

Signaling Mechanisms Involved in Protease-Activated Receptor-1-Mediated Interleukin-6 Production by Human Gingival Fibroblasts

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Received March 18, 2004; accepted June 21, 2004

ABSTRACT

Human gingival fibroblasts (HGFs) express protease-activated receptor-1 (PAR-1) at high levels. In cultured HGFs, we studied the signaling pathway of thrombin-induced interleukin-6 (IL-6) production. The PAR-1 agonist peptide SFLLRN mimicked the thrombin-induced IL-6 production in the presence of amastatin, an aminopeptidase inhibitor. Thrombin or a combination of SFLLRN and amastatin also strikingly induced the expression of IL-6 mRNA. Although continuous exposure of HGFs to thrombin rapidly desensitized Ca^{2+} signaling, the cells did not lose their ability to produce IL-6 in response to thrombin. Similarly, although treatment of HGFs with BAPTA-AM [1,2-bis(O-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid-acetoxymethyl ester], an intracellular Ca^{2+} chelator, markedly attenuated the thrombin-induced increase in intracellular Ca^{2+} concentration,

the same treatment did not suppress the thrombin-induced IL-6 production. However, thrombin-induced IL-6 production was strongly inhibited by the p38 mitogen-activated protein (MAP) kinase and tyrosine kinase inhibitors, and Western blotting analyses showed that thrombin stimulates p38 MAP kinase phosphorylation. Specific inhibitors that inhibit extracellular signal-regulated kinase 1/2 kinase, phosphatidylinositol 3-kinase, and RhoA kinase also partially suppressed the thrombin-induced IL-6 production, but the effects were smaller than those of the p38 MAP and tyrosine kinase inhibitors. Thus, thrombin induces HGFs to produce IL-6 by activating PAR-1, and the tyrosine kinase- and p38 MAP kinase-dependent pathways, rather than the Ca^{2+} signaling pathway, may play a crucial role in the IL-6 production.

Protease-activated receptors (PARs) are unique members of the G protein-coupled receptor superfamily (Kawabata and Kuroda, 2000; Macfarlane et al., 2001; Hollenberg and Compton, 2002). The activation of the PARs involves the proteolytic unmasking of an N-terminal receptor sequence by serine proteases. The newly generated N terminus then acts as a tethered ligand that binds to the second extracellular loop of PAR, resulting in the activation of the cleaved recep-

tor. To date, four PAR family members have been identified. PAR-1, PAR-3, and PAR-4 are activated mainly by thrombin (TB), whereas PAR-2 is activated by trypsin and mast cell tryptase, but not by thrombin. PARs are widely distributed in the body and have been demonstrated to be involved in a variety of biological responses, including cell growth, tissue repair, coagulation, secretion, and inflammation.

Human gingival fibroblasts (HGFs), which predominate in periodontal tissues, can produce various proinflammatory cytokines, such as interleukin (IL)-1, IL-6, and IL-8 (Bartold and Haynes, 1991; Takada et al., 1991; Agarwal et al., 1995). The inflammatory cytokines derived from HGFs have been demonstrated to play an important role in the initiation and progression of periodontal diseases. IL-6 is a major mediator

This study was partially supported by the Academic Science Frontier Project of the Ministry of Education, Culture, Sports, Science and Technology, Japan.

Article, publication date, and citation information can be found at <http://jpet.aspetjournals.org>.
doi:10.1124/jpet.104.068569.

ABBREVIATIONS: PAR, protease-activated receptor; TB thrombin; HGF, human gingival fibroblast; IL, interleukin; MAP kinase, mitogen-activated protein kinase; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1*H*-imidazole; PD98059, 2'-amino-3'-methoxyflavone; LPS, lipopolysaccharide; U0126, 1,4-diamino-2,3-dicyano-1,4-bis(2-methylthio)butadiene; SB 202474, 4-(ethyl)-2-(4-methoxyphenyl)-5-(4-pyridyl)-1*H*-imidazole; Y-27632, (*R*)-(+)-*trans*-*N*-(4-pyridyl)-4-(1-aminoethyl)-cyclohexanecarboxamide; SB 202190, 4-(4-fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)-1*H*-imidazole; Ro-31-8220, bisindolylmaleimide IX, 3-[1-[3-(amidinothio)propyl-1*H*-indol-3-yl]-3-(1-methyl-1*H*-indol-3-yl)] maleimide; BAPTA-AM, 1,2-bis(O-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid-acetoxymethyl ester; α -MEM, α -minimum essential medium; ELISA, enzyme-linked immunosorbent assay; RT, reverse transcriptase; PCR, polymerase chain reaction; DMSO, dimethyl sulfoxide; ERK1/2, extracellular signal-regulated kinase 1/2; PI, phosphatidylinositol; PKC, protein kinase C.

of the host defense to injury and infection because it induces plasma cell differentiation and antibody production. However, IL-6 can also be pathogenic because it stimulates osteoclastic bone resorption by promoting osteoclast formation (Kurihara et al., 1990; Tamura et al., 1993). Gingival bleeding occurs commonly in chronic gingivitis and periodontitis, resulting in the generation of thrombin at the coagulation sites in inflamed gingival tissues. Hou et al. (1998) have reported that thrombin stimulates IL-6 production in cultured HGFs. Because HGFs express PAR-1 at high levels in vitro (Hou et al., 1998; Chang et al., 2001; Tanaka et al., 2003), it may be that thrombin is involved in the pathogenesis of periodontal diseases, perhaps by activating PAR-1.

There is little information regarding the signaling mechanisms by which thrombin induces HGFs to produce IL-6. It is known that the activation of PAR-1 stimulates Ca^{2+} mobilization from intracellular Ca^{2+} stores via phosphoinositide hydrolysis, but this Ca^{2+} signaling is rapidly desensitized by continuous exposure to thrombin (Tanaka et al., 2003). That the thrombin-induced increase in intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) is transient is also incompatible with the fact that stimulation with thrombin can increase the expression of IL-6 mRNA for at least 8 h in HGFs (Hou et al., 1998). In addition, it has been noted that although the transient increase in $[\text{Ca}^{2+}]_i$ induced by thrombin is mimicked by the PAR-1 agonist peptide SFLLRN (Tanaka et al., 2003), the agonist induces much less IL-6 production than that of thrombin (Sower et al., 1995; Hou et al., 1998). Therefore, it is unclear whether thrombin-induced IL-6 production is mediated by Ca^{2+} signaling or whether it involves other signaling mechanisms. We investigated the signaling pathways underlying thrombin-induced IL-6 production by HGFs and show here that the IL-6 production is largely due to PAR-1 activation and that p38 mitogen-activated protein (MAP) kinase- and protein tyrosine kinase-dependent mechanisms, but not Ca^{2+} signaling, may be involved in the IL-6 production.

Materials and Methods

Reagents. Human α -thrombin was purchased from Haematologic Technologies, Inc. (Essex Junction, VT). The PAR agonist peptides SFLLRN and SLIGKV were from Bachem (Bubendorf, Switzerland). Tyrphostin 23, SB203580, PD98059, staurosporine, K252a, lipopolysaccharide (LPS) from *Escherichia coli*, and soybean trypsin inhibitor were from Sigma-Aldrich (St. Louis, MO). Recombinant hirudin, U0126, SB 202190, SB 202474, and Ro-31-8220 were obtained from Calbiochem (San Diego, CA). Y-27632 was from BIOMOL Research Laboratories (Plymouth Meeting, PA). Genistein, histamine, wortmannin, phenylmethylsulfonyl fluoride, aprotinin, and leupeptin were from Wako Pure Chemicals (Osaka, Japan). Amastatin was from Peptide Institute, Inc. (Osaka, Japan). Fura-2-AM and the acetoxymethyl ester of 1,2-bis(*O*-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid (BAPTA-AM) were from Dojin Laboratories (Kumamoto, Japan).

Cell Culture. HGFs were prepared from explants of healthy gingival tissues obtained from patients during teeth extraction. Informed consent was obtained from the patients before tooth extraction. The explants were cultured in α -minimum essential medium (α -MEM) (Sigma-Aldrich) supplemented with 10% fetal calf serum (Invitrogen, Carlsbad, CA), 2 mM glutamine, 100 U/ml penicillin, and 100 μg of streptomycin (Invitrogen) at 37°C in an atmosphere of 5% CO_2 . After confluent monolayers of migrating cells formed, the cells were detached by 0.25% trypsin/1 mM EDTA and then grown to

confluence. After the third passage, the cells displayed the typical properties of spindle-shaped fibroblasts. Thus, for this study, HGFs were used between passages 5 and 8.

Measurement of IL-6 Released by HGFs. HGFs were cultured in 24-well tissue culture plates in α -MEM supplemented with fetal calf serum, glutamine, penicillin, and streptomycin. When the monolayers were confluent, the HGFs were incubated in serum-free α -MEM for 24 h. The medium was then removed and replaced with serum-free α -MEM containing various test agents. The culture supernatant fluids were collected at the indicated times and stored at -70°C until the IL-6 levels could be determined with an enzyme-linked immunosorbent assay (ELISA) kit (BioSource International, Inc., CA).

Determination of IL-6 mRNA Levels by Reverse Transcriptase (RT)-Polymerase Chain Reaction (PCR). Confluent HGFs were incubated in serum-free α -MEM for 24 h and then exposed to various stimuli. The total cellular RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's suggested protocol. First strand cDNA was synthesized from 1 μg of RNA using the oligo(dT)16 primer (Roche Diagnostics, Mannheim, Germany) and ReverTra Ace (Toyobo Engineering, Osaka, Japan). The primer set used was 5'-GCAGATGAGTACAAAAGTCCTG-3' (forward) and 5'-CCTTTCACCGATACGTCAAACTT-3' (reverse), corresponding to nucleotides 501 to 522 and 958 to 979 of the human IL-6 cDNA sequence. The reaction mixture was subjected to 30 cycles of denaturation for 1 min at 94°C, primer annealing for 1 min at 60°C, and chain elongation for 1 min at 72°C using a Gene Amp PCR system 2400 (PerkinElmer Life and Analytical Sciences, Boston, MA). Glyceraldehyde-3-phosphate dehydrogenase mRNA, used as an internal control to evaluate total RNA input, was subjected to RT-PCR as previously described (Tanaka et al., 2003). The amplified DNA was then electrophoresed on a 2% agarose LO3 gel (Takara, Kyoto, Japan) containing ethidium bromide and analyzed on a UV transilluminator using an ATTO Cool Sever (ATTO, Tokyo, Japan).

Measurements of $[\text{Ca}^{2+}]_i$. HGFs were grown in sample chambers consisting of 13- × 13-mm plastic cylinders glued to glass coverslips. When the cells had grown to semiconfluence, the culture medium was changed to Hanks' balanced salt solution buffered with 20 mM HEPES-NaOH (pH 7.4), and the cells were incubated with 2 μM fura-2-AM for 45 min at room temperature. The fura-2 fluorescence images were acquired as described previously (Tanaka et al., 2003) using an ARGUS HiSCA imaging system (Hamamatsu Photonics, Shizuoka, Japan) attached to a Diaphot inverted fluorescence microscope (Nikon, Tokyo, Japan). The changes in $[\text{Ca}^{2+}]_i$ are shown by the 340-/380-nm fluorescence ratio.

Western Blotting. HGFs on a culture dish (10 × 10 cm) were scraped into 200 μl of ice-cold extraction buffer (50 mM HEPES-NaOH, pH 7.4, 1% Triton-X, 2 mM sodium orthovanadate, 100 mM NaF, 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 100 $\mu\text{g}/\text{ml}$ aprotinin, and 10 $\mu\text{g}/\text{ml}$ leupeptin) and transferred to a microcentrifuge tube. The samples were sonicated briefly and then centrifuged to collect the supernatants. After being treated with sample buffer containing SDS, the samples (15 $\mu\text{g}/\text{lane}$) were subjected to electrophoresis on 3 to 8% NuPAGE Tris-acetate gels (Novel Experimental Technology, San Diego, CA) and then transferred to nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). The nitrocellulose membranes were blocked for 1 h in Tris-buffered saline containing 5% skim milk powder and then incubated for 3 h at room temperature, or overnight at 4°C, with anti-phospho-p38 MAP kinase antibody or anti-total p38 MAP kinase antibody (1:1000 dilution) (Cell Signaling Technology Inc., Beverly, MA) in Tris-buffered saline. Blots were then incubated for 1 h with peroxidase-conjugated goat anti-rabbit IgG (1:10,000 dilution) in 10% Block Ace. Immunoreactive bands were visualized using the Super Signal West Dura substrate (Pierce Chemical, Rockford, IL) and a Bio/Chem-Luminescence imaging system (Light Capture; ATTO).

Statistical Analysis. Results are expressed as mean \pm S.E.M. Statistical differences were determined by one-way analysis of variance followed by Fisher's protected least significant difference.

Results

Thrombin Induces HGFs to Produce IL-6. HGFs were stimulated with various concentrations of thrombin in serum-free medium, and the IL-6 levels in the culture supernatants were determined 48 h later by ELISA. The treatment with thrombin induced the HGFs to secrete IL-6 in a concentration-dependent manner (Fig. 1). Histamine and LPS, which are important factors that induce the proinflammatory and pathogenic activity of HGFs, also stimulated IL-6 production but at much lower levels compared with the effect of 10 nM thrombin. Figure 2A shows the time-dependent production of IL-6 that is evoked by 10 nM thrombin. Increased IL-6 production was detected 8 h after thrombin was applied, and these levels increased continuously in a linear fashion for at least 48 h. Furthermore, when we examined the thrombin-induced expression of IL-6 mRNA by RT-PCR analysis, we observed enhanced expression of IL-6 mRNA 1 h after stimulation with 10 nM thrombin and that this increased expression did not change until 24 h after the stimulation (Fig. 2B).

Hirudin, a thrombin antagonist, prevents thrombin from binding and cleaving the thrombin receptor (Vu et al., 1991). To show an essential contribution by the thrombin catalytic region, HGFs were stimulated for 24 h with 10 nM thrombin in the presence or absence of 10 U/ml recombinant hirudin. Coincubation with hirudin almost completely inhibited the thrombin-induced IL-6 production (data not shown). The IL-6 levels were 109 ± 14 pg/ml (mean \pm S.E.M., $n = 3$) in the presence of hirudin and 8892 ± 347 pg/ml in the absence of hirudin. This result suggests that the thrombin-induced IL-6 production requires the receptor activation by proteolytic cleavage.

The PAR-1 Agonist Peptide SFLLRN Also Induces IL-6 Production. Synthetic peptides corresponding to the tethered ligand sequence of PAR-1 function as PAR-1 agonists (Hollenberg and Compton, 2002). To demonstrate that the thrombin-induced IL-6 production is mediated by activating PAR-1, HGFs were incubated for 24 h with the PAR-1

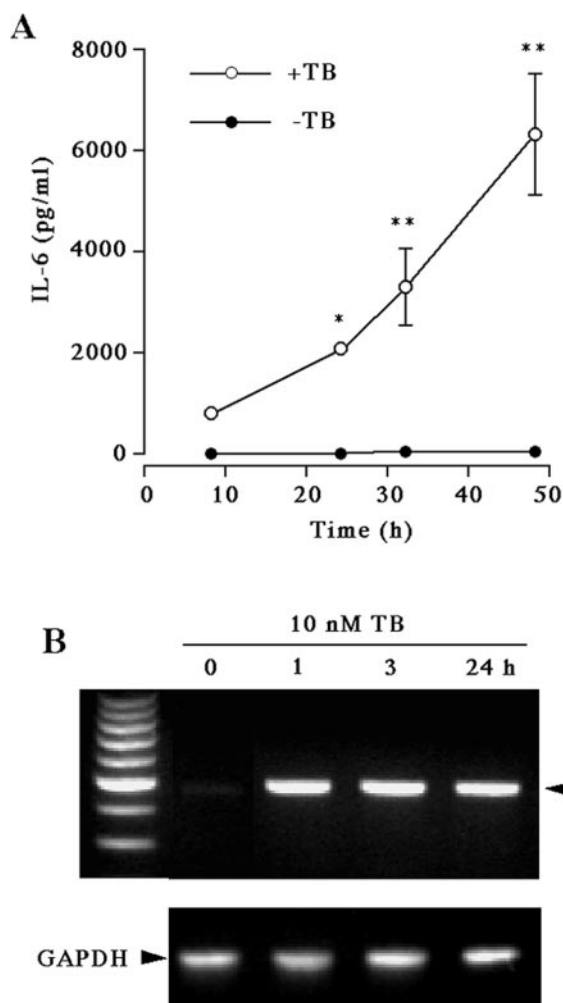
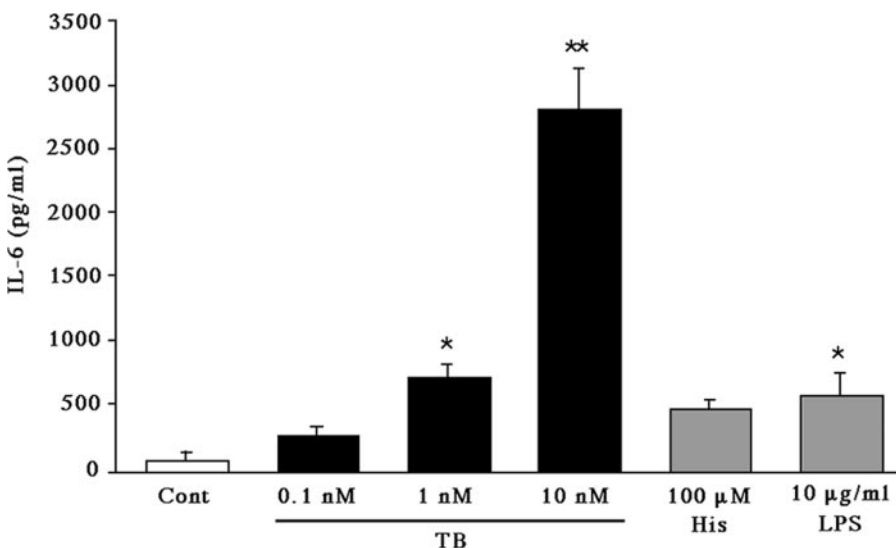


Fig. 2. Time course of TB-induced IL-6 production and IL-6 mRNA expression. A, IL-6 production. HGFs were stimulated with 10 nM TB, and the supernatants were harvested at 8, 24, 32, and 48 h. The IL-6 levels were determined by ELISA. Data represent mean \pm S.E.M. of triplicate wells. *, $P < 0.05$; **, $P < 0.001$, significantly different from the control value in the absence of TB. B, IL-6 mRNA expression. HGFs were stimulated with 10 nM TB for the time periods indicated, and the total RNA was subjected to RT-PCR. The bottom panel shows the levels of glyceraldehyde-3-phosphate dehydrogenase mRNA.

Fig. 1. IL-6 production induced by thrombin (TB), histamine (His), and LPS. HGFs were stimulated for 48 h with TB, His, or LPS, and the levels of IL-6 in the supernatants were measured by ELISA. Data represent mean \pm S.E.M. of triplicate wells. *, $P < 0.05$; **, $P < 0.001$, significantly different from the control value without stimuli (Cont).

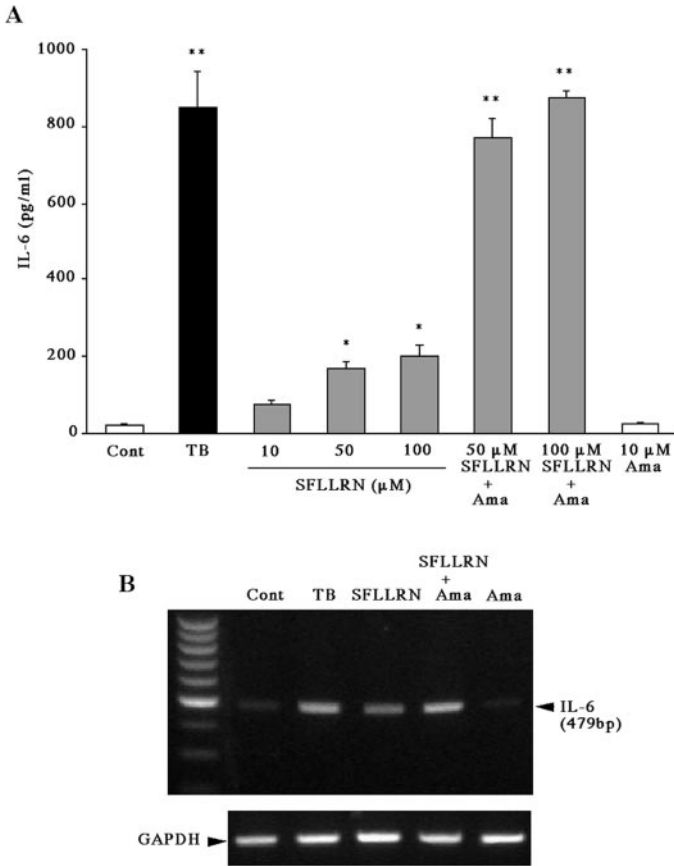


Fig. 3. IL-6 production and IL-6 mRNA expression induced by the PAR-1 agonist peptide SFLLRN in the presence and absence of amastatine. **A**, IL-6 production. HGFs were stimulated for 24 h with 10 nM TB or various concentrations of SFLLRN in the presence or absence of 10 μM amastatine (Ama). The IL-6 levels in the supernatants were determined by ELISA. Data represent mean ± S.E.M. of triplicate wells. *, $P < 0.05$; **, $P < 0.001$, significantly different from the control value in the absence of agonists (Cont). **B**, IL-6 mRNA expression. HGFs were stimulated for 6 h with 10 nM TB, 50 μM SFLLRN, or a combination of 50 μM SFLLRN and 10 μM amastatine, and their total RNAs were subjected to RT-PCR.

agonist peptide SFLLRN (10, 50, and 100 μM) (Fig. 3A). Incubation with SFLLRN stimulated the production of IL-6 in concentration-dependent manner but at much lower levels compared with 10 nM thrombin. To assess the possibility that the poor ability of SFLLRN to induce IL-6 production may be due to its degradation by aminopeptidases on the HGF plasma membranes, HGFs were incubated with a combination of SFLLRN and amastatin, an aminopeptidase inhibitor. The combination of 50 or 100 μM SFLLRN and 10 μM amastatin induced IL-6 levels that are comparable with those evoked by 10 nM thrombin (Fig. 3A). Amastatin itself had no effect on IL-6 production. Because SFLLRN not only activates PAR-1 but also weakly stimulates PAR-2 (Blackhart et al., 1996), we tested the effect of the PAR-2 agonist peptide SLIGKV. However, incubation with 100 μM SLIGKV for 24 h did not stimulate IL-6 production (data not shown).

We also examined the IL-6 mRNA levels in HGFs 6 h after incubating them with SFLLRN in the presence or absence of amastatin (Fig. 3B). Stimulation with 50 μM SFLLRN in the absence of amastatin induced IL-6 mRNA expression but at lower levels compared with 10 nM thrombin. However, a combination of 50 μM SFLLRN and 10 μM amastatin markedly enhanced IL-6 mRNA levels, although amastatin itself had no effect on the expression. Thus, the effect of amastatin on the SFLLRN-induced IL-6 mRNA expression was well compatible with its effect on the IL-6 production.

Thrombin Induces IL-6 Production Even after Desensitization of Ca^{2+} Signaling. It is known that PAR-1-mediated Ca^{2+} signaling is rapidly desensitized after stimulation (Hammes and Coughlin, 1999; Ubl et al., 2000; Tanaka et al., 2003). To determine whether the desensitization of Ca^{2+} signaling reduces the thrombin-induced IL-6 production, HGFs were stimulated for 2 h with 10 nM thrombin, then washed once with a thrombin-free medium and exposed to a second stimulation of thrombin for an additional 22 h (Fig. 4). The second challenge with 10 nM thrombin failed to increase the $[Ca^{2+}]_i$ (data not shown). Nevertheless, the IL-6 levels at the end of the second 22-h culture period were almost identical to those produced after continuous stimula-

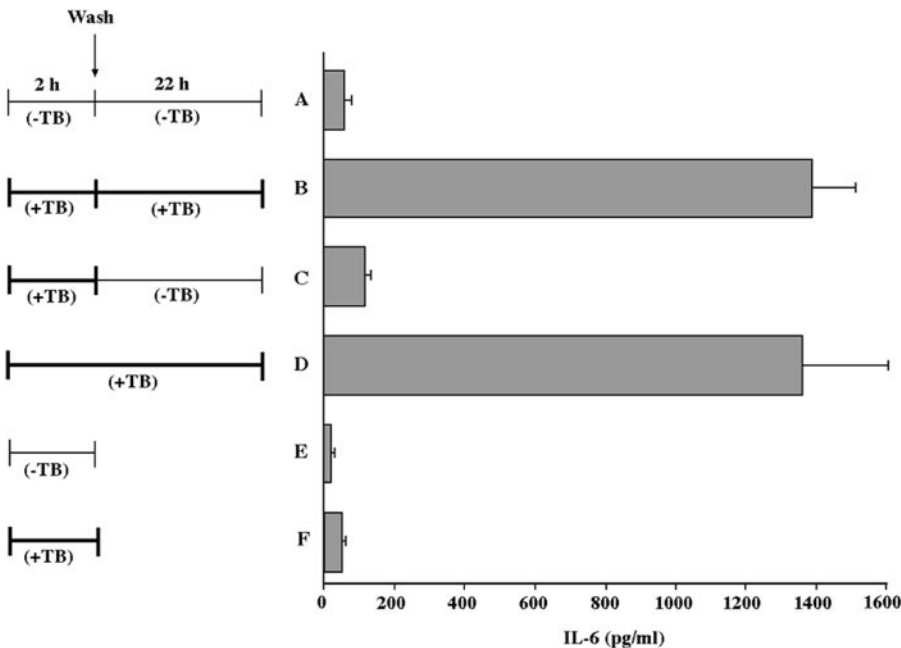


Fig. 4. IL-6 production in HGFs pretreated with TB. **A** to **C**, HGFs were exposed to medium containing 10 nM thrombin (+TB) or without TB (-TB) for 2 h, washed once with TB-free medium, and then re-exposed to TB-containing (+TB) or -free (-TB) medium for a further 22 h. **D**, HGFs were continuously incubated for 24 h with TB. **E** and **F**, HGFs were incubated for 2 h in the presence or absence of TB. Data represent mean ± S.E.M. of quadruplicate wells.

tion for 24 h (Fig. 4, B and D). To examine the possibility that the transient Ca^{2+} signaling induced by the first stimulation may be sufficient to trigger IL-6 production, the culture medium was switched to a thrombin-free medium after the first 2 h of thrombin stimulation. In the absence of thrombin during the last 22 h, little or no IL-6 was produced (Fig. 4C). Also, the stimulation for the first 2 h with thrombin induced little IL-6 production (Fig. 4, E and F). Thus, continuous exposure to thrombin is necessary to maintain the PAR-1-mediated IL-6 production by HGFs.

Treatment with BAPTA-AM Has No Effect on Thrombin-Induced IL-6 Production. To provide further evidence that Ca^{2+} signaling is not essential to the production of IL-6, we investigated the effects of the intracellular Ca^{2+} chelator BAPTA-AM on thrombin-induced IL-6 production. HGFs were loaded with BAPTA by being incubated for 60 min in the presence of 10 μM BAPTA-AM and then stimulated with 10 nM thrombin. Thrombin transiently increased the $[\text{Ca}^{2+}]_i$ in control cells preincubated with 0.05% DMSO (vehicle) (Fig. 5A), and this $[\text{Ca}^{2+}]_i$ response was almost completely inhibited by the BAPTA-AM treatment (Fig. 5B). However,

when the BAPTA-loaded HGFs were stimulated for 24 h with 10 nM thrombin, they produced IL-6 at equivalent levels as the control cells that had been preincubated without BAPTA-AM (Fig. 5C).

Effect of Protein Kinase Inhibitors on Thrombin-Induced IL-6 Production. Since many studies have demonstrated that p38 MAP kinase is involved in regulating inflammatory cytokine biosynthesis in a variety of cell types (Lee et al., 1994; Beyaert et al., 1996; Rawadi et al., 1998; Rincon et al., 1998; Baldassare et al., 1999), we investigated whether treatment with 10 nM thrombin stimulates the phosphorylation of p38 MAP kinase in HGFs. Western blot analysis showed that p38 MAP kinase was rapidly phosphorylated 5 min after stimulation (Fig. 6), suggesting that thrombin activates the p38 MAP kinase pathway in HGFs. Furthermore, the effect of p38 MAP kinase inhibitors on thrombin-induced IL-6 production was tested. As shown in Fig. 7, A and B, addition of SB203580, which is extensively used to inhibit p38 MAP kinase activity, strongly inhibited the IL-6 production by HGFs. A more potent inhibitor of p38 MAP kinase, SB 202190 (10 μM), also suppressed the throm-

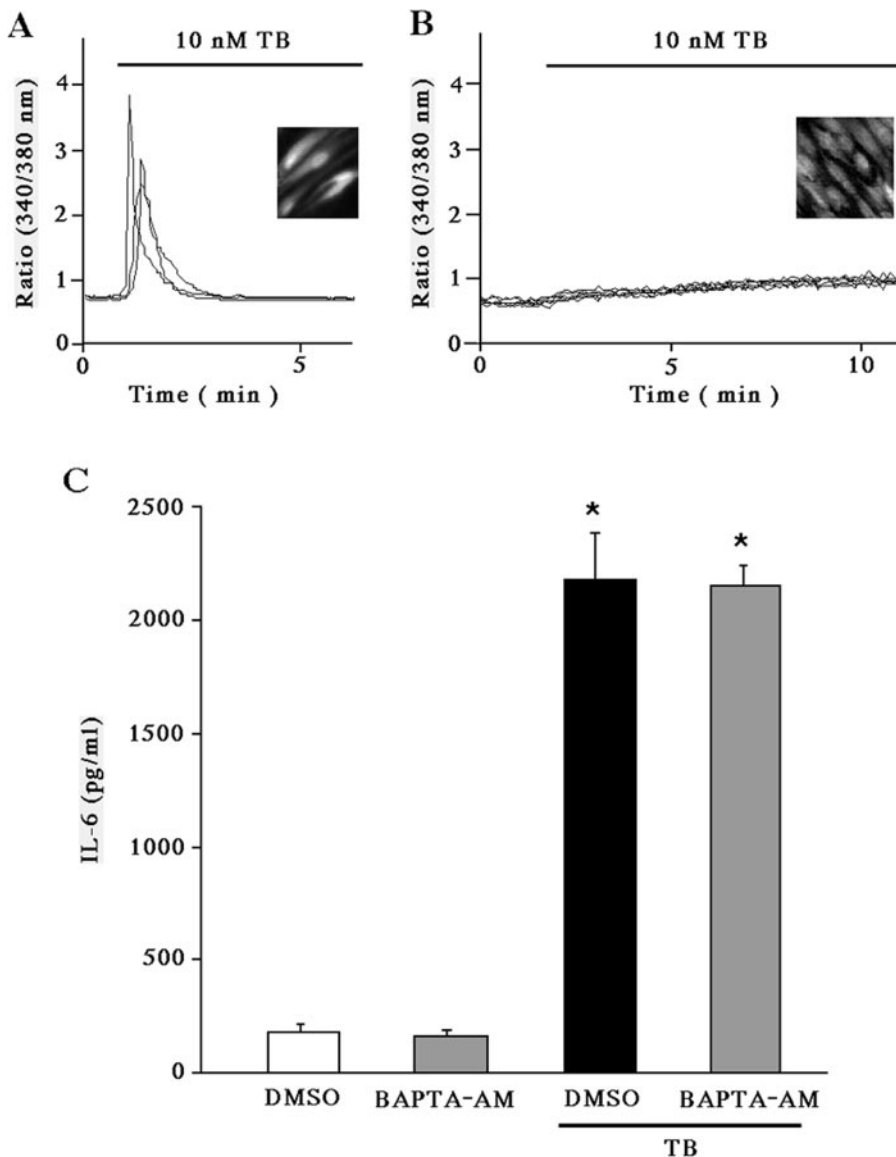


Fig. 5. Effect of BAPTA-AM treatment on the transient elevation in $[\text{Ca}^{2+}]_i$ (A and B) and the IL-6 production (C) induced by TB. A and B, $[\text{Ca}^{2+}]_i$ levels. Fura-2-loaded HGFs were preincubated for 60 min with 0.05% DMSO (vehicle) (A) or 10 μM BAPTA-AM (B). After washing with BAPTA-AM-free medium, the cells were exposed to 10 nM TB for the period indicated by the horizontal bar at the top of each panel. The fluorescence ratio (340/380 nm) was recorded from each cell indicated in the fluorescence image (inset). C, IL-6 production. HGFs were preincubated for 60 min with 0.05% DMSO (vehicle) or 10 μM BAPTA-AM. BAPTA-AM was removed, and the cells were exposed to fresh medium containing 10 nM TB for 24 h. Data represent mean \pm S.E.M. of quadruplicate wells. *, $P < 0.001$, significantly different from the value obtained in the absence of thrombin.

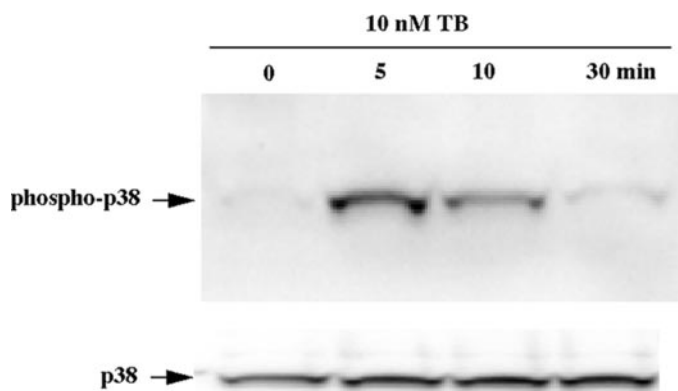


Fig. 6. TB-induced phosphorylation of p38 MAP kinase in HGFs. HGFs were exposed to 10 nM TB for the time period indicated. Phosphorylated p38 MAP kinase was detected by Western blotting with an anti-phospho-p38 MAP kinase antibody (top panel). The bottom panel shows the total p38 MAP kinase levels that were detected by Western blotting with an anti-p38 MAP kinase antibody.

bin-induced IL-6 production (Table 1), whereas addition of 10 μ M SB 202474, an inert analog of SB203580, had little or no effect on the thrombin-induced IL-6 production.

Many protein kinases, i.e., tyrosine kinases, extracellular signal-regulated kinase ERK1/2 (p44/42 MAP kinases), phosphatidylinositol (PI)-3 kinase, and Rho kinase, have been suggested to be involved in PAR-1-mediated biological events (Coughlin, 2000). Thus, we also tested the effects of various other kinase inhibitors on thrombin-induced IL-6 production. The tyrosine kinase inhibitors genistein (50 μ M) and tyrphostin 23 (50 μ M) had an inhibitory effect that was comparable with that of SB203580 (Fig. 7A). PD98059 (20 and 40 μ M), which specifically inhibits p44/42 MAP kinase kinase, also significantly inhibited the IL-6 production, but its effect was much smaller than that of SB203580 (Fig. 7B). Another MAP kinase kinase inhibitor, U0126 (10 μ M), did not inhibit the thrombin-induced IL-6 production (Table 1). Wortmannin (10 and 100 nM), a PI-3 kinase inhibitor, and Y-27632 (10 μ M), a RhoA kinase inhibitor, only partially inhibited the IL-6 production (Fig. 7, C and D). The protein kinase C (PKC) inhibitor staurosporine considerably inhibited the thrombin-induced IL-6 production (Fig. 7C), whereas Ro-31-8220 (5 μ M) and K252a (1 μ M), more specific PKC inhibitors, had only a weak or no effect on the thrombin-induced IL-6 production (Table 1).

It was assessed whether the p38 MAP kinase and tyrosine kinase inhibitors inhibit SFLLRN-induced IL-6 production. HGFs were stimulated with a combination of 50 μ M SFLLRN and 10 μ M amastatin in the presence and absence of the inhibitors. The IL-6 production was significantly suppressed by coinubation with either SB203580 or SB 202190 (Fig. 8). Genistein and tyrphostin 23 also strongly inhibited the IL-6 production.

Discussion

The main purpose of the present study was to assess the signaling mechanisms that underlie the thrombin-induced IL-6 production of HGFs. We first demonstrated that the synthetic PAR-1 agonist peptide, SFLLRN, induces HGFs to secrete significant levels of IL-6. Although SFLLRN alone had a much smaller effect on IL-6 production than thrombin, this

does not mean that other thrombin receptors apart from PAR-1 are involved because the SFLLRN-induced IL-6 production was markedly enhanced by the presence of the aminopeptidase inhibitor amastatin. This enhancement by amastatin strongly suggests that the poor ability of SFLLRN to produce IL-6 on its own is the result of rapid degradation of the PAR-1 agonist peptide by HGF-derived aminopeptidase activity. SFLLRN can also weakly stimulate PAR-2 (Blackhart et al., 1996), but previous studies have demonstrated that HGFs do not express PAR-2 and PAR-4 (Hou et al., 1998; Tanaka et al., 2003). Moreover, we found that the specific PAR-2 agonist peptide SLIGKV failed to stimulate HGFs to produce IL-6. Thus, PAR-2 does not contribute to the SFLLRN-induced IL-6 production in HGFs. PAR-3 mRNA is slightly detectable in HGFs (Chang et al., 2001; Tanaka et al., 2003). However, the thrombin-induced IL-6 production was completely mimicked by a combination of the PAR-1 agonist peptide and amastatin, suggesting that it is unlikely that PAR-3 plays an important role in the IL-6 production. Supporting this are recent studies that have shown that murine PAR-3 does not generate an intracellular signal but rather acts as a cofactor for PAR-4 activation (Nakanishi-Matsui et al., 2000). Thus, we conclude that the IL-6 produced by HGFs in response to thrombin is exclusively mediated by PAR-1 activation.

It has been described that thrombin stimulates production of IL-6 by a variety of cell types (Sower et al., 1995; Hou et al., 1998; Johnson et al., 1998; Shin et al., 1999; Gordon et al., 2000; Chi et al., 2001; Loubakos et al., 2001; Asokanathan et al., 2002), but the intracellular mechanisms regulating this are not well understood. Since PAR-1 is coupled to $G_{q/11}$, the activation of which results in Ca^{2+} mobilization via phosphoinositide hydrolysis, the Ca^{2+} signaling pathway has been suggested to play a crucial role in PAR-1-induced IL-6 production (Chi et al., 2001; Loubakos et al., 2001; Asokanathan et al., 2002). However, several lines of evidence presented here raise doubts about the involvement of Ca^{2+} signaling in the PAR-1-mediated IL-6 production by HGFs. First, although treatment with the intracellular Ca^{2+} chelating agent BAPTA-AM almost completely blocked the increase in $[Ca^{2+}]_i$ following exposure to thrombin, it did not reduce the IL-6 production induced by thrombin. Second, stimulation with thrombin resulted in the continuous production of IL-6 by HGFs, which contrasts with the finding that exposure to thrombin rapidly desensitizes Ca^{2+} signaling in the cells (Tanaka et al., 2003). Moreover, we found that even when Ca^{2+} signaling had been desensitized by thrombin treatment, a second stimulation with thrombin could still induce marked IL-6 production. In addition, although histamine is known to continuously increase $[Ca^{2+}]_i$ in HGFs without any desensitization (Niisato et al., 1996; Tanaka et al., 2003), we found that it generated much lower levels of IL-6 than thrombin. These observations suggest that PAR-1-mediated IL-6 production does not necessarily require Ca^{2+} mobilization and that signal transduction pathways that do not involve phosphoinositide hydrolysis through $G_{q/11}$ may regulate IL-6 production by HGFs.

It is unlikely that a transient activation of PAR-1 functions as a trigger to initiate IL-6 production because exposure to thrombin for a short period (2 h) followed by the removal of thrombin was not associated with the accelerated production of IL-6 that occurred when the cells continued to be exposed

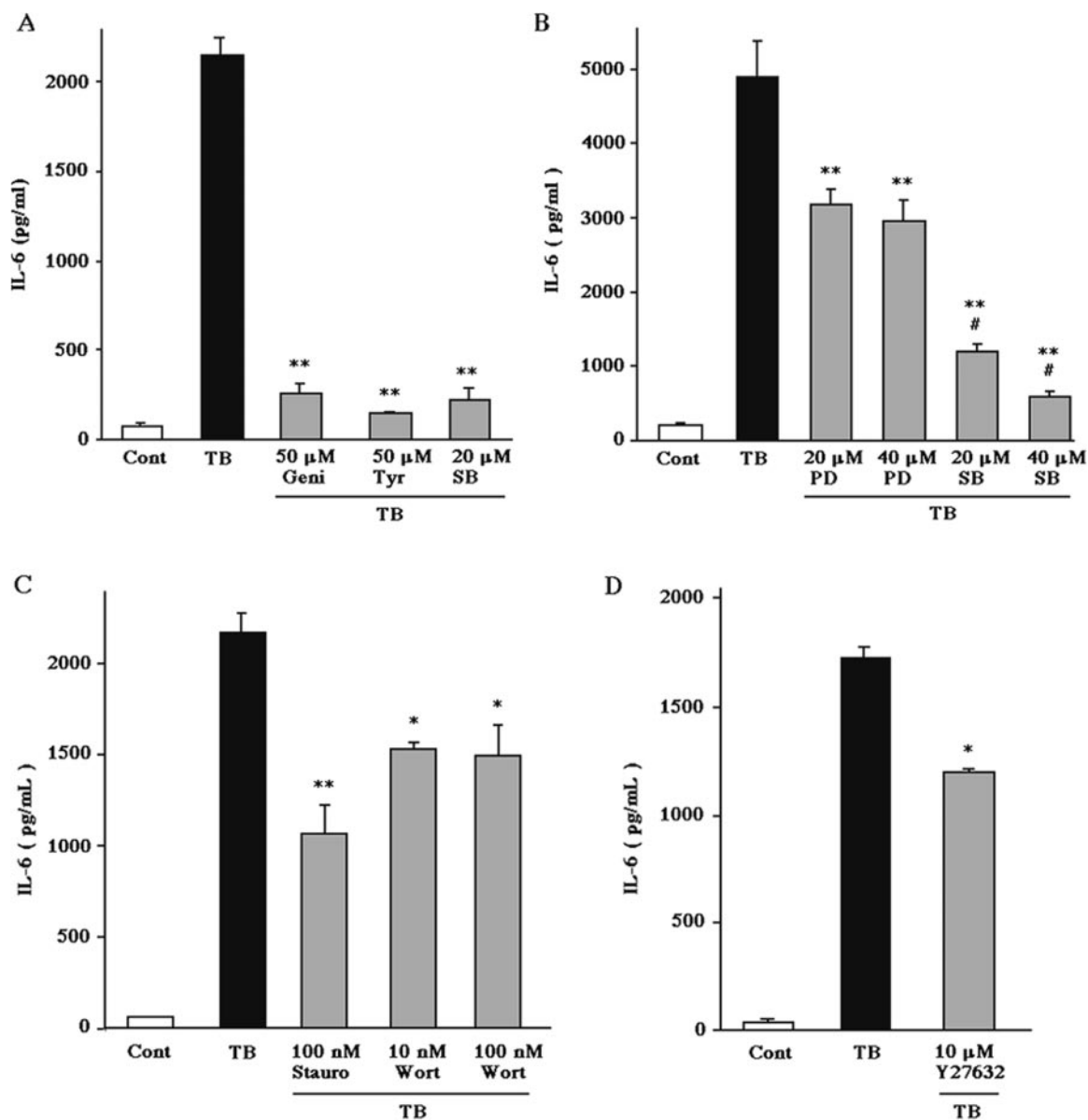


Fig. 7. Effect of protein kinase inhibitors on TB-induced IL-6 production. HGFs were stimulated for 24 h with 10 nM TB or a combination of TB and one of the following kinase inhibitors: SB203580 (SB), genistein (Geni), or tyrphostin 23 (Tyr) (A); PD98059 (PD) or SB (B); staurosporine (Stauro) or wortmannin (Wort) (C); or Y-27632 (D). Data represent mean \pm S.E.M. of triplicate or quadruplicate wells. *, $P < 0.05$; **, $P < 0.001$, significantly different from the value induced by TB in the absence of inhibitors. #, $P < 0.001$, significantly different from the value induced by a combination of TB and PD.

to thrombin past the initial 2 h. This indicates that a continuous production of IL-6 requires the sustained activation of PAR-1 and that intracellular signals for IL-6 production are generated even after the termination of Ca^{2+} signaling. In some cell types, PAR-1 appears to have an intracellular pool from which cell surface receptors can be efficiently replenished (Hollenberg and Compton, 2002). Activation of surface receptors may initiate a continuous transport of intracellular receptors to the cell surface, thereby maintaining responsiveness to thrombin.

Recent studies have demonstrated that PAR-1 couples not only with the $G_{q/11}$ family but also with the G_i and $G_{12/13}$ families (Coughlin, 2000; Hollenberg and Compton, 2002) and that PAR-1 stimulation appears to activate a variety of protein kinase pathways that lie downstream of G_i and $G_{12/13}$ activation (Coughlin, 2000; Macfarlane et al., 2001). At

present, however, there is little or no information about the protein kinase pathways involved in PAR-1-mediated IL-6 production. We have shown here that stimulation with thrombin induces the phosphorylation of p38 MAP kinase and that the specific p38 inhibitors, SB203580 and SB 202190, significantly inhibits the thrombin- and SFLLRN-induced IL-6 productions. This observation is compatible with many studies that suggest the p38 MAP kinase pathway is involved in the biosynthesis of pro-inflammatory cytokines (Lee et al., 1994; Beyaert et al., 1996; Rawadi et al., 1998; Rincon et al., 1998; Baldassare et al., 1999). Ridley et al. (1997) have reported that SB203580 significantly inhibits the IL-1-stimulated IL-6 production by human fibroblasts and human endothelial cells. Thus, it is likely that p38 MAP kinase may be a common signal molecule that mediates the IL-6 production induced in response to various stimuli.

TABLE 1

Effect of analogs of SB 203580, U0126, and PKC inhibitors on thrombin-induced IL-6 production

HGFs were stimulated for 24 h with 10 nM TB in the presence or absence of the inhibitors. Data represent mean \pm S.E.M. of triplicate or quadruplicate wells.

Culture Conditions	IL-6 <i>pg/ml</i>
Experiment 1	
Control	49 \pm 5
TB	2479 \pm 150
TB + 10 μ M SB 202190	1927 \pm 72*
TB + 10 μ M SB 202474	2422 \pm 185
Experiment 2	
Control	45 \pm 1
TB	8469 \pm 410
TB + 10 μ M U0126	8710 \pm 735
Experiment 3	
Control	35 \pm 4
TB	2357 \pm 197
TB + 5 μ M RO-31-8220	1782 \pm 38*
TB + 1 μ M K252a	2291 \pm 35

* $P < 0.05$, significantly different from the value induced by TB in the absence of inhibitors.

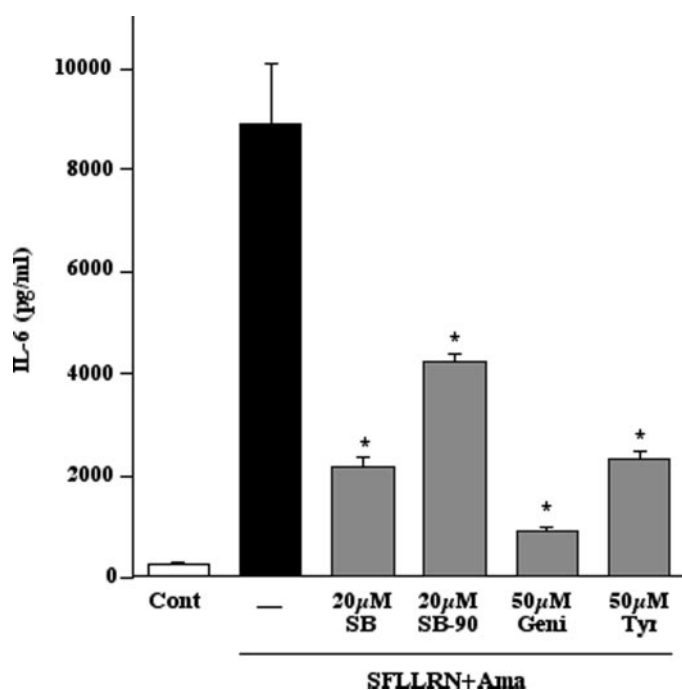


Fig. 8. Effect of p38 MAP kinase and tyrosine kinase inhibitors on SFLLRN-induced IL-6 production. HGFs were stimulated for 24 h with a combination of 50 μ M SFLLRN and 10 μ M amastatine (Ama) in the presence or absence of one of the following kinase inhibitors: SB203580 (SB), SB 202190 (SB-90), genistein (Geni), or tyrphostin 23 (Tyr). Data represent \pm S.E.M. of quadruplicate wells. *, $P < 0.001$, significantly different from the value induced by a combination of SFLLRN and Ama in the absence of inhibitors.

Nonreceptor tyrosine kinases such as Src family have been found to be involved in PAR-1-mediated signaling pathways in a variety of cell types (Chen et al., 1994; Li et al., 1995; Ellis et al., 1999). Since we found that the PAR-1-mediated IL-6 production of HGFs is also very sensitive to the tyrosine kinase inhibitors genistein and tyrphostin 23, it is possible that protein tyrosine phosphorylation participates in the IL-6 production regulatory pathway upstream of p38 MAP kinase. Genistein and tyrphostin 23 are nonselective tyrosine kinase inhibitors that can inhibit various types of tyrosine kinases, and identification of the specific tyrosine kinases that are

involved in the PAR-1-mediated IL-6 production of HGFs remains to be determined in further studies. We also found that staurosporine strongly inhibited thrombin-induced IL-6 production by HGFs. As staurosporine is known to inhibit a variety of kinases, including tyrosine kinases, at concentrations that are close to those required to inhibit PKC (Fallon, 1990), its inhibitory effect on IL-6 production may arise from the inhibition of protein kinases other than PKC. Supporting this is that Ro-31-8220 and K252a, more specific PKC inhibitors, did not cause a marked inhibition of the IL-6 production. However, we cannot completely rule out a PKC involvement, which is upstream of the p38 pathway, because Ro-31-8220 had an inhibitory effect that was comparable with that of SB 202190. Therefore, further studies are necessary to determine whether PKC plays a role in thrombin-induced IL-6 production.

The inhibitors of p44/42 MAP kinase (ERK1/2) kinase, PI-3 kinase, and Rho kinase also significantly inhibited the thrombin-induced IL-6 production of HGFs. This suggests that some types of kinase molecules are partially involved in IL-6 production and that the PAR-1-mediated IL-6 production may result from the cooperative activation of multiple transducing pathways. In mouse mast cells, the PI-3 kinase pathway is intimately involved in thrombin-induced IL-6 secretion (Gordon et al., 2000). Moreover, the ERK1/2 and PI-3 kinase have been shown to be key signaling molecules for the PAR-1-induced proliferation of fibroblasts and astrocytes (Pages et al., 1993; Trejo et al., 1996; Wang et al., 2002). In addition, Rho-dependent cytoskeletal responses are involved in PAR-1 activation-induced platelet shape changes and endothelial cell hyperpermeability (van Nieuw Amerongen et al., 2000; Dorsam et al., 2002). Thus, PAR-1 appears to activate a network of multiple signaling pathways in even a single cell type, thereby generating a variety of biological events.

In summary, we have demonstrated that the thrombin-induced IL-6 production by HGFs is mediated by PAR-1 activation. The Ca^{2+} signaling does not appear to play an essential role in the PAR-1-mediated IL-6 production since PAR-1 activation continues to induce IL-6 production even after Ca^{2+} signaling has ceased. The results presented here suggest that p38 MAP and tyrosine kinases are important signaling molecules in the PAR-1-mediated IL-6 production of HGFs. The precise network of the signaling pathways involved in the PAR-1-mediated IL-6 production remains to be determined in detail.

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