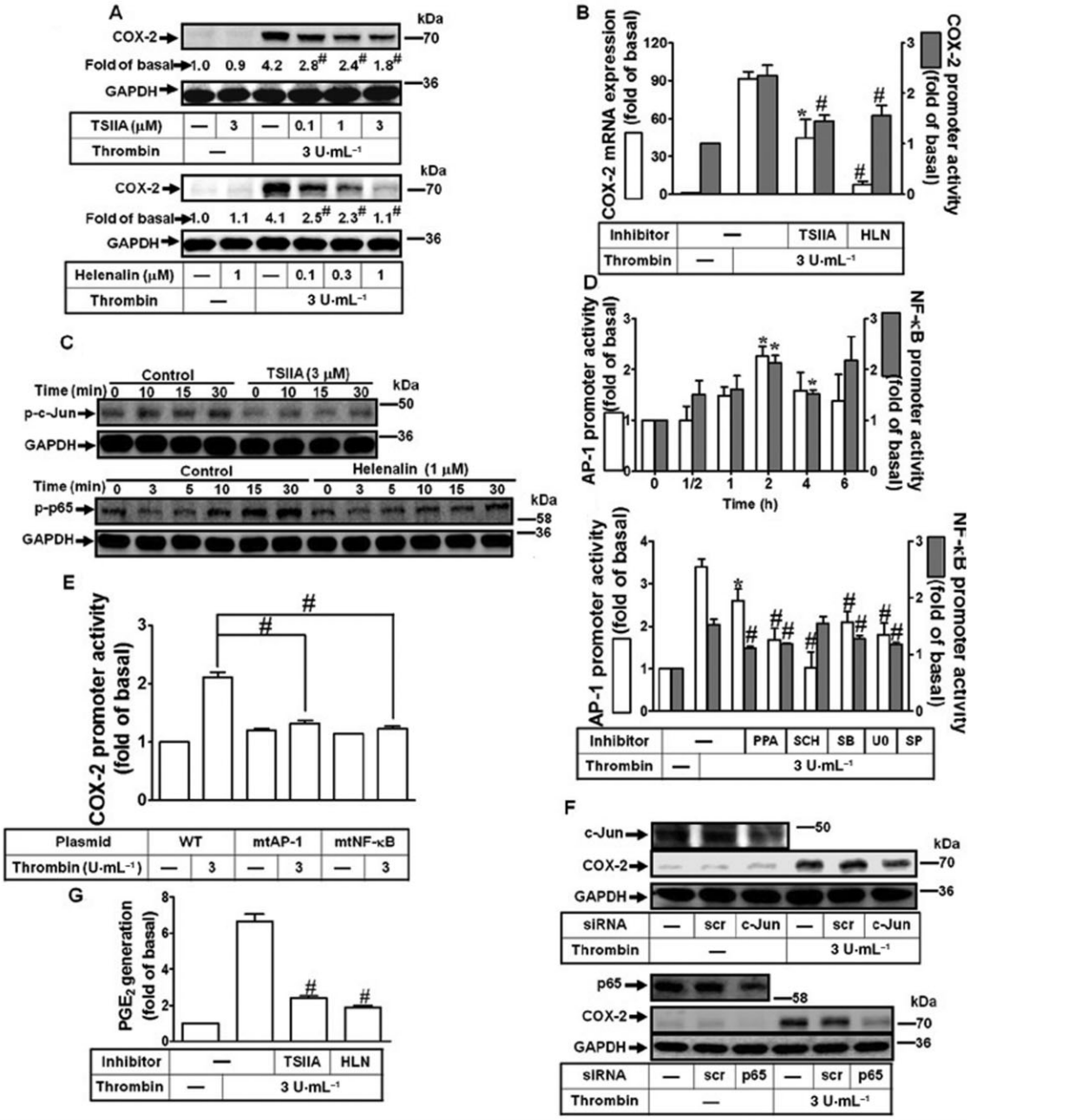

Figure 4

AP-1 and NF-κB are required for thrombin-induced COX-2 expression. (A) Cells were pretreated with tanshinone IIA (TSIIA) or helenalin (HLN) for 1 h and then incubated with thrombin for 16 h. The COX-2 protein expression was determined by Western blot. (B) Cells were pretreated with TSIIA (3 μM) or HLN (1 μM) for 1 h and then incubated with thrombin for 4 h. The COX-2 mRNA levels and promoter luciferase activity were detected. (C) Cells were pretreated with TSIIA (3 μM) or HLN (1 μM) for 1 h and then challenged with thrombin (3 U·mL^{-1}) for the indicated time intervals. The cell lysates were analysed by Western blot using an anti-phospho-c-Jun, anti-phospho-p65 or anti-GAPDH (as an internal control) antibody. (D) Cells were transfected with AP-1 or NF-κB report gene and treated with thrombin for the indicated time intervals. The luciferase activity was detected and normalized to β-galactosidase activity. (E) Cells were transfected with wild-type (WT), mutated AP-1 (mtAP-1) or mutated NF-κB (mtNF-κB) COX-2 promoter gene and then challenged with thrombin (3 U·mL^{-1}) for 2 h. (F) Cells were transfected with siRNA for scramble (scr), c-Jun or p65 and then incubated with thrombin for 16 h. The COX-2 protein expression was determined by Western blot. (G) Cells were pretreated with TSIIA (3 μM), or HLN (1 μM) for 1 h and then incubated with thrombin for 16 h. The conditioned media were collected for PGE_2 generation analysed by an EIA kit. Data are expressed as mean ± SEM (n = 3). *P < 0.05; #P < 0.01, significantly different from cells incubated thrombin alone.

Involvement of PAR1-mediated activation of MAPKs in thrombin-stimulated AP-1 and NF-κB

We demonstrated that PAR1, p38 MAPK, Erk1/2, JNK1/2, AP-1 and NF-κB are required for thrombin-induced COX-2 expression in human cardiomyocytes. Next, to investigate the relationships among these signalling molecules, their respective inhibitors were used. As shown in Figure 5A, pretreatment with PPACK or SCH79797 reduced phosphorylation of p38 MAPK, Erk1/2 and JNK1/2, suggesting that thrombin stimulated MAPKs phosphorylation via a PAR1-dependent manner. To further investigate whether thrombin-stimulated phosphorylation of c-Jun/AP-1 and p65/NF-κB is mediated through PAR1-dependent MAPKs cascade, as shown in Figure 5B, thrombin-stimulated c-Jun phosphorylation was attenuated by PPACK, SCH79797, SB202190, U0126 or SP600125. Moreover, thrombin-stimulated p65 phosphorylation was attenuated by PPACK, SCH79797, U0126 or SP600125, but not SB202190. To ensure the phosphorylation of these MAPKs was due to activation of PAR1, we found that TFLLR-NH_2 also stimulated p38 MAPK, Erk1/2, JNK1/2, p65 and c-Jun phosphorylation in a time-dependent manner, which was attenuated by pretreatment with SCH79797 (Supporting Information Fig. S1C). These results suggested that thrombin-stimulated AP-1 and NF-κB activation is mediated through PAR1-mediated MAPKs and leading to COX-2 expression in human cardiomyocytes.

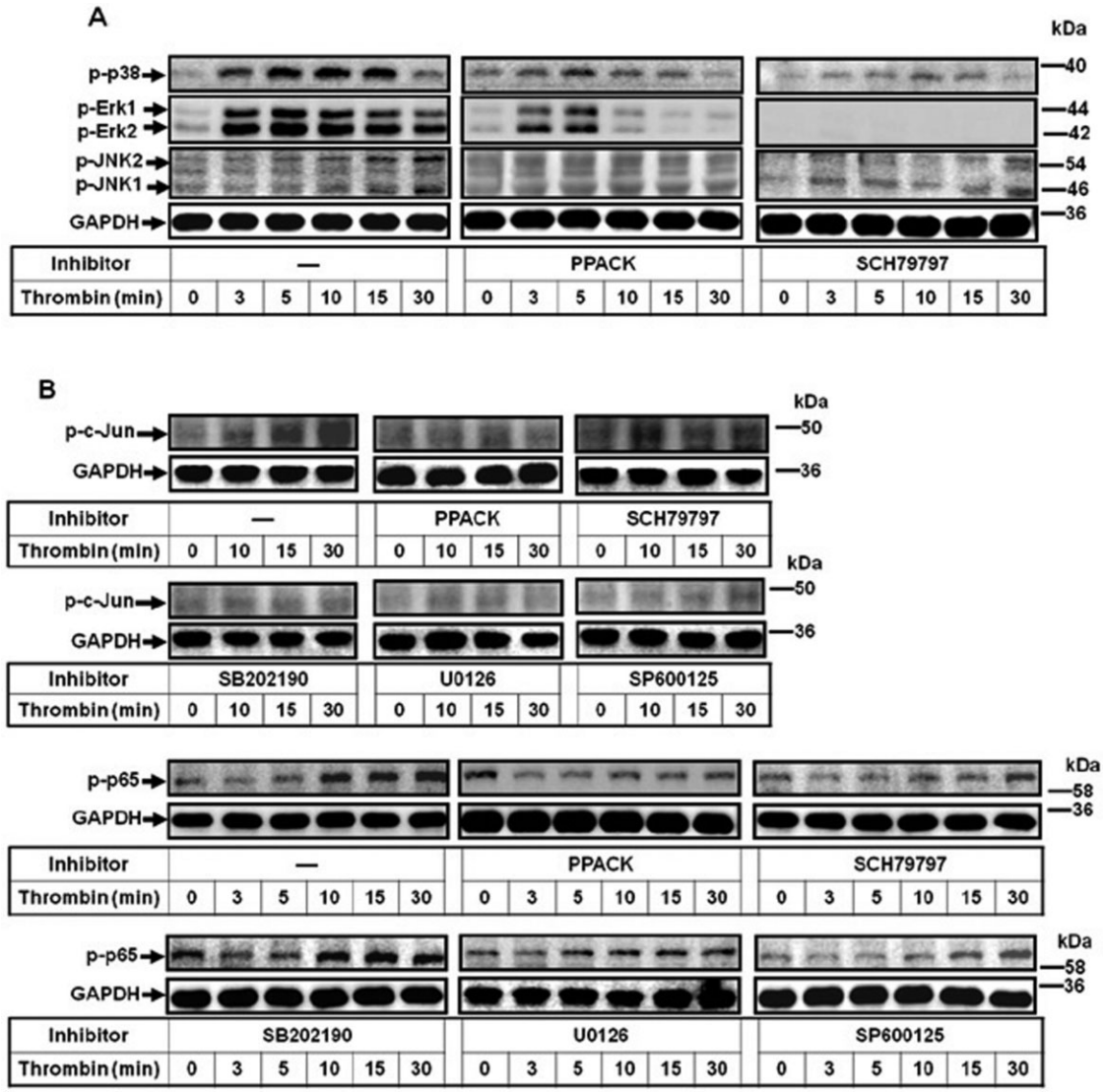

Figure 5

Thrombin stimulates MAPK-dependent activation of AP-1 and NF-κB via PAR1. (A) Cells were pretreated with PPACK (1 μM) or SCH79797 (10 μM) for 1 h and then challenged with thrombin (3 U·mL^{-1}) for the indicated time intervals. The phosphorylation of p38 MAPK, Erk1/2, and JNK1/2 were determined by Western blot. (B) Cells were pretreated with PPACK (1 μM), SCH79797 (10 μM), SB202190 (1 μM), U0126 (10 μM) or SP600125 (10 μM) for 1 h and then challenged with thrombin (3 U·mL^{-1}) for the indicated time intervals. The phosphorylation of c-Jun and p65 were determined by Western blot (n = 3).

Thrombin induces proliferation of human cardiomyocytes via PAR1-dependent MAPKs, AP-1 and NF-κB pathways

Thrombin has been shown to regulate several cellular functions such as proliferation in various cells (Isenovic *et al.*, 2010). Hence, we investigated the effects of COX-2/PGE_2 up-regulation by thrombin on human cardiomyocyte proliferation. The results showed that thrombin induced cell proliferation (Figure 6A), which was inhibited by pretreatment with COX-2 inhibitors, celecoxib and NS-398,as well as PPACK and SCH79797. Moreover, pretreatment with SB202190, U0126, SP600125, tanshinone IIA or helenalin also inhibited thrombin-induced cell proliferation, suggesting that COX-2/PGE_2 may contribute to thrombin/PAR1-induced cardiomyocyte proliferation. Moreover, TFLLR-NH_2 also induced cell proliferation (Supporting Information Fig. S1D). We further assayed the levels of proliferation

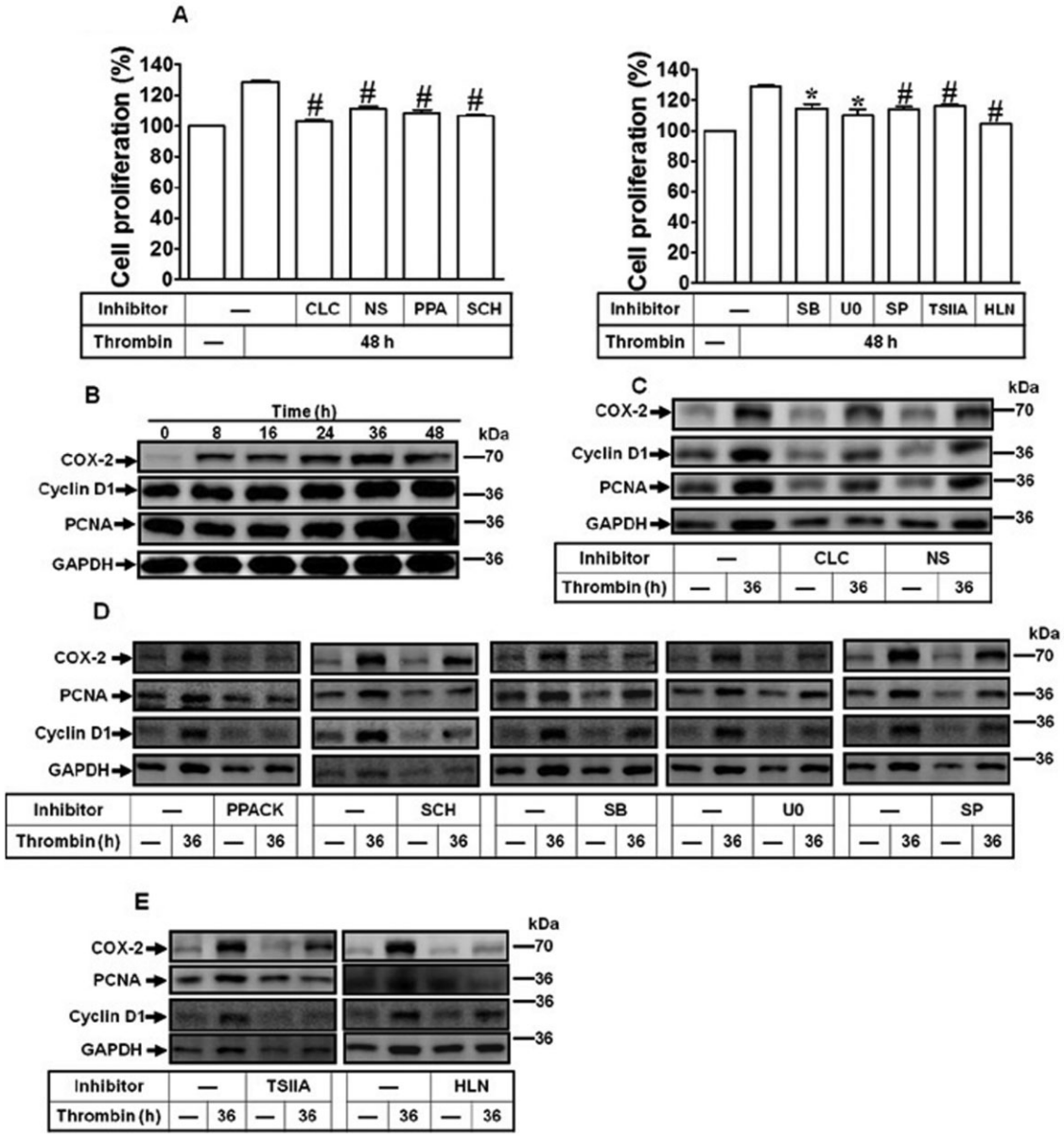

Figure 6

Thrombin-induced COX-2 expression contributes to cell proliferation. (A) The proliferating effects of thrombin on human cardiomyocytes were analysed by a CCK-8 kit. Cells were pretreated with CLC (10 μM), NS-398 (NS, 10 μM), PPACK (PPA, 1 μM), SCH79797 (SCH, 1 μM), SB202190 (SB, 1 μM), U0126 (U0, 1 μM), SP600125 (SP, 0.1 μM), tanshinone IIA (TSIIA; 3 μM) or helenalin (HLN; 1 μM) for 1 h and then incubated with thrombin for 48 h. (B) Cells were incubated with thrombin (3 $U \cdot mL^{-1}$) for the indicated time intervals. (C–D) Cells were pretreated with CLC or NS-398 (C), PPACK, SCH79797, SB202190, U0126, SP600125, TSIIA or HLN (D) for 1 h and then incubated with thrombin for 36 h. The COX-2, PCNA and cyclin D1 protein expression were determined by Western blot. Data are expressed as mean ± SEM (n = 3). $^{*}P < 0.05$; $^{\#}P < 0.01$, significantly different from thrombin alone.

marker molecules including a cell cycle regulator cyclin D1 and proliferating cell nuclear antigen (PCNA) in human cardiomyocytes challenged with thrombin. As shown in Figure 6B, thrombin time-dependently up-regulated cyclin D1 and PCNA expression, with a significant response within 36–48 h, and GAPDH expression was used as an indicator of internal protein control. Moreover, pretreatment with either of the COX-2 inhibitors attenuated thrombin-induced cyclin D1 and PCNA expression (Figure 6C). To further examine the effects of these signalling molecules of COX-2 induction by thrombin on cardiomyocyte proliferation (Figure 6D and 6E), pretreatment with PPACK, SCH79797, SB202190, U0126, SP600125, tanshinone IIA or helenalin attenuated thrombin-induced PCNA and cyclin D1 expression. To further confirm the proliferating response, the cell counting assay was also performed. The results showed that thrombin or TFLLR-NH_2 increased cell numbers in a time-dependent manner (Supporting Information Fig. S2A). Collectively, these results indicated that thrombin induced COX-2/PGE_2-mediated cell proliferation via PAR1-dependent MAPKs linking to AP-1 and NF-κB pathways in human cardiomyocytes.

Thrombin-induced cell proliferation is mediated through the PG EP_2 receptor

The prostanoid EP receptors mediate PGE_2-activated biological events, including cell proliferation, in rat neonatal cardiomyocytes (Harding and Murray, 2011; Zhang *et al.*, 2013). To examine whether EP receptors were involved in thrombin-triggered responses, we first used RT-PCR to analyse whether EP receptors are expressed in human cardiomyocytes. As shown in Figure 7A, the EP_2 receptor was predominantly expressed in human cardiomyocytes. Next, pretreatment with an EP_2 receptor antagonist AH6809 attenuated thrombin-induced cell proliferation at 48 h (Figure 7B). Moreover, when human cardiomyocytes were directly treated with PGE_2 for the indicated time intervals. cell proliferation was induced in a time-dependent manner (Figure 7C), which was attenuated by pretreatment with AH6809 (Figure 7D). These results were also confirmed by a cell counting assay (Supporting Information Fig. S2). Pretreatment with AH6809 also attenuated thrombin-induced expression of proliferating markers such as PCNA and Cyclin D1 at 36 h (Figure 7E). Taken together, our reulst show that EP_2 receptors played an important role in thrombin-induced human cardiomyocyte proliferation.

Discussion and conclusions

Several factors have been implicated in heart failure, with an up-regulation of many proteases and inflammatory mediators (Yang *et al.*, 2013). Among them, IL-1β, TNF-α and thrombin are elevated and highly related to heart failure progression (Li and Olshansky, 2011). COX-2 up-regulation is also detected in patients with chronic heart failure (Saito and Giaid, 1999). However, little is known about the detailed mechanisms of COX-2 up-regulation by thrombin in human cardiomyocytes. Thus, we investigated the molecular mechanisms underlying thrombin-induced COX-2 expression in human cardiomyocytes. Here, we demonstrated that in human cardiomyocytes, activation of PAR1-dependent MAPKs, c-Jun/AP-1 and NF-κB, were essential for thrombin-induced COX-2 expression and PGE_2 production, and enhancement of cell proliferation. The results suggested that thrombin-induced COX-2 expression, PGE_2 production and cell proliferation may contribute to heart inflammation and situations such as heart failure.

COX-2 is an inducible mediator of inflammation, which can be up-regulated via various signalling pathways and is involved in various pathological states and inflammatory diseases (Streicher *et al.*, 2010). COX-2 is induced by many pro-inflammatory mediators in many different cell types (Kang *et al.*, 2007). Several transcriptional factors have been identified to regulate human COX-2 gene expression, including PPAR, NF-κB, AP-1 and cyclic AMP-binding protein (CREB), leading to a complex signal coordination for COX-2 expression. In vascular smooth muscle cells and murine macrophages, thrombin triggers COX-2 induction via a PAR1-dependent pathway (Hsieh *et al.*, 2008; Lo *et al.*, 2009). Here, we first showed that, in human cardiomyocytes, thrombin induced the COX-2/PGE_2 system, including expression of protein and mRNA, and PGE_2 generation via a PAR1-dependent cascade. Moreover, the involvement of PAR1 in thrombin-induced responses via MAPKs was also confirmed by using a PAR1 activation peptide, which was attenuated by SCH79797 in human cardiomyocytes. However, in the A549 cell line, activation of PAR-2 also led to COX-2 expression via Erk1/2 and p38 MAPK and thus shared a mechanism, similar to those of PAR1 (Kawao *et al.*, 2005; Wang *et al.*, 2008). Further work will be necessary to determine whether PAR-2 can induce COX-2 expression. Moreover, PAR 4 was not expressed in human cardiomyocytes, consistent with a previous report using the same primers of PAR-4 (Kreda *et al.*, 2010). Furthermore, pretreatment with a PAR-4 antagonist, Tcy-NH2, had no inhibitory effect on thrombin-induced COX-2 expression. Based on these data, we suggested that PAR1 may be critical for thrombin-induced COX-2 expression in human cardiomyocytes.

Examination of the mechanisms of increased potential risk factors contributing to patients combined stroke with heart failure showed that pro-coagulation factors, particularly thrombin, are up-regulated (Jug *et al.*, 2009). Several proteases have been implicated in activation of PARs *in vivo* (Soh *et al.*, 2010). These observations imply that thrombin may act as an important factor in the pathogenesis of heart failure. Thrombin activates PARs, which mediate diverse intracellular signals for cellular functions, including atrial natriuretic factor (ANF) generation and cardiac hypertrophy (Sabri *et al.*, 2003). A recent study shows that PAR1 and thrombin are up-regulated in left atria and ventricle in patients with atrial fibrillation (Ito *et al.*, 2013). Nevertheless, little is known for the expression and role of PARs in human cardiomyocytes. In this study, we confirmed that thrombin-induced COX-2 expression was attenuated by a proteolytic activity inhibitor PPACK, suggesting that the proteolytic activity of thrombin is involved in COX-2 expression. Furthermore, we confirmed that human cardiomyocytes expressed PAR1, PAR-2 and PAR-3. PAR1 is the predominant prototype of PAR-mediated thrombin responses. Moreover, we confirmed the role of PAR1 in thrombin-induced responses by transfection with a PAR1 siRNA. These results are consistent with previous

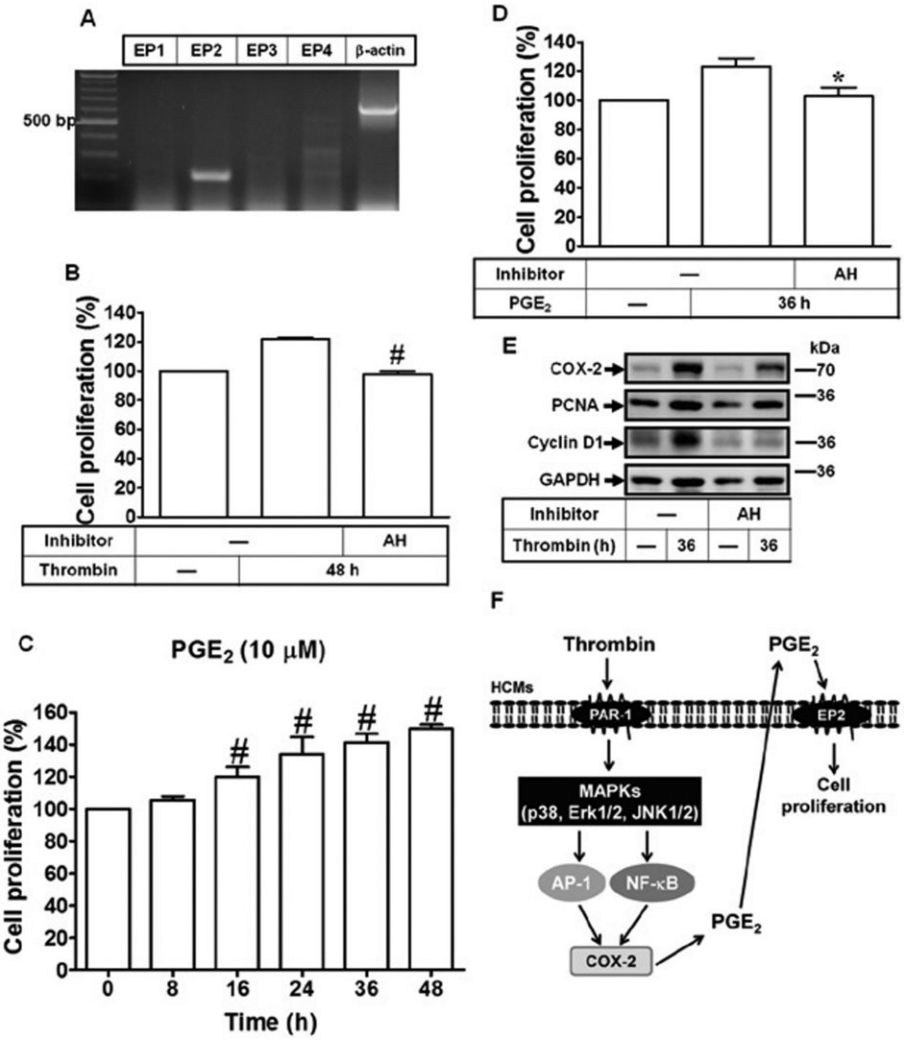

Figure 7

EP_2 receptors mediate thrombin-induced cell proliferation. (A) Expression of EP receptor subtypes in human cardiomyocytes was analysed by RT-PCR. (B) The proliferating effects of thrombin on human cardiomyocytes were analysed by a CCK-8 kit. Cells were pretreated with AH6809 (AH, 10 μM) for 1 h prior to thrombin treatment for 48 h. (C) Cells were treated with PGE_2 (10 μM) for the indicated times and analysed the proliferating effects by a XTT kit. (D) Cells were pretreated with AH6809 for 1 h and then incubated with thrombin for 36 h. (E) The COX-2, PCNA and cyclin D1 protein expression were determined by Western blot. Data are expressed as mean ± SEM ($n = 3$). $^*P < 0.05$; $^\#P < 0.01$, significantly different from thrombin alone. (F) Each solid line and arrow represents a step in an activating pathway. Thrombin binds and activates its PAR1 receptor on the surface of primary human cardiomyocytes and relays intracellular signalling transduction cascades including MAPKs (i.e. p38, ERK1/2 and JNK1/2), subsequently initiated the transcription factors AP-1 and NF-κB pathways and ultimately leading to COX-2/PGE_2-dependent EP_2 receptor-activated cell proliferation in primary human cardiomyocytes.

reports indicating that PAR1 contributes to COX-2 induction in rat gastric epithelial cells and murine macrophages (Lo *et al.*, 2009; Sekiguchi *et al.*, 2011).

MAPKs have been shown to mediate various cellular functions, including proliferation, differentiation, motility, survival and apoptosis in response to various stimuli (Ravingerova *et al.*, 2003). Activation of MAPKs has been well defined at different stages of heart diseases such as cardiac hypertrophy (Wang, 2007). Moreover, activation of PARs may increase MAPK activity and cause cardiac hypertrophy, although, the definite roles of each MAPK in thrombin-mediated responses in human cardiomyocytes remain to be investigated. Earlier work showed that thrombin could activate Erk1/2, p38 MAPK and JNK1/2 leading to cardiac hypertrophy and ANF expression (Jaffre *et al.*, 2012). Here, we further confirmed that in human cardiomyocytes, thrombin-stimulated activation of MAPKs was involved in COX-2 induction, which was attenuated by their respective MAPKs inhibitors or siRNA, consistent with previous reports indicating that MAPKs are crucial to thrombin-induced responses in several other cell types (Lo *et al.*, 2009; Sekiguchi *et al.*, 2011). Moreover, phosphorylation of MAPKs by thrombin was attenuated by pretreatment with PPACK and SCH79797, indicating that thrombin stimulated activation of MAPKs via a PAR1-mediated manner. These results are consistent with earlier data showing PAR1 stimulated MAPK pathways

leading to PGE_2 formation in RGM1 cells (Sekiguchi *et al.*, 2007).

The transcription factor AP-1, a complex of c-Jun, c-Fos and ATF subunits, regulates gene expression in response to various stimuli, such as cytokines, stress or growth factors. Previous studies demonstrate that AP-1 is engaged in endoplasmic reticulum stress-induced heart failure and participates in oxidant-induced cardiac hypertrophy (Tu *et al.*, 2003; Sawada *et al.*, 2010). Our data showed that pretreatment with tanshinone IIA reduced thrombin-induced c-Jun phosphorylation, COX-2 protein and mRNA expression, and PGE_2 generation, suggesting that AP-1 was required for thrombin-induced COX-2 expression. These results are consistent with earlier work demonstrating that thrombin-induced COX-2 expression was mediated through activation of AP-1 binding to COX-2 promoter in vascular smooth muscle cells (Hsieh *et al.*, 2008). Moreover, we showed that phosphorylation of c-Jun/AP-1 was attenuated by various inhibitors used in this study, suggesting that thrombin activates c-Jun/AP-1 via a PAR1-dependent MAPK activation pathway in human cardiomyocytes.

In the heart, the major form of NF-κB is the p50/p65 heterodimer associated with I-κB and, in a resting state, this remains in the cytoplasm which can be triggered by a variety of stimuli-related to inflammatory responses, mainly via transcription activation (Norman *et al.*, 1998). Moreover, NF-κB activation has been shown to aggravate cardiac diseases through activation of pro-inflammatory pathways and enhancing heart failure (Kawamura *et al.*, 2005). Here, we used an NF-κB inhibitor, helenalin to investigate its role in COX-2 induction, indicating that NF-κB is required for thrombin-induced COX-2 expression and PGE_2 generation in human cardiomyocytes. Moreover, thrombin-stimulated p65 phosphorylation was mediated through PAR1, Erk1/2 and JNK1/2 in human cardiomyocytes, consistent with the results indicating that thrombin and PAR1 induce COX-2 expression via coordination of MAPKs and NF-κB in human umbilical vascular endothelial cells (Syeda *et al.*, 2006).

Thrombin can induce cardiofibroblast proliferation via PAR-dependent signalling pathways (Sabri *et al.*, 2002). However, proliferation of cardiomyocytes still remains controversial. As it is believed that cardiomyocytes are incapable of proliferating and are terminally undifferentiated, it is unlikely that adult cardiomyocytes have proliferating capability. A previous study demonstrated that cardiomyocytes in human failing hearts showed dividing cells by ki67 staining, implying that cardiomyocytes may be able to form new cells (Nadal-Ginard *et al.*, 2003). In our study, we found that the pro-proliferation effects of thrombin on human cardiomyocytes occurred within 36–48 h, consistent with increased PCNA and cyclin D1 expression during embryonic heart development and disease states. The mitochondria-formazan used as a proliferation index still remains controversial. Therefore, cell counting was also performed to show that thrombin and PAR1 agonist did increase cell number, which was significantly but not completely attenuated by SCH79797 and AH6809, implying that PAR1-independent pathways may be also involved in proliferation in our model. Herber *et al.* demonstrated that endothelial cell growth was mediated through proteolytic- and non-proteolytic-dependent paths (Herbert *et al.*, 1994). Synthetic thrombin receptor peptides activated the GPCR but were incapable of inducing mitogenesis (Vouret-Craviari *et al.*, 1992). These different results may be due to cell specificity and various experimental conditions. During a variety of heart diseases, measurement of mitotic index revealed cell proliferation, even under electron micrograph showing mild mitosis (Nadal-Ginard *et al.*, 2003; Chang *et al.*, 2010). Although injuries were observed for the loss of pre-existing cells, the intact left ventricle recovered within 6 months, suggesting that human cardiomyocyte proliferation could be activated by various factors involved in pathological progression, and one of potential candidates may be thrombin.

The compensatory responses in failing hearts comprise not only hypertrophy but also the growth responses induced by thrombin. Here, we demonstrated that thrombin induced cell proliferating events via PAR1-dependent MAPKs/AP-1 and NF-κB linking to induction of the COX-2/PGE_2 system. EP receptors have been identified in cardiomyocytes in mice, in contrast to our finding that human cardiomyocytes only possess EP_2 receptors (Xiao *et al.*, 2004). These receptors are known for their proliferative effects in many types of cells through downstream signalling pathways (Guo *et al.*, 2011; Liu *et al.*, 2012; Lee *et al.*, 2013). In this study, we demonstrated that the EP_2 receptor antagonist, AH6809, inhibited thrombin-induced cell proliferation and cell cycle-dependent protein expression in human cardiomyocytes. Moreover, the cell counting and XTT assays showed that PGE_2 alone promoted cell proliferation, which was also observed in other cell types as well (Jang *et al.*, 2012; Lee *et al.*, 2013). In a post-infarction model, PGE_2 promotes replenishment of newly formed cardiomyocytes through both direct and indirect EP_2 receptor pathways (Hsueh *et al.*, 2014). Collectively, these observations provide a novel insight into COX-2/PGE_2 regulation in heart. As there is a loss of proliferating ability of cardiomyocytes following myocardial infarction, exploration of the factors driving cardiomyocyte regeneration is essential. There is evidence that proliferation of cardiomyocytes is triggered by various exogenous factors, such as fibroblast growth factor, PDGF or IL-6 (Kardami *et al.*, 2003; Hinrichsen *et al.*, 2007; Lu *et al.*, 2008). The increased cell number in our neonatal cell model is likely due to transitory capacity being maintained. Optimization of thrombin given in an appropriate time and dose could be beneficial for tissue repair. However, the origin of increased cells could not rule out the effects of cardiac progenitor cells, which need to be clarified. These results merit further investigation as the proliferation could serve as a strategy to maintain or protect the loss of cardiomyocytes following a pathological loss of cells.

In conclusion, we demonstrated that thrombin induced COX-2 expression in human cardiomyocytes. Thrombin/PAR1-dependent COX-2 up-regulation was mediated through activation of MAPKs, AP-1 and NF-κB, and resulted in increased cell proliferation. Based on published data and our findings, Figure 7F provides a model for the molecular mechanisms underlying thrombin-induced COX-2 expression and cell proliferation in human cardiomyocytes. These findings highlight the critical role of COX-2/PGE_2/EP_2 system in PAR-mediated interventions in heart disease. Pharmacological approaches suggest that targeting the COX-2/PGE_2 system or PAR-dependent downstream signalling components may yield useful therapeutic targets for heart failure.

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Author contributions

P. T-Y. C., H-L. H., P-L. C. and C-M. Y. developed and designed experiments, and analysed and interpreted data. P. T-Y. C. and P-L. C. conducted the experiments and collected data. This article was drafted by P. T-Y. C., H-L. H., P-L. C. and C-M. Y.

Conflict of interest

The authors declare no conflict of interest.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

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Figure S1 Effects of TFLLR-NH_2 in human cardiomyocytes. (A) Cells were pretreated with SCH79797 (1 μM) and incubated with TFLLR-NH_2 (50 μM) for the indicated time intervals. The cell lysates were subjected to Western blot analysis. (B) Cells were pretreated wth SCH79797 (10 μM) and incubated with TFLLR-NH_2 (50 μM) for the indicated times. The COX-2 mRNA and promoter luciferase activity were detected by real-time PCR (open bar) and promoter assay (black bar). (C) Cells were pretreated with SCH79797 (10 μM) and then stimulated with TFLLR-NH_2 (50 μM) for indicated time intervals. The cell lysates were analysed by Western blot using an anti-phospho-p38, phospho-Erk1/2, phospho-JNK1/2, phospho-p65, phospho-c-Jun, or GAPDH (as an internal control) antibody. (D) The proliferating effects of thrombin on human cardiomyocytes were analysed by a XTT kit. Cells were pretreated with SCH79797 (SCH, 1 μM) for 1 h and then incubated with TFLLR-NH_2 (50 μM) for 48 h. Data are expressed as mean ± SEM (n = 3). $^{\#}P < 0.01$, significantly different from TFLLR-NH_2 alone (C).

Figure S2 Thrombin, TFLLR-NH_2 and PGE_2 induce cell proliferation. (A) Cells were treated with thrombin (3 $U \cdot mL^{-1}$) or TFLLR-NH_2 (50 μM) in the presence or absence of AH6809 (10 μM) and the cell number of human cardiomyocytes were counted by cell counting assay. $^{*}P < 0.05$, as compared with thrombin or TFLLR-NH_2 stimulation alone. (B) Cells were stimulated with PGE_2 (10 μM) for the indicated times. After stimulation, the cell number of human cardiomyocytes was counted by cell counting assay. $^{*}P < 0.05$; $^{\#}P < 0.01$, significantly different from vehicle (B) or thrombin and TFLLR-NH_2 (A) alone (n = 3).