ARTICLE
Journal of Cellular Biochemistry 108:1143–1152 (2009)

Journal of Cellular Biochemistry

Thrombin Induces Cyclooxygenase-2 Expression and Prostaglandin E_2 Release Via PAR1 Activation and ERK1/2- and p38 MAPK-Dependent Pathway in Murine Macrophages

Huey-Ming Lo,[1,2] Chih-Li Chen,[1] Yih-Jeng Tsai,[3] Pi-Hui Wu,[1] and Wen-Bin Wu[1*]

[1] *School of Medicine, Fu-Jen Catholic University, Taipei, Taiwan*

[2] *Section of Cardiology, Department of Internal Medicine, Shin Kong Wu Ho-Su Memorial Hospital, Taipei, Taiwan*

[3] *Department of Otolaryngology, Shin Kong Wu Ho-Su Memorial Hospital, Taipei, Taiwan*

ABSTRACT

Thrombin levels increase at sites of vascular injury and during acute coronary syndromes. It is also increased several fold by sepsis with a reciprocal decrease in the anti-thrombin III levels. In this study we investigate the effects of thrombin on the induction of cyclooxygenase-2 (COX-2) and prostaglandin (PG) production in macrophages. Thrombin-induced COX-2 protein and mRNA expression in RAW264.7 and primary cultured peritoneal macrophages. A serine proteinase, trypsin, also exerted a similar effect. The inducing effect by thrombin in macrophages was not affected by a lipopolysaccharide (LPS)-binding antibiotic, polymyxin B, excluding the possibility of LPS contamination. The increase of COX-2 expression by thrombin was functionally linked to release of PGE_2 and PGI_2 but not thromboxane A_2 into macrophage culture medium. Thrombin-induced COX-2 expression and PGE_2 production were significantly attenuated by PD98059 and SB202190 but not by SP600125, suggesting that ERK1/2 and p38 MAPK activation were involved in this process. This was supported by the observation that thrombin could directly activate ERK1/2 and p38 MAPK in macrophages. A further analysis indicated that the proteinase-activated receptor 1 (PAR1)-activating agonist induced effects similar to those induced by thrombin in macrophages and the PAR1 antagonist-SCH79797 could attenuate thrombin-induced COX-2 expression and PGE_2 release. Taken together, we provided evidence demonstrating that thrombin can induce COX-2 mRNA and protein expression and PGE_2 production in macrophages through PAR1 activation and ERK1/2 and p38 MAPK-dependent pathway. The results presented here may explain, at least in part, the possible contribution of thrombin and macrophages in these pathological conditions. J. Cell. Biochem. 108: 1143–1152, 2009. © 2009 Wiley-Liss, Inc.

KEY WORDS: CYCLOOXYGENASE; MACROPHAGE; MAPK; PAR; PROSTAGLANDIN; PGE; THROMBIN

Thrombin, a serine protease of the trypsin family, is a key enzyme of the blood coagulation system. It is commonly involved in two independent (e.g., contact and extrinsic systems) activation pathways. The coagulation pathway involves a series of reactions, which culminate in the production of sufficient thrombin. Thrombin converts fibrinogen to fibrin and participates in regulating numerous physiological and pathological processes [Furie and Furie, 1988; Esmon, 2000].

Thrombin levels increase at sites of vascular injury [Hatton et al., 1989] and during acute coronary syndromes [Merlini et al., 1994]. It is also increased several fold by sepsis with a reciprocal decrease in the anti-thrombin III levels [Nielsen, 1998; Mavrommatis et al., 2001]. Thrombin has powerful proinflammatory effects on vascular endothelial cells, smooth muscle cells, platelets, monocytes, and macrophages that promote the development of vascular diseases such as atherosclerosis [Croce and Libby, 2007]. It is acting on cells through protease-activated receptors (PARs). PARs belong to a subfamily of G-protein coupled receptors with seven transmembrane domains activated via proteolytic cleavage by serine proteinases [Dery et al., 1998; Coughlin, 2000; Hollenberg and

Abbreviations used: COX, cyclooxygenase; ERK, extracellular matrix-regulated kinase; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; PAR, proteinase-activated receptor; PG, prostaglandin.

Additional Supporting Information may be found in the online version of this article.

Grant sponsor: Shin Kong Wu Ho-Su Memorial Hospital, Taipei, Taiwan; Grant number: SKH-FJU-97-06.

*Correspondence to: Dr. Wen-Bin Wu, School of Medicine, Fu-Jen Catholic University, No. 510, Chung-Cheng Road, Hsinchuang, Taipei, Taiwan. E-mail: wenbin@mail.fju.edu.tw

Received 26 February 2009; Accepted 11 August 2009 • DOI 10.1002/jcb.22341 • © 2009 Wiley-Liss, Inc.
Published online 8 September 2009 in Wiley InterScience (www.interscience.wiley.com).

Compton, 2002]. PAR1, 3, and 4 have been demonstrated to be activated by thrombin and trypsin, whereas PAR2 is only activated by trypsin and tryptase in vitro but in vivo is yet to be demonstrated [Alexander et al., 2006].

Macrophages are central cellular effectors of thrombotic and inflammatory signals in atherosclerosis. In vitro studies have demonstrated that human monocytes and macrophages differentially express PAR-1, 2, and 3 [Colognato et al., 2003] and activation of which by thrombin initiates calcium signaling as well as monocyte-chemoattractant protein-1 (MCP-1), interleukin (IL)-6, IL-8, and matrix metalloproteinase production [Mazzucato et al., 1998; Raza et al., 2000; Colognato et al., 2003; Johansson et al., 2005].

The cyclooxygenases (COX) catalyze the rate-limiting step in prostaglandin (PG) synthesis. It has been known that COX-1 is constitutively expressed in tissues and involved in homeostatic prostanoid biosynthesis, whereas COX-2 is the predominant form in the inflammatory response [Smith et al., 1996]. In macrophages, the most known inducer is lipopolysaccharide (LPS), an endotoxin that causes the induction of induction on COX-2 and inducible nitric-oxide synthase (iNOS) [Akarasereenont et al., 1994]. Thrombin has been shown to induce inflammatory mediators that stimulate macrophages include MCP-1 and RANTES, which serve as potent monocyte and macrophage chemoattractants, and platelet factor 4, which increases macrophage oxidative burst and production of reactive oxygen species [Pervushina et al., 2004]. It also has been shown that thrombin induces inducible NO synthase (iNOS) via Gα12/13-coupled protein kinase C and JNK-dependent pathway in murine macrophages [Kang et al., 2003]. Thus, thrombin appears to influence monocyte/macrophage biology during vascular diseases through direct and indirect paracrine inflammatory pathways. Recently, Kim et al. [2008] have shown that retinoic acid and thrombin can induce COX-2 expression in brain microglia, the resident brain macrophages. However, the effect of thrombin on COX-2 in other macrophages has not been determined and its underlying mechanism remains unclear.

Considering the significance of macrophages for pathological processes, such as chronic inflammation, atherosclerosis, and septic shock, we investigated thrombin effects on macrophages in the present study. We demonstrated that thrombin can induce COX-2 mRNA and protein expression in RAW264.7 and primary cultured peritoneal macrophages, whereas it did not affect COX-1 expression. Moreover, prostaglandin E_2 (PGE_2) release in these two macrophages was increased by thrombin. The possible underlying mechanisms were investigated in this study.

METHODS

MATERIALS

Thrombin, trypsin, LPS (*Escherichia coli* 026:B6), PD98059, SB202190, SP600125, and actinomycin D were purchased from Sigma Chemical Co. (St. Louis, MO). The antibodies (Abs) raised against COX-1 and phospho-ERK1/2 were from Santa Cruz Biotechnology (Santa Cruz, CA). The Ab raised against COX-2 was from BD Biosciences (Franklin Lakes, NJ). The Abs raised against phospho-IκB, IκB, phospho-p38, and phospho-JNK were from New England Biolabs, Inc. (Beverly, MA). The Abs for and total p38 and ERK1/2 were from R&D Systems, Inc. (MN). PAR1-activating peptide H-Thr-Phe-Leu-Leu-Arg-NH_2 ((Thr^1)-TRAP-5 amide (TFLLR amide)) was from Bachem Bioscience, Inc. (King of Prussia, PA). PAR1 antagonist-SCH79797 [(N^3-cyclopropyl-7-{[4-(1-methylethyl)phenyl]methyl}-7H-pyrrolo[3,2-f]quinazoline-1,3-diamine dihydrochloride)] was from Tocris Cookson Ltd. (Bristol, BS, UK). The Ab for α-tubulin was purchased from Calbiochem EMD Bioscience, Inc. (San Diego, CA).

CELL CULTURE

RAW264.7 macrophages and human THP-1 monocytes were obtained from Food Industry Research and Development Institute (Hsinchu, Taiwan). RAW264.7 macrophages were cultured in 90% Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS supplemented with 4 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate and 4.5 g/L glucose, penicillin (100 U/ml), streptomycin (100 μg/ml), and fungizone (250 ng/ml) (Invitrogen Life Technologies, Carlsbad, CA). Murine resident peritoneal macrophages were isolated from ICR mice (aged 6–8 weeks) according to the previously described methods [Zhang et al., 2008] and the animal experimental procedures have been approved by the Fu-Jen Animal Experiment Committee. Briefly, ICR mice were euthanized by decapitation or CO_2 asphyxiation. The syringe was filled with the harvest medium (10 ml/mouse) and attached needle was inserted through peritoneal wall at the midline. The cells were centrifuged (300*g*, 15 min at 4°C). After several washes, cells were allowed to adhesion to culture dish and cultured in the same medium for RAW264.7 before a further analysis. Cells reaching 80–90% of confluency were starved and synchronized in DMEM containing 0.5% FBS at 37°C for 24 h and then subjected to further analysis.

CELL LYSATE PREPARATION AND WESTERN BLOT ANALYSIS

For cell lysate preparation, macrophages were washed with prechilled PBS and lysed in radioimmunoprecipitation assay buffer (20 mM Tris–HCl, pH 7.4, 137 mM NaCl, 2 mM EDTA. One millimolar sodium fluoride, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 10% glycerol, 1 mM sodium orthovanadate, 1 mM PMSF, and 1 μg/ml aprotinin and leupeptin (freshly prepared)). After sonication, the lysate was centrifuged (14,000*g* for 10 min at 4°C), and supernatant was transferred to a tube. The protein content was quantified by Pierce protein assay kit (Pierce, Rockford, IL). In some experiment, cytosolic and nuclear fraction of cell lysates were prepared by using Panomics' Nuclear extraction kit (Panomics, Inc., Redwood City, CA) according to the previously described protocol [Hung et al., 2008]. Total protein was separated by electrophoresis on SDS–polyacrylamide gels and the proteins were electroblotted onto PVDF membranes and then probed using primary anti-phospho-ERK, p38, JNK, and anti-COX mAbs. Immunoblots were detected by enhanced chemiluminescence (ECL, Chemiluminescence Reagent Plus from NEN, Boston, MA). For some experiments, membranes were stripped with a striping buffer (62.5 mM Tris–HCl,

pH 6.7, 2% SDS and 100 mM β-mercaptoethanol), washed, and reprobed with anti-COX-1, α-tubulin or other total protein antibodies and developed as described above.

REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION (RT-PCR) ANALYSIS OF COX mRNA EXPRESSION

Oligonucleotide PCR primers targeting to murine COX-1 and COX-2 were synthesized. The forward primer for COX-1 is 5′-ACTGGCTCTGGGAATTTGTG-3′ and the reverse primer is 5′-AGAGCCGCAGGTGATACTGT-3′. The forward primer for COX-2 is 5′-CAGCAAATCCTTGCTGTTCC-3′ and the reverse primer is 5′-TGGGCAAAGAATGCAAACATC-3′. Total RNA of macrophages was extracted by Trizol reagents (Invitrogen Life Technologies) and reverse transcription reaction was performed by using Superscript III First-Strand Synthesis System (Invitrogen Life Technologies). Briefly, aliquots of 3 μg total RNA were incubated with random hexaprimers for 10 min at 65°C and chilled on ice shortly. After primer annealing, RNA was reverse transcribed by the reverse transcriptase. Reactions were stopped and RNase H was added to remove RNA. Aliquots of transcribed cDNA were subjected to PCR in 25 μl of reaction mixture containing reaction buffer, dNTP, primers, and *Taq* DNA polymerase (Ab Peptides, St. Louis). PCR was performed with a hot start at 94°C for 5 min and then with 30 cycles of denaturation at 94°C for 1 min, annealing at 56°C for 1 min, and elongation at 72°C for 1.5 min on the ABI 7200 Thermal Cycler (Applied Biosystems, Foster City, CA). The amplification products were then analyzed by gel electrophoresis in 2% agarose.

PGE_2, PGI_2, AND TXB_2 RELEASE ASSAY

PGE_2, PGI_2, and TXB_2 released by macrophages were determined by PGE_2, PGI_2, and TXB_2 EIA kits, respectively (Cayman Chemical Company, Ann Arbor, MI) according to the manufacturer's protocol. Briefly, macrophages were treated with PBS or thrombin for 16 h. The cultured media were collected, centrifuged and PGE_2 and TXB_2 release was measured by the acetylcholine esterase competitive enzyme immunoassay. The product of this enzymatic reaction was yellowish color and absorbs strongly at 412 nm. The intensity of this color is proportional to the amount of PGE_2, PGI_2, and TXB_2 tracer bound to the well, which is inversely proportional to the mount of free PGE_2, PGI_2, and TXB_2 present in the well during the incubation. PG concentrations in macrophage cultured medium were calculated from the standard curve.

COX-2 REPORTER CONSTRUCT, TRANSFECTION, AND LUCIFERASE ASSAY

The wild-type COX-2 promoter fragment spanning nucleotides −724 to +7 of the COX-2 promoter cloned into pXP2 luciferase reporter plasmid was a gift kindly provided by Dr. Herschman (Molecular Biology Institute and Department of Biological Chemistry, UCLA, LA, California, USA). The COX-2 reporter construct has been used for luciferase assay in RAW264.7 macrophages [Wadleigh et al., 2000]. Briefly, cells at approximately 80% confluence in 6-well culture plate (Costar) were transfected with 12 μg of total DNA, using lipofectamine (Invitrogen Life Technologies) for 6 h in OPTI medium (Invitrogen Life Technologies) according to the manufacturer's protocol. All DNAs were prepared using endotoxin-free plasmid preparation kits (Qiagen). All transient transfections included 5 μg of psk2 plasmid, 5 μg of COX-2 reporter construct, and 2 μg of pSV-β-galactosidase control vector (used as a transfection efficiency control; from Promega). Following transfection, cells were washed once with endotoxin-free medium and then allowed to grow for 16 h in complete medium containing antibiotics. COX-2 reporter firefly luciferase values were obtained by analyzing 1 ml of purified cell extract according to standard instructions provided by the Luciferase Kit (Promega) in a Wallac Victor 3 1420 multilabel counter (Perkin Elmer, Turku, Finland). β-galactosidase (β-gal) activity assay using ONPG (*O*-nitrophenyl-β-D-galactopyranoside) as substrate was determined by the previously described method [Sambrook et al., 1989]. Relative luciferase activity of purified cell extracts was typically represented as firefly luciferase value (ffLU)/β-gal.

STATISTICAL ANALYSIS

Data were expressed as mean ± standard error mean (SEM). Comparison of means of two groups of data was made by using the unpaired, two-tailed Student's *t*-test.

RESULTS

THROMBIN INDUCES COX-2 PROTEIN EXPRESSION IN RAW264.7 AND PERITONEAL MACROPHAGES

To investigate if thrombin-induced COX-2 protein expression in murine macrophages, Western blotting was performed. Figure 1 showed that thrombin induced an increase in COX-2 protein expression at 8 and 24 h but not at 48 h in RAW264.7 macrophages (Fig. 1A). Moreover, the induction of COX-2 protein by thrombin was in a concentration-dependent manner and the identity was corresponded to that induced by LPS, a potent bacterial toxin that is known to induce COX-2 expression in macrophages (Fig. 1B). In contrast, thrombin did not affect COX-1 and α-tubulin protein expression (Fig. 1A,B). We then examined whether trypsin, a serine proteinase that is known to activate PAR, had a similar effect on macrophages. Figure 1C shows that addition of trypsin to macrophages for 8 and 16 h also caused an increase in COX-2 protein expression in macrophages.

We next examined whether thrombin caused a similar effect on primary cultured murine peritoneal macrophages. Figure 2A shows that thrombin also caused an increase in COX-2 expression in primary cultured peritoneal macrophages. However, thrombin did not affect COX-2 expression in monocytes even at concentration of 5 U/ml (data not shown). It has been reported that an antibiotic polymyxin B can bind to lipid A portion of LPS and interfere with LPS function [Morrison and Jacobs, 1976]. To exclude the possibility that the increase of COX-2 expression was due to LPS contamination, we performed a similar experiment in the presence of polymyxin B. Figure 2B shows that polymyxin B alone neither influenced basal COX-2 expression nor thrombin-induced COX-2 expression; however it inhibited LPS-induced COX-2 expression in macrophages.

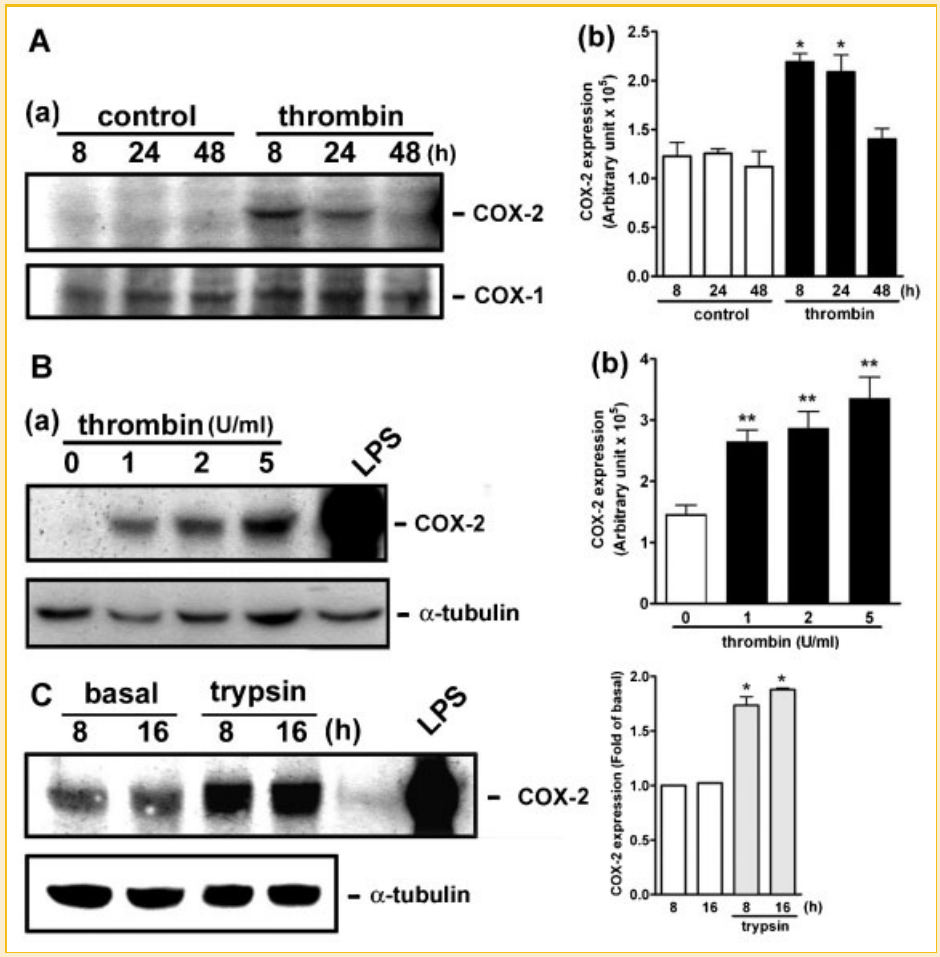

Fig. 1. Thrombin and trypsin induce COX-2 protein expression in murine macrophage cell line. RAW264.7 macrophages were treated with (A) thrombin (1 U/ml), (C) trypsin (100 U/ml) for the indicated time points, or (B) the indicated concentrations of thrombin for 8 h. COX-2 expression in macrophages stimulated by LPS (100 ng/ml) was taken as a positive control (right lane in B and C). Cell lysates were analyzed by Western blotting and the data were quantified by densitometry ($n = 3–4$). $^{*}P < 0.05$, $^{**}P < 0.01$ as compared with control.

Since thrombin/trypsin can activate PAR [Alexander et al., 2006], we used the PAR1 antagonist to examine whether PAR activation was involved in thrombin-induced COX-2 expression. Figure 2C shows that a quantitative analysis of data from Western blotting revealed that SCH79797 inhibited COX-2 expression induced by a higher concentration of thrombin. Taken together, thrombin and trypsin can induce COX-2 protein expression in RAW264.7 and primary cultured peritoneal macrophages but not in monocytes. The induction is dependent on PAR1 activation and is not due to LPS contamination.

THROMBIN TRANSCRIPTIONALLY REGULATES COX-2 EXPRESSION IN MURINE MACROPHAGES

To further examine whether thrombin-induced COX-2 mRNA expression, macrophages were treated with thrombin and COX (COX-1 and COX-2) mRNA expression at 4 and 8 h was evaluated by RT-PCR. As shown in Figure 3A, COX-2 mRNA was upregulated by thrombin at 4 and 8 h and was increased in a concentration-dependent manner, while COX-1 mRNA expression was not affected, suggesting that thrombin induces COX-2 expression in macrophages through a transcriptional regulation. To confirm this hypothesis, a gene transcription inhibitor actinomycin D was used to examine its effect on thrombin-induced COX-2 expression. The effect of actinomycin D on LPS-induced COX-2 expression was taken as a positive control because LPS has been demonstrated to act through the transcriptional regulation [Wu, 2006]. Actinomycin D treatment did not affect macrophage viability (data not shown). However, it inhibited both LPS- and thrombin-induced COX-2 expression (Fig. 3B), suggesting that thrombin-induced COX-2 expression through a transcriptional regulation. The possibility of thrombin acted through affecting the stability of COX-2 mRNA was excluded by the following experiment. Cells were treated with thrombin for 4 h, followed by a rapid wash and incubation with actinomycin D (1 μM) in the absence or presence of thrombin for an additional 1 h. It was found that thrombin-induced COX-2 mRNA level was slightly decayed after addition of actinomycin D. However, thrombin had no effect on COX-2 mRNA level in the presence of actinomycin D (Fig. 3C).

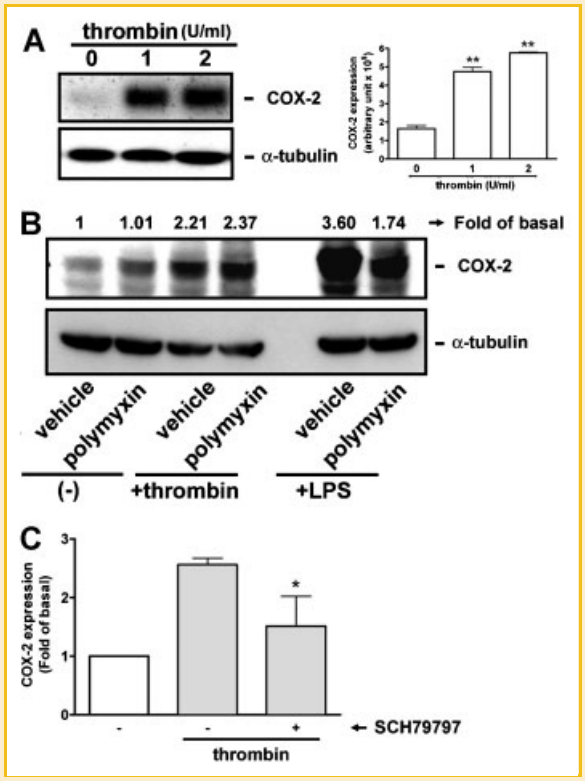

Fig. 2. Effect of polymyxin B and PAR1 antagonist on thrombin-induced COX-2 protein expression in murine macrophages. A: Primary cultured peritoneal macrophages were stimulated with the indicated concentrations of thrombin for 8 h. Cell lysates were analyzed by Western blotting and data analysis was performed by densitometry ($n = 3$). B: Peritoneal macrophages were pretreated with DMSO or polymyxin B (10 μg/ml) for 30 min and followed by the stimulation of thrombin (2 U/ml) or LPS (100 ng/ml) for 8 h. Cell lysates were analyzed by Western blotting. COX-2 level was expressed as the fold of basal and was ratio of COX-2 to α-tubulin intensity by densitometry. $^{**}P < 0.01$ versus control. C: Effect of PAR1 antagonist on thrombin-induced COX-2 expression. RAW264.7 macrophages were pretreated with SCH79797 (5 μM) and followed by addition of thrombin (5 U/ml) for 8 h. Cell lysates were analyzed by Western blotting and expressed as a histogram ($n = 3$).

THROMBIN INDUCES PROSTAGLANDIN E_2 (PGE_2), PGI_2, BUT NOT THROMBOXANE A_2 (TXA_2) RELEASE IN MURINE MACROPHAGES

We next investigated the functional link between COX-2 expression and PG synthesis in macrophages. PGs content in culture medium was analyzed by ELISA, which measures PG content based on the competition between PG and a PG-acetylcholinesterase conjugate for a limited amount of PG monoclonal Ab. Therefore, to exclude the possibility that thrombin might interfere with this assay, we first performed this assay in the presence of thrombin. Figure 4A demonstrates that thrombin alone (without cells) did not significantly increase PGE_2 production (open bar). However, in the presence of macrophages, thrombin concentration-dependently increased PGE_2 release into culture medium (closed bars). Thrombin also induced PGI_2 production in macrophage culture medium, although at a less extent than PGE_2 (Fig. 4B). In contrast, TXB_2, a stable TXA_2 metabolite, was not increased in macrophages challenged with thrombin (Fig. 4C), suggesting that macrophages primarily release PGE_2 in response to thrombin.

EFFECT OF MAPK INHIBITORS ON THROMBIN-INDUCED COX-2 EXPRESSION, PGE_2 RELEASE, AND COX-2 REPORTER ACTIVITY IN MACROPHAGES

It has been reported that thrombin induces iNOS expression in murine RAW264.7 macrophages through a JNK-dependent pathway [Kang et al., 2003]. To investigate the possible signaling pathway involved in the induction of COX-2 by thrombin, three MAPK inhibitors were used, including PD98059, SB202190, and SP600125, which target ERK1/2 upstream kinase-MEK, p38 mitogen-activated protein kinase (MAPK) and JNK, respectively. It was shown that the increase of COX-2 protein expression by thrombin was significantly inhibited by PD98059 and SB202190 but not by SP600125, as determined by Western blotting and densitometry (Fig. 5A). This was also observed in peritoneal macrophages (data not shown). Interestingly, these inhibitors appeared to differentially affect thrombin-induced COX-2 and iNOS expression in macrophages, suggesting a distinct pathway was involved in induction of these two proteins by thrombin in macrophages (Fig. 5A).

We next examined whether PD98059, SB202190, and SP600125 had a similar effect on thrombin-induced PGE_2 release. Figure 5B shows that thrombin-induced PGE_2 release was also significantly inhibited by PD98059 and SB202190 but not by SP600125, while the basal level was not affected by these MAPK inhibitors. To further examine MAPK inhibitors on COX-2 transcription, we performed a COX-2 luciferase reporter assay using COX-2 reporter construct [Wadleigh et al., 2000]. The COX-2 reporter construct was transiently transfected into subconfluent RAW264.7 macrophages. To test efficiency of transfection, cells were subsequently induced with LPS. As previously reported [Wadleigh et al., 2000], LPS induced about threefold increases in luciferase activity in macrophages (Fig. 5C, left panel). In this regard, thrombin also increased luciferase activity and it was attenuated by PD98059 and SB202190 but not by SP600125 (Fig. 5C, right panel). Taken together, ERK1/2 and p38 MAPK activation are required for thrombin-induced COX-2 mRNA transcription and protein expression and PGE_2 release in macrophages.

THROMBIN DIRECTLY INDUCES MAPK ACTIVATION IN MURINE MACROPHAGES

We next examined whether thrombin could directly activate signaling pathway in macrophages. Figure 6A shows that thrombin markedly activated ERK1/2 and p38 MAPK both in RAW264.7 and peritoneal macrophages. It was found that thrombin-induced p38 MAPK activation was in a two-phase fashion, which was activated at 15 min but returned to basal level at 30 and 60 min and followed by the increase at 120 and 180 min. On the other hand, thrombin appeared to slightly activated JNK at the tested time points in RAW264.7 and peritoneal macrophages. To further test whether COX-2 expression by thrombin was simultaneously increased with p38 MAPK activation, a time-chasing experiment on COX-2 expression was performed. Unlike p38 MAPK, it appeared that COX-2 expression by thrombin was not induced in a similar way (Fig. 6B). It has been reported that thrombin can induce IκB

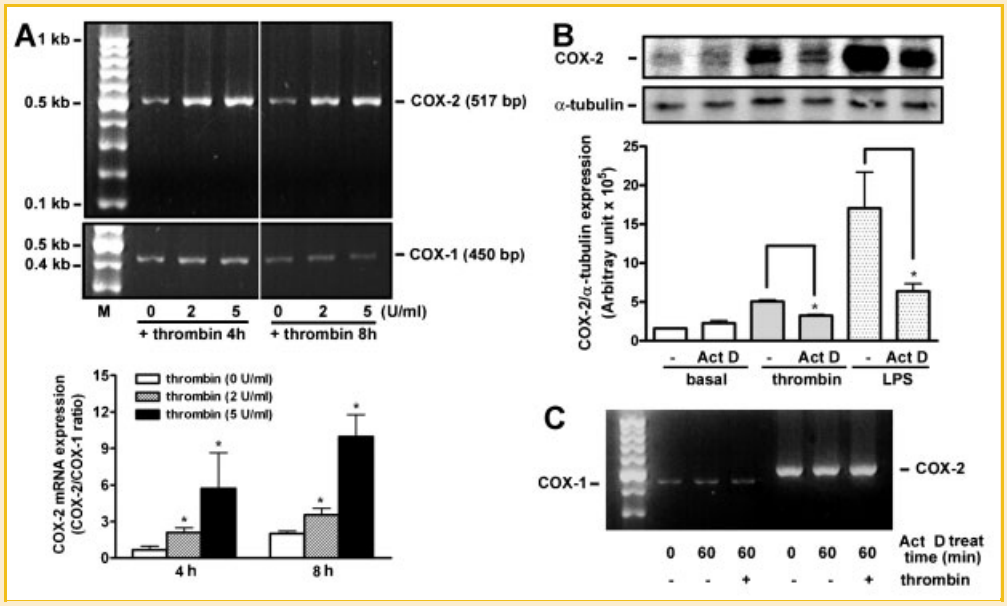

Fig. 3. Thrombin transcriptionally regulates COX-2 expression in macrophages. A: Effect of thrombin on COX-2 mRNA expression in macrophages. RAW264.7 macrophages were treated with the indicated concentrations of thrombin for 4 and 8 h. At the end of incubation, cells were collected and total RNA was analyzed by RT-PCR. The PCR products for COX-2 and COX-1 were indicated. Data from similar experiments were quantified by densitometry. Results were expressed as ratio of COX-2 to COX-1 intensity ($n = 3$). M, 100-bp marker. B: Effect of the transcription inhibitor on thrombin-induced COX-2 expression. RAW264.7 macrophages were pretreated with actinomycin D (Act D, 1 μM) for 30 min and followed by the addition of thrombin (5 U/ml) or LPS (1 μg/ml) for 2 h. Cell lysates were analyzed by Western blotting ($n = 3$). $^*P < 0.05$ versus control. C: Effect on thrombin on COX-2 mRNA stability. RAW264.7 macrophages were treated as described in the text. COX-2 mRNA level was analyzed by RT-PCR ($n = 3$).

phosphorylation and degradation in macrophages [Kang et al., 2003], however, in our system although IκBα phosphorylation was slightly increased by thrombin at a longer incubation (120 and 180 min), total IκB protein level in macrophages was not obviously changed at the tested time points. Moreover, NF-κB p65 subunit translocation to nucleus, which is required for the regulation of transcription, was not observed in macrophages in response to thrombin (Fig. 6C).

EGF transactivation has been reported to be involved in thrombin-regulated COX-2 and PGE_2 production in vascular smooth muscle cells (VSMCs) [Hsieh et al., 2008] and in rat gastric mucosal epithelial cells [Sekiguchi et al., 2007]. Since thrombin induced two-phase p38 MAPK activation in macrophages, we tested whether AG1478, an EGF receptor antagonist, affected thrombin-induced COX-2 expression in macrophages. The specificity and effectiveness of AG1478 was demonstrated by its inhibitory effects on EGF-induced COX-2 expression in macrophages and ERK1/2 activation in macrophages and VSMCs (Supplementary Data, panel A). However, AG1478 did not affect thrombin-induced COX-2 expression (panel B), suggesting that EGF transactivation was not involved in this process.

EFFECT OF PAR1 AGONIST AND ANTAGONIST ON COX-2 EXPRESSION, PGE_2 RELEASE, AND SIGNALING IN MACROPHAGES

We have demonstrated that PAR1 activation by thrombin was required for the induction of COX-2 expression in macrophages (Fig. 2C). A synthetic PAR1 agonist was further used to investigate PAR1 activation on COX-2 expression and signaling in macrophages. As shown in Figure 7A, the PAR1 selective agonist obviously induced ERK1/2 and p38 MAPK activation and slightly activated JNK activation in macrophages. The induction profile is similar to those activated by thrombin (Fig. 6A). A further analysis revealed that PAR1 agonist induced COX-2 protein expression and it was also attenuated by PD98059 and SB202190 in macrophages (Fig. 7B), indicating that PAR1 activation was involved in thrombin-induced COX-2 expression. We next examined whether PAR1 selective antagonist-SCH79797 could functionally affect thrombin-induced PGE_2 production. The specificity of SCH79797 toward PAR1 was demonstrated by the observation that it could inhibit thrombin and PAR1 agonist-induced ERK1/2 and p38 activation in macrophages (Fig. 7C, right panel). Taken together, we suggest that PAR1 activation is involved in thrombin-induced signaling, COX-2 expression and PGE_2 release in murine macrophages.

DISCUSSION

In this study, we demonstrate that thrombin could induce COX-2 mRNA and protein expression in murine RAW264.7 macrophage cell line and primary cultured peritoneal macrophages (Figs. 1–3). Moreover, trypsin, a serine proteinase that can activate PARs, induced COX-2 expression in macrophages (Fig. 1C). We also demonstrate that thrombin induced PGE_2, PGI_2, but not TXB_2 release from macrophages (Fig. 4), suggesting that the increase of COX-2 was functionally link to the increase of PGE_2 and PGI_2. The most concern was whether the induction of COX-2 expression by thrombin in macrophages resulted from LPS contamination. The possibility was excluded according to our observations that

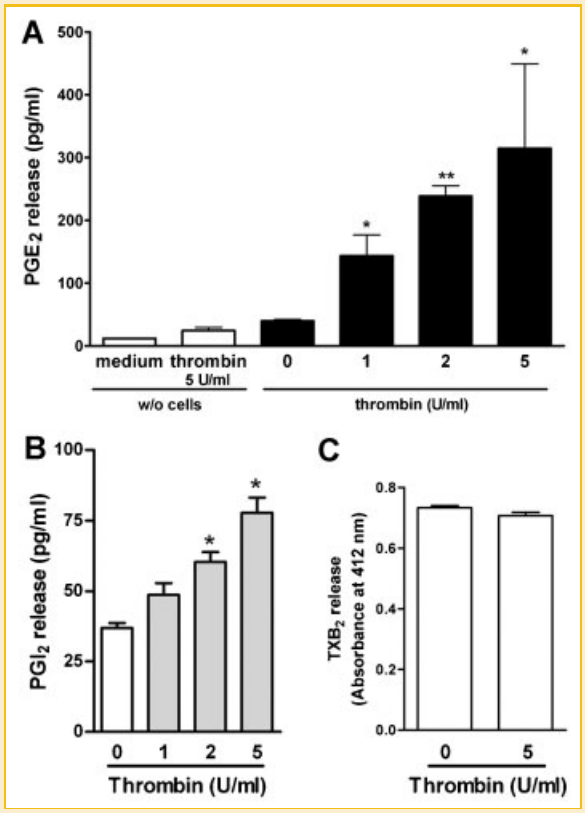

Fig. 4. Thrombin causes PGE_2 and PGI_2 but not TXB_2 production in macrophage culture medium. The 6-well culture plate was seeded with or without RAW264.7 macrophages. Media in the wells in the absence (w/o cells) or presence of macrophages were added with the indicated concentrations of thrombin for 16 h. Culture media were collected and PGE_2, PGI_2, or TXB_2 released from macrophages were measured by ELISA as described in the Methods Section. Data were mean ± SEM (n = 3–4). $^{*}P < 0.05$, $^{**}P < 0.01$ versus control.

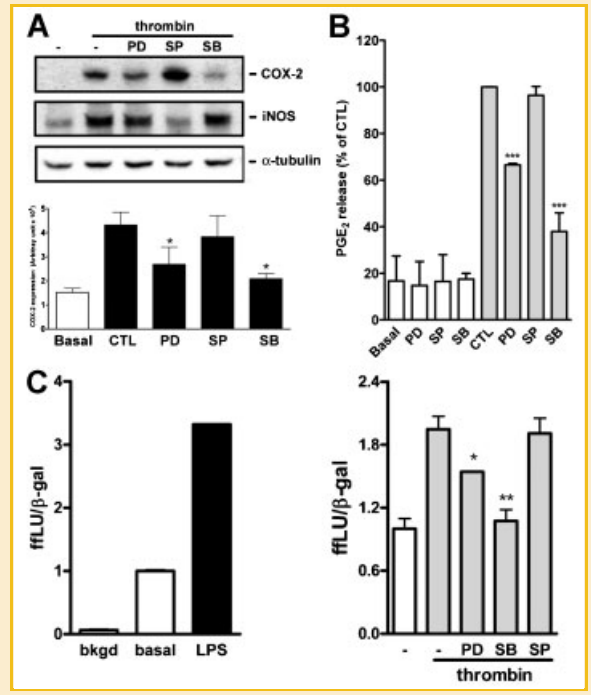

Fig. 5. Effect of MAPK inhibitors on thrombin-induced COX-2 expression, PGE_2 production and COX-2 reporter activity in macrophages. A: RAW264.7 macrophages were pretreated with DMSO or the indicated MAPK inhibitors (10 μM for each, PD: PD98059, SP: SP600125 and SB: SB202190) for 30 min and followed by the addition of thrombin (1 U/ml) for 8 h. After incubation, cells were analyzed by Western blotting. COX-2 expression was determined by densitometry (n = 3–5). B: The culture media from (A) were analyzed by ELISA (n = 4). $^{*}P < 0.05$, $^{***}P < 0.001$ versus control. C: The COX-2 reporter construct was transiently transfected into RAW264.7 macrophages, along with β-galactosidase plasmid as transfection efficiency control for 6 h, followed by growth for 16 h in endotoxin-free medium. Cells were treated with LPS (1 μg/ml) for 6 h or thrombin (5 U/ml) for 4 h in the presence or absence of MAPK inhibitors and then lysed. Firefly luciferase value (ffLU) and β-galactosidase activity (β-gal) were determined as described in the Methods Section (n = 2–3). Bkgd: Cells only transfected with control and β-galactosidase plasmids. $^{*}P < 0.05$, $^{**}P < 0.01$ versus control.

treatment of macrophages with polymyxin B, an antibiotic known to inhibit LPS activity by binding to the lipid A moiety, was ineffective in suppressing thrombin-induced COX-2 expression but had a marked inhibitory effect on LPS-induced COX-2 expression (Fig. 2B).

It has been shown that a cyclic AMP response element (CRE) located at −53/−59 is absolutely essential for basal and induced COX-2 transcription as mutation of this site renders COX-2 promoter completely silent and unable to respond to exogenous stimuli. Moreover, a number of transactivators have been shown to be involved in COX-2 transcriptional activation by cytokines, LPS and growth factors. They include c-Jun/c-Fos (AP-1), p65/p50 NF-κB, C/EBPβ and C/EBPδ, NF-AT, SP-1, CREB-2/ATF2 [Wu, 2006]. However, it was recently reported that some drugs such as aspirin can enhance the stability of COX-2 mRNA in human intestinal myofibroblasts treated with IL-1 [Mifflin et al., 2004]. In the present study, we suggest that thrombin induces COX-2 expression through transcriptional regulation, which is evidenced by the following findings. In Figure 3, thrombin enhanced COX-2 mRNA transcription and a gene transcription inhibitor-actinomycin D could attenuate thrombin-induced COX-2 protein expression. In addition, thrombin did not affect COX-2 mRNA stability. In Figure 5, a luciferase reporter analysis indicated that thrombin could increase luciferase activity in macrophages transfected with the COX-2 reporter construct.

Since thrombin-induced COX-2 expression and PGE_2 release were significantly inhibited by PD98059 and SB202190 but not by SP600125, we suggest ERK1/2 and p38 MAPK activation are involved in the induction of COX-2 expression and PGE_2 release (Fig. 5). Several lines of evidence support this hypothesis. Firstly, MTT assay demonstrated that cell viability was not affected in macrophages after addition of these inhibitors (data not shown), excluding the possibility that the inhibition is due to cytotoxicity of these inhibitors. Secondly, these inhibitors have differential effects on thrombin-induced COX-2 and iNOS expression in macrophages. Notably, the SB202190 (p38 MAPK inhibitor) inhibited thrombin-induced COX-2 but not iNOS expression, whereas the SP600125 (JNK inhibitor) inhibited thrombin-induced iNOS but not COX-2 expression (Fig. 5A). A similar effect of these inhibitors on

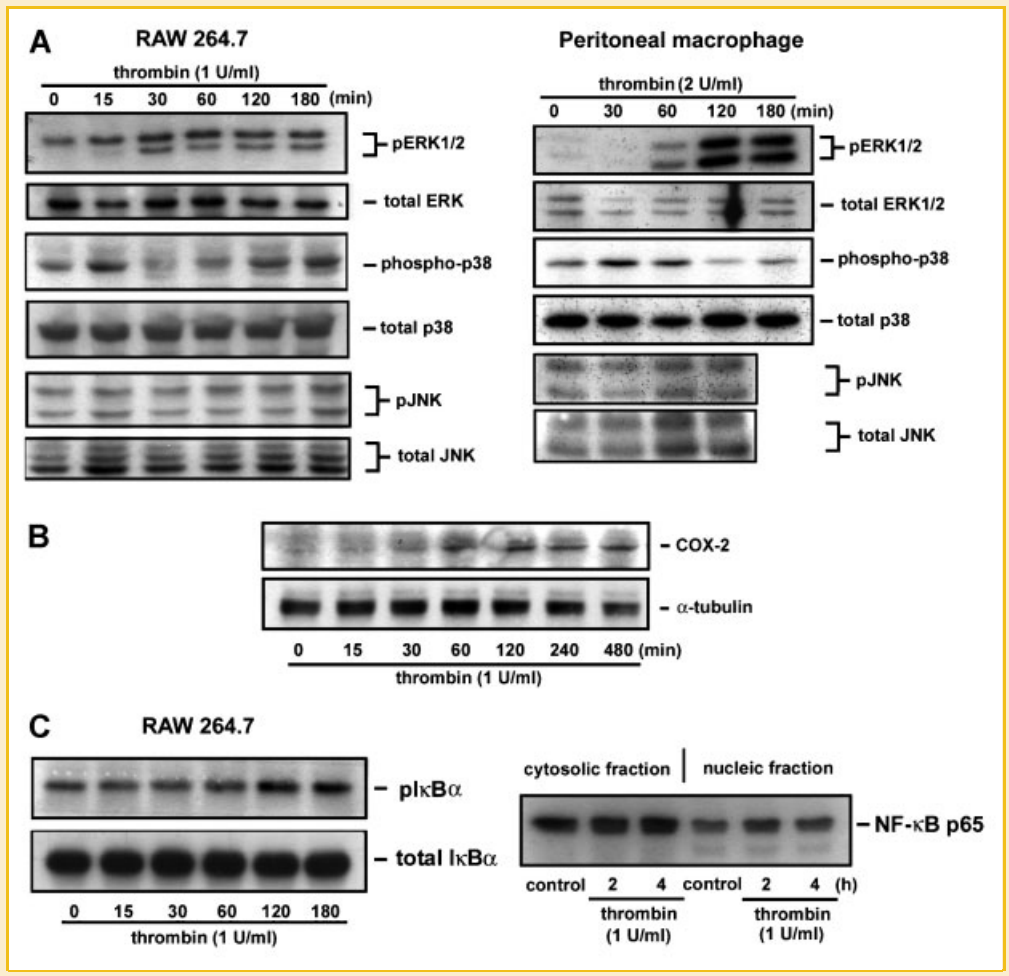

Fig. 6. Effect of thrombin on MAPKs, IκB and NF-κB p65 activation and COX-2 expression in macrophages. RAW264.7 or peritoneal macrophages were treated with thrombin for the indicated time points. Cell lysates, cytosolic or nucleic fraction were analyzed by Western blotting. Each blot was representative from three experiments.

thrombin-induced iNOS expression has also been described by Kang et al. [2003]. Thirdly, thrombin could directly and markedly activate ERK1/2 and p38 MAPK in both RAW264.7 and peritoneal macrophages (Fig. 6). Fourthly, PAR1-selective agonist activated ERK1/2 and p38 MAPK and induced COX-2 expression in macrophages. The inhibitor targets to each MAPK also attenuated PAR1 agonist-induced COX-2 expression (Fig. 7B). However, the COX-2 induction and MAPK activation by thrombin seems not to be mediated by EGF transactivation because AG1478 (an EGF receptor antagonist) did not affect COX-2 expression (Supplementary Data). As some transcription factors that drive COX-2 transcription such as ATF-2, CREB, and c-Fos are activated by phosphorylated (activated) ERK1/2 and p38 MAPK, it is possible that activation of these two MAPKs by thrombin is responsible for the increase of COX-2 mRNA and protein expression.

Thrombin and trypsin can activate PAR1, 3, and 4 [Alexander et al., 2006]. In our study we found that PAR1-selective agonist, TFLLR amide, not only could induce signaling transduction similar to those by thrombin but also an increase in COX-2 expression in macrophages (Fig. 7A,B). Moreover, thrombin-induced COX-2 expression and PEG_2 production was inhibited by the PAR1 antagonist-SCH79797 (Fig. 7C). Based on these observations, we suggest that PAR1 activation is involved in thrombin-induced COX-2 expression. This is different from that thrombin acts independent of PAR1 to induce growth and proinflammatory actions in human cultured airway smooth muscle cells [Tran and Stewart, 2003]. Although human monocytes mainly expressed PAR1 while differentiated macrophages expressed PAR1, 2, and 3 [Colognato et al., 2003], PAR expression in murine macrophages has not been determined. The well established is that mouse platelets express PAR3 and 4, but not PAR1 and thrombin binding to PAR3 transiently anchors the enzyme to the cell surface where it can cleave and activate PAR4 [Nakanishi-Matsui et al., 2000]. However, there is a report showing that PAR3 peptides based on either the human or murine PAR3-derived tethered ligand sequences do not activate PAR3, but rather activate PAR1 and 2 in Jurkat cells [Hansen et al., 2004]. Therefore we did not test whether thrombin can also activate signaling and COX-2 expression through other PARs in macrophages.

PGs belong to autacoids that can induce many physiological/pharmacological responses on vascular tone, platelet aggregation, etc. They also participate in the development of many diseases such

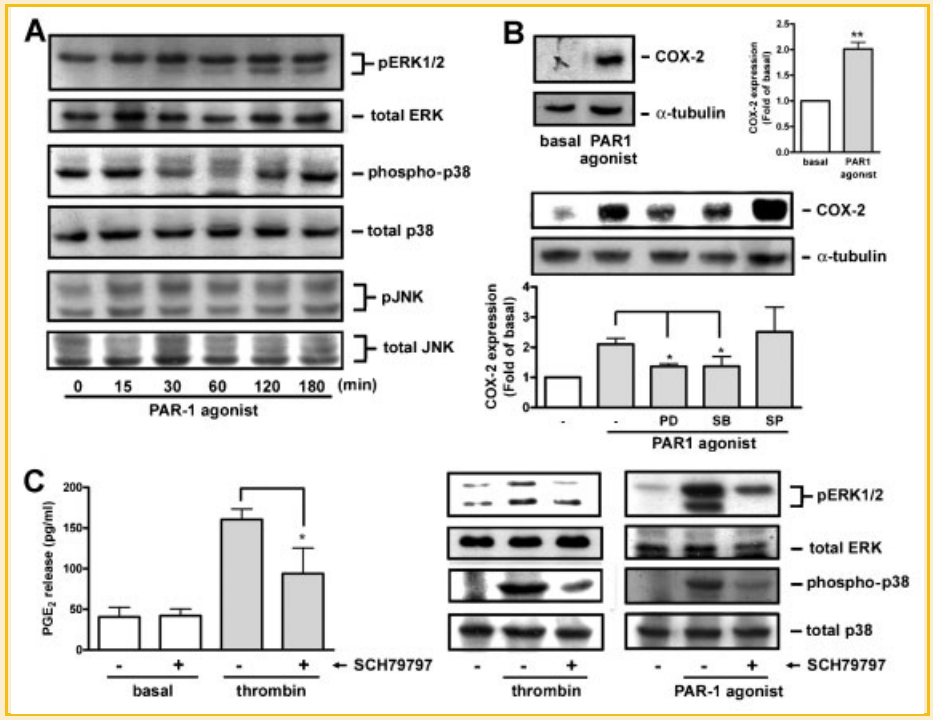

Fig. 7. Effect of PAR1 agonist and antagonist on signaling, COX-2 expression, and PGE_2 production in macrophages. A: PAR1 agonist activates MAPK. RAW264.7 macrophages were treated with PAR1 selective peptide (100 μM) for the indicated time points. B: Effect of MAPK inhibitors on PAR1 agonist-induced COX-2 expression. RAW264.7 macrophages were treated with PAR1 selective peptide (100 μM) for 8 h in the absence or presence of MAPK inhibitors (10 μM for each, PD: PD98059, SB: SB202190, and SP: SP600125). Cell lysates were analyzed by Western blotting and the quantitative analysis was performed by densitometry ($n = 3–4$). C: Effect of PAR1 antagonist on thrombin-induced PGE_2 production. Left panel: RAW264.7 macrophages were treated with thrombin (5 U/ml) for 8 h in the absence or presence of PAR1 antagonist-SCH79797 (5 μM) and PGE_2 in culture medium was analyzed by ELISA. Right panel indicated that SCH79797 (5 μM) abolished MAPK activation in macrophages in response to thrombin (1 U/ml) and PAR1 agonist (100 μM) at 120 min. $^*P < 0.05$ versus control.

as cancer and cardiovascular diseases. In the present study, we show that thrombin/trypsin enhanced COX-2 expression and PGE_2 and PGI_2 production in macrophages. It has been demonstrated that selective inhibition of COX-2 (by rofecoxib and NS-398) and elimination of COX-2 ($COX\text{-}2^{-/-}$) from macrophages significantly reduces early atherosclerotic lesion formation in apoE-deficient and C57BL/6 mice [Burleigh et al., 2005]. This suggests that thrombin-induced COX-2 expression may contribute to atherogenesis. However, PGE_2 and PGI_2 can also induce vasorelaxation and inhibit platelet aggregation in vessels response to injury. It was reported that TXA_2 promotes and PGI_2 prevents the initiation and progression of atherogenesis through control of platelet activation and leukocyte–endothelial cell interaction [Kobayashi et al., 2004]. Therefore, it is possible that PGE_2 and PGI_2 production in macrophages stimulated by thrombin have a protective role during atherosclerosis and other vascular diseases.

In conclusion, in the present study we demonstrated that thrombin can induce COX-2 but not COX-1 mRNA and protein expression in RAW264.7 and primary cultured peritoneal macrophages through an ERK1/2 and p38 MAPK-dependent pathway. At least PAR1 activation is involved in this process. More importantly, the induction of COX-2 expression by thrombin functionally leads to the increase of PGE_2 and PGI_2 but not TXA_2 release from macrophages. Since macrophages play an important role in development of vascular diseases such as atherosclerosis and septic shock, the results presented here may explain, at least in part, the possible contribution of thrombin and macrophages in these pathological conditions.

ACKNOWLEDGMENTS

We thank Dr. Herschman (Molecular Biology Institute and Department of Biological Chemistry, UCLA, LA, California, USA) for providing wild-type COX-2 reporter construct. The work was supported by the research grant (SKH-FJU-97-06) from Shin Kong Wu Ho-Su Memorial Hospital, Taipei, Taiwan.

REFERENCES

Akarasereenont P, Mitchell JA, Appleton I, Thiemermann C, Vane JR. 1994. Involvement of tyrosine kinase in the induction of cyclo-oxygenase and nitric oxide synthase by endotoxin in cultured cells. Br J Pharmacol 113: 1522–1528.

Alexander SP, Mathie A, Peters JA. 2006. Guide to receptors and channels, 2nd edition. Br J Pharmacol 147(Suppl 3):S1–S168.

Burleigh ME, Babaev VR, Yancey PG, Major AS, McCaleb JL, Oates JA, Morrow JD, Fazio S, Linton MF. 2005. Cyclooxygenase-2 promotes early atherosclerotic lesion formation in ApoE-deficient and C57BL/6 mice. J Mol Cell Cardiol 39:443–452.

Colognato R, Slupsky JR, Jendrach M, Burysek L, Syrovets T, Simmet T. 2003. Differential expression and regulation of protease-activated receptors in human peripheral monocytes and monocyte-derived antigen-presenting cells. Blood 102:2645–2652.

Coughlin SR. 2000. Thrombin signalling and protease-activated receptors. Nature 407:258–264.

Croce K, Libby P. 2007. Intertwining of thrombosis and inflammation in atherosclerosis [Miscellaneous Article]. Curr Opin Hematol 14:55–61.

Dery O, Corvera CU, Steinhoff M, Bunnett NW. 1998. Proteinase-activated receptors: Novel mechanisms of signaling by serine proteases. Am J Physiol 274:C1429–C1452.

Esmon CT. 2000. Regulation of blood coagulation. Biochim Biophys Acta 1477:349–360.

Furie B, Furie BC. 1988. The molecular basis of blood coagulation. Cell 53:505–518.

Hansen KK, Saifeddine M, Hollenberg MD. 2004. Tethered ligand-derived peptides of proteinase-activated receptor 3 (PAR3) activate PAR1 and PAR2 in Jurkat T cells. Immunology 112:183–190.

Hatton MW, Moar SL, Richardson M. 1989. Deendothelialization in vivo initiates a thrombogenic reaction at the rabbit aorta surface. Correlation of uptake of fibrinogen and antithrombin III with thrombin generation by the exposed subendothelium. Am J Pathol 135:499–508.

Hollenberg MD, Compton SJ. 2002. International Union of Pharmacology. XXVIII. Proteinase-activated receptors. Pharmacol Rev 54:203–217.

Hsieh HL, Sun CC, Wang TS, Yang CM. 2008. PKC-delta/c-Src-mediated EGF receptor transactivation regulates thrombin-induced COX-2 expression and PGE(2) production in rat vascular smooth muscle cells. Biochim Biophys Acta 1783:1563–1575.

Hung CF, Huang TF, Chen BH, Shieh JM, Wu PH, Wu WB. 2008. Lycopene inhibits TNF-[alpha]-induced endothelial ICAM-1 expression and monocyte-endothelial adhesion. Eur J Pharmacol 586:275–282.

Johansson U, Lawson C, Dabare M, Syndercombe-Court D, Newland AC, Howells GL, Macey MG. 2005. Human peripheral blood monocytes express protease receptor-2 and respond to receptor activation by production of IL-6, IL-8, and IL-1{beta}. J Leukoc Biol 78:967–975.

Kang KW, Choi SY, Cho MK, Lee CH, Kim SG. 2003. Thrombin induces nitric-oxide synthase via Galpha 12/13-coupled protein kinase C-dependent I-kappa Balpha phosphorylation and JNK-mediated I-kappa Balpha degradation. J Biol Chem 278:17368–17378.

Kim B, Lee JH, Yang MS, Jou I, Joe EH. 2008. Retinoic acid enhances prostaglandin E2 production through increased expression of cyclooxygenase-2 and microsomal prostaglandin E synthase-1 in rat brain microglia. J Neurosci Res 86:1353–1360.

Kobayashi T, Tahara Y, Matsumoto M, Iguchi M, Sano H, Murayama T, Arai H, Oida H, Yurugi-Kobayashi T, Yamashita JK, Katagiri H, Majima M, Yokode M, Kita T, Narumiya S. 2004. Roles of thromboxane A(2) and prostacyclin in the development of atherosclerosis in apoE-deficient mice. J Clin Invest 114:784–794.

Mavrommatis AC, Theodoridis T, Economou M, Kotanidou A, El AM, Christopoulou-Kokkinou V, Zakynthinos SG. 2001. Activation of the fibrinolytic system and utilization of the coagulation inhibitors in sepsis: Comparison with severe sepsis and septic shock. Intensive Care Med 27: 1853–1859.

Mazzucato M, De Marco L, Masotti A, Pradella P, Bahou WF, Ruggeri ZM. 1998. Characterization of the initial alpha—Thrombin interaction with glycoprotein Ibalpha in relation to platelet activation. J Biol Chem 273: 1880–1887.

Merlini PA, Bauer KA, Oltrona L, Ardissino D, Cattaneo M, Belli C, Mannucci PM, Rosenberg RD. 1994. Persistent activation of coagulation mechanism in unstable angina and myocardial infarction. Circulation 90: 61–68.

Mifflin RC, Saada JI, Di Mari JF, Valentich JD, Adegboyega PA, Powell DW. 2004. Aspirin-mediated COX-2 transcript stabilization via sustained p38 activation in human intestinal myofibroblasts. Mol Pharmacol 65:470–478.

Morrison DC, Jacobs DM. 1976. Binding of polymyxin B to the lipid A portion of bacterial lipopolysaccharides. Immunochemistry 13:813–818.

Nakanishi-Matsui M, Zheng YW, Sulciner DJ, Weiss EJ, Ludeman MJ, Coughlin SR. 2000. PAR3 is a cofactor for PAR4 activation by thrombin. Nature 404:609–613.

Nielsen JD. 1998. The effect of antithrombin on the systemic inflammatory response in disseminated intravascular coagulation. Blood Coagul Fibrinolysis 9(Suppl 3):S11–S15.

Pervushina O, Scheuerer B, Reiling N, Behnke L, Schroder J, Kasper B, Brandt E, Bulfone-Paus S, Petersen F. 2004. Platelet factor 4/CXCL4 induces phagocytosis and the generation of reactive oxygen metabolites in mononuclear phagocytes independently of Gi protein activation or intracellular calcium transients. J Immunol 173:2060–2067.

Raza SL, Nehring LC, Shapiro SD, Cornelius LA. 2000. Proteinase-activated receptor-1 regulation of macrophage elastase (MMP-12) secretion by serine proteinases. J Biol Chem 275:41243–41250.

Sambrook J, Fritsch EF, Maniatis T. 1989. Book3, Chapter 16. Expression of Cloned Genes in Cultured Mammalian Cells. Molecular cloning: A Laboratory Manual. New York: CSHL Press. 16.66–16.67.

Sekiguchi F, Saito S, Takaoka K, Hayashi H, Nagataki M, Nagasawa K, Nishikawa H, Matsui H, Kawabata A. 2007. Mechanisms for prostaglandin E2 formation caused by proteinase-activated receptor-1 activation in rat gastric mucosal epithelial cells. Biochem Pharmacol 73:103–114.

Smith WL, Garavito RM, DeWitt DL. 1996. Prostaglandin endoperoxide H synthases (cyclooxygenases)-1 and -2. J Biol Chem 271:33157–33160.

Tran T, Stewart AG. 2003. Protease-activated receptor (PAR)-independent growth and pro-inflammatory actions of thrombin on human cultured airway smooth muscle. Br J Pharmacol 138:865–875.

Wadleigh DJ, Reddy ST, Kopp E, Ghosh S, Herschman HR. 2000. Transcriptional activation of the cyclooxygenase-2 gene in endotoxin-treated RAW 264.7 macrophages. J Biol Chem 275:6259–6266.

Wu KK. 2006. Transcription-based COX-2 inhibition: A therapeutic strategy. Thromb Haemost 96:417–422.

Zhang X, Goncalves R, Mosser DM. 2008. The isolation and characterization of murine macrophages. Curr Protoc Immunol 83:14.1.1–14.1.9.