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Involvement of p38 MAPK and ERK/MAPK pathways in staurosporine-induced production of macrophage inflammatory protein-2 in rat peritoneal neutrophils

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Abstract

Stimulation of rat peritoneal neutrophils with staurosporine (64 nM) induced production of macrophage inflammatory protein-2 (MIP-2) and phosphorylation of p38 mitogen-activated protein kinase (MAPK) and extracellular signal-regulated kinase/MAP kinase (ERK/MAPK). The staurosporine-induced MIP-2 production at 4 h was inhibited by the highly specific p38 MAPK inhibitor SB 203580 and the MAPK/ERK kinase (MEK-1) inhibitor PD 98059 in a concentration-dependent manner. By treatment with SB 203580 (1 μM) or PD 98059 (50 μM), the staurosporine-induced increase in the levels of mRNA for MIP-2 was only partially lowered, although the staurosporine-induced MIP-2 production was completely inhibited. Consistent with the inhibition by the protein synthesis inhibitor cycloheximide, SB 203580 and PD 98059 inhibited MIP-2 production at 4 h either when added simultaneously with staurosporine or 2 h after stimulation with staurosporine. In contrast, the DNA-dependent RNA polymerase inhibitor actinomycin D did not inhibit MIP-2 production at 4 h when it was added 2 h after staurosporine stimulation. Dot blot analysis demonstrated that treatment with SB 203580 or PD 98059 down-regulates the stability of MIP-2 mRNA. These results suggested that p38 MAPK and ERK/MAPK pathways are involved in translation of MIP-2 mRNA to protein and stabilization of MIP-2 mRNA.

Keywords: Staurosporine; Chemotaxis; Neutrophil; Macrophage inflammatory protein-2; Extracellular signal-regulated kinase/mitogen-activated protein kinase; p38 mitogen-activated protein kinase

1. Introduction

Chemokines represent an increasing superfamily of structurally and functionally related proteins which play important roles in inflammation and immune responses. They are classified into CC, CXC, CX_3C and C chemokine subfamilies according to whether the first two cysteines are separated or adjacent. In humans, interleukin (IL)-8 is the major CXC chemokine [1]. In rats, cytokine-induced neutrophil chemoattractants (CINCs) [2] including CINC-1, CINC-2α, CINC-2β and macrophage inflammatory protein-2 (MIP-2 also known as CINC-3) [3–8] are important chemoattractants for neutrophils.

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Previously, we found [9] that the production of MIP-2 by leukocytes infiltrating the pouch fluid in the air pouch-type allergic inflammation model in rats is inhibited by the protein kinase C (PKC) inhibitor H-7, and the tyrosine kinase inhibitor genistein, but not by the cyclic adenosine 3′,5′-monophosphate (cyclic AMP)-dependent protein kinase inhibitor H-89. Furthermore, we found [10] that staurosporine, generally used as a non-specific protein kinase inhibitor, stimulates MIP-2 production in rat peritoneal neutrophils, and the staurosporine-induced MIP-2 production is inhibited by the PKC inhibitors H-7 and calphostin C, and the tyrosine kinase inhibitor genistein, suggesting that staurosporine activates some PKC isozymes and tyrosine kinase which lead to MIP-2 production. Recently, it has been reported that p38 mitogen-activated protein kinase (MAPK) and extracellular signal-regulated kinase (ERK)/MAPK cascade pathways are involved in the regulation of IL-6 and IL-8 production [11–13]. The purpose of the present study was to clarify whether MAPK signaling pathways are involved in the staurosporine-induced MIP-2 production in rat peritoneal neutrophils.

2. Materials and methods

2.1. Reagents

The drugs used were staurosporine (Kyowa Medex, Tokyo, Japan), PD 98059 (Daiichi Pure Chemicals, Tokyo, Japan), SB 203580 (Smithkline Beecham Pharmaceuticals, King of Prussia, PA, USA), actinomycin D (Wako Pure Chemicals, Osaka, Japan), and cycloheximide (Sigma, St. Louis, MO, USA). They were dissolved in dimethylsulfoxide, and an aliquot of each solution was added to medium. The final concentration of the vehicle in medium was adjusted to 0.1% (v/v). The control medium contained the same amount of the vehicle.

2.2. Preparation of rat peritoneal neutrophils

Male Sprague-Dawley strain rats, specific pathogen-free (Charles River Japan, Kanagawa, Japan) were used. The rats were treated in accordance with procedures approved by the Animal Ethics Committee of the Faculty of Pharmaceutical Sciences, Tohoku University, Sendai, Japan. Rat peritoneal neutrophils were harvested 16 h after intraperitoneal injection of 40 ml of Ca^{2+}-free Krebs-Ringer solution containing 1% casein (casein from milk, vitamin-free, Wako Pure Chemicals) which had been sterilized by autoclaving at 120°C for 15 min [10]. The peritoneal cells were washed twice with Ca^{2+}-free phosphate-buffered saline (PBS, pH 7.4) and finally suspended in RPMI 1640 medium (Nissui Seiyaku, Tokyo, Japan) containing 0.25% (w/v) bovine serum albumin (BSA, Sigma) at 2×10^7 cells/ml. The purity of neutrophils was more than 97% as assessed by May Grünwald-Giemsa staining.

2.3. Cell culture

Rat peritoneal neutrophils (1×10^7 cells) were incubated in 1 ml of RPMI 1640 medium containing 0.25% BSA (w/v) and the indicated concentrations of drugs at 37°C for 4 h. The cells were then centrifuged at $1500\times g$ and 4°C for 5 min. The supernatant obtained was diluted with RPMI 1640 medium containing 0.25% (w/v) BSA, and used for the measurement of neutrophil chemotactic activity and for the determination of MIP-2. After treatment with drugs, the viability of the neutrophils was examined by the ability of mitochondrial succinate dehydrogenase to cleave 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) to the blue compound formazan [14], and no significant changes in viability of the cells was observed.

2.4. Western blot analysis of p38 MAPK and ERK/MAPK phosphorylation

After stimulation of neutrophils (5×10^6 cells) with staurosporine (64 nM) at 37°C for 1, 3, 5, 10, 15, 30 and 60 min, the cells were lysed in 100 μl of ice-cold lysis buffer (20 mM HEPES, pH 7.3, 1% (v/v) Triton X-100, 1 mM EDTA, 50 mM NaF, 2.5 mM *p*-nitrophenylphosphate, 1 mM Na_3VO_4, 10 μg/ml leupeptin and 10% (v/v) glycerol). Proteins in the cell lysate were resolved by 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany). The membranes were blocked in Block Ace (Dainippon Pharmaceut-

ical, Osaka, Japan) at room temperature for 1 h and then incubated overnight at 4°C with phospho-specific p38 MAPK (Thr180/Tyr182) antibody or phospho-p44/42 MAPK (Thr202/Tyr204) antibody (New England Biolabs, Beverly, MA, USA), which was diluted 1/1000 in 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% (v/v) Tween 20 (TBST) containing 1% BSA. After extensive washes, the membranes were incubated with 1/2000 diluted biotinylated anti-rabbit IgG (Vector Laboratories, Burlingame, CA, USA) for 1 h at room temperature. After three washes in TBST, the membranes were treated with enhanced chemiluminescence reagents (ECL, Amersham Life Science, Buckinghamshire, UK), and the phosphorylated p38 MAPK and ERK/MAPK were detected by autoradiography with Kodak Scientific Imaging Films (Eastman Kodak, Rochester, NY, USA).

To confirm that equal amounts of protein were loaded in each lane, the membranes were incubated in stripping buffer (62.5 mM Tris-HCl, pH 6.7, 2% SDS and 100 mM β-mercaptoethanol) at 50°C for 30 min to remove the complex of the primary antibody and the secondary antibody. The blots were blocked in Block Ace, and reprobed with p38 (C-20) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or anti-rat MAPK R2 (Upstate Biotechnology, Lake Placid, NY, USA), followed with alkaline phosphatase-conjugated anti-rabbit IgG (Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA). Proteins were visualized using a nitroblue tetrazolium/5-bromo-4-chloro-3-indolylphosphate system (Promega, Madison, WI, USA).

2.5. Measurement of neutrophil chemotactic activity

The chemotactic activity of MIP-2 in the supernatant fraction of the conditioned medium was determined using modified Boyden chambers and expressed as migration index according to the procedure described previously [15].

2.6. Determination of MIP-2 in the conditioned medium by ELISA

Concentrations of MIP-2 in the conditioned medium were measured by the enzyme-linked immunosorbent assay (ELISA) system which we have developed. Briefly, we produced GST-fusion rat MIP-2 protein and prepared its polyclonal antibody using rabbits as described previously [8]. The enzyme-labeled antibody was produced by conjugation of peroxidase (Toyobo, Tokyo, Japan) to anti-MIP-2 polyclonal antibody. 3,3′,5,5′-Tetramethylbenzidine (TMBZ) (Dojindo Laboratories, Kumamoto, Japan) was used as a substrate for peroxidase. The detection range for rat MIP-2 is between 6.25 and 400 pg/ml without cross-reaction with CINC-1, CINC-2α and CINC-2β.

2.7. Northern blot and dot blot analysis of MIP-2 mRNA

Rat peritoneal neutrophils (8×10^7 cells) were incubated at 37°C for the periods indicated in 8 ml of RPMI 1640 medium containing 0.25% (w/v) BSA in the presence or absence of drugs. Total RNA was then prepared from neutrophils by acid guanidinium-phenol-chloroform extraction [16]. For Northern blotting, 15 μg of total RNA from each sample was loaded and separated on a 1% agarose gel containing 1.7% formaldehyde and blotted onto a Hybond-N^+ membrane (Amersham Life Science). For dot blotting, 10 μg of total RNA was denatured in 1×SSC (0.15 M NaCl, 15 mM sodium citrate, pH 7.0) containing 50% formamide and 7% formaldehyde at 68°C for 15 min, and blotted onto a Hybond-N^+ membrane using a dot blot apparatus (ATTO, Tokyo, Japan). Probes for MIP-2 cDNA and β-actin cDNA were labeled with [α-^{32}P]dCTP (3000 Ci/mmol) (NEN Life Science Products, Boston, MA, USA) using a Ready-To-Go DNA labeling kit (Pharmacia Biotech, USA), and chromatographed through G-50 Sephadex columns (Boehringer Mannheim, Indianapolis, IN, USA). The blots were prehybridized for 3–6 h and then hybridized for 16–18 h at 42°C in 5×SSPE buffer (0.75 M NaCl, 50 mM $NaH_2PO_4\cdot H_2O$, 5 mM EDTA, pH 7.4) containing 10×Denhardt's solution, 50% formamide, 2% SDS, and 100 μg/ml freshly denatured, sheared salmon sperm DNA (Wako Pure Chemicals). After hybridization, the blots were rinsed three times at room temperature for 15 min in 2×SSC containing 0.05% SDS, then washed three times at 55°C for 40 min each in 0.1×SSC containing 0.1% SDS. The membrane was exposed to Kodak Scientific Imaging

Films (Eastman Kodak) at −80°C. For rehybridization, the hybridized probe was stripped from the blot by incubating the blot in sterile H_2O containing 0.5% SDS at 90–100°C for 10 min.

2.8. Statistical analysis

The results are presented as means ± S.E.M. from at least four samples. Comparisons were performed by Student's unpaired *t*-test.

3. Results

3.1. Stimulation of MIP-2 production by staurosporine

Concentrations of MIP-2 in the conditioned medium of the staurosporine-stimulated neutrophils was determined by ELISA. As shown in Fig. 1A, MIP-2 concentrations in the conditioned medium in the absence of staurosporine were negligible, but were increased time-dependently in the presence of 64 nM of staurosporine. Significant increase in MIP-2 production at 4 h was observed at 6.4–210 nM of staurosporine in a concentration-dependent manner (data not shown). Northern blot analysis demonstrated that the accumulation of MIP-2 mRNA at 4 h in non-stimulated neutrophils was very low. Following incubation with staurosporine (64 nM), the MIP-2 mRNA level increased rapidly reaching a maximum at 3 h and remained unchanged at 4 h (Fig. 1B).

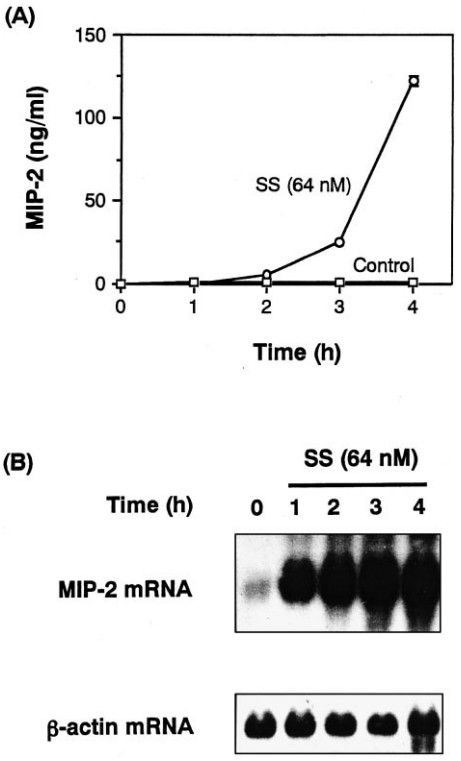

Fig. 1. Time changes in the production of MIP-2 protein and the accumulation of MIP-2 mRNA in staurosporine-stimulated neutrophils. (A) Rat peritoneal neutrophils (1×10^7 cells) were incubated at 37°C for the periods indicated in 1 ml of medium in the presence or absence of staurosporine (SS, 64 nM). Concentrations of MIP-2 in the conditioned medium were determined by ELISA. Values are the means ± S.E.M. from four samples. The results were confirmed by three separate experiments. (B) Rat peritoneal neutrophils (8×10^7 cells) were incubated at 37°C for the periods indicated in 8 ml of medium in the presence of staurosporine (64 nM). Northern blot analysis for each MIP-2 mRNA and β-actin mRNA was performed using 15 μg of total RNA extracted from neutrophils as described in Section 2.4.

3.2. Effects of the p38 MAPK inhibitor SB 203580 and the MEK-1 inhibitor PD 98059 on the staurosporine-induced MIP-2 production

We then determined whether p38 MAPK and ERK/MAPK are involved in the staurosporine-stimulated MIP-2 production. Stimulation of rat peritoneal neutrophils with staurosporine (64 nM) activated p38 MAPK and ERK/MAPK, as revealed by increased phosphorylation (Fig. 2). The phosphorylation of p38 MAPK was observed at 5 min, reached a maximum at 10 min and then declined at 60 min (Fig. 2A). The phosphorylation of ERK/MAPK by staurosporine was detectable at 5 min and increased with time till 60 min (Fig. 2B). When neutrophils were incubated with staurosporine (64 nM) at 37°C for 4 h, the neutrophil chemotactic activity in the conditioned medium increased significantly. The staurosporine-induced increase in neutrophil chemotactic activity in the conditioned medium was lowered by the p38 MAPK inhibitor SB 203580 and the MEK-1 inhibitor PD 98059 in a concentration-dependent manner (Fig. 3A,B). Consistent with the results obtained by chemotaxis determination (Fig. 3A,B), stimulation of neutrophils with staurosporine (64 nM) markedly increased MIP-2 production at 4 h (Fig. 3C,D). The staurosporine-induced MIP-2 production was inhibited by SB 203580 and

PD 98059 in a concentration-dependent manner, and almost complete inhibition was obtained with 1 μM SB 203580 and 50 μM PD 98059 (Fig. 3C,D).

3.3. Involvement of p38 MAPK and ERK/MAPK in the regulation of MIP-2 protein synthesis

We then examined the effects of SB 203580 and PD 98059 on MIP-2 mRNA accumulation at 2 h after staurosporine stimulation. Although staurosporine-induced production of MIP-2 protein at 4 h was almost completely inhibited by 1 μM SB 203580 or 50 μM PD 98059 (Fig. 3C,D), MIP-2 mRNA accumulation was only partially inhibited (Fig. 4A). As positive and negative controls, we examined the effects of actinomycin D and cycloheximide, which inhibit transcription and translation, respectively. By treatment with actinomycin D (8 μM), staurosporine-induced accumulation of MIP-2 mRNA at 2 h was almost completely abolished, but treatment with cycloheximide (3.6 μM) did not affect the staurosporine-induced increase in MIP-2 mRNA levels at 2 h (Fig. 4A). These findings suggested that the inhibition of staurosporine-induced MIP-2 production by the inhibitors of p38 MAPK and ERK/MAPK is expressed at the post-transcriptional level. To further determine whether p38 MAPK and ERK/MAPK are key mediators of the stimulation of MIP-2 protein synthesis by staurosporine, SB 203580 (0.1 μM) or PD 98059 (10 μM) was added simultaneously with staurosporine, or 2 h after staurosporine (64 nM) stimulation, and the levels of MIP-2 production 4 h after staurosporine stimulation were examined. Although levels of MIP-2 mRNA were strongly elevated 2 h after stimulation by staurosporine (64 nM) (Figs. 1B and 4A), only a small amount of MIP-2 protein was synthesized at 2 h (Fig. 1A). Addition of SB 203580 (0.1 μM) or PD 98059 (10 μM) 2 h after stimulation with staurosporine inhibited MIP-2 production at 4 h, and the inhibitory activity was almost the same as that when the inhibitors were added simultaneously with staurosporine (Fig. 4B). Almost the same inhibition of MIP-2 production at 4 h was observed when cycloheximide (0.108 μM) was added 2 h after stimulation or simultaneously with staurosporine (Fig. 4B). In contrast, addition of actinomycin D (0.8 μM) 2 h after stimulation with staurosporine did not inhibit MIP-2 production at 4 h. However, addition of actinomycin D (0.8 μM) together with SB 203580 (0.1 μM) or PD 98059 (10 μM) 2 h after staurosporine stimulation inhibited MIP-2 production at 4 h (Fig. 4B). These findings suggested that p38 MAPK and ERK/MAPK are involved in the regulation of MIP-2 protein synthesis at the post-transcriptional level.

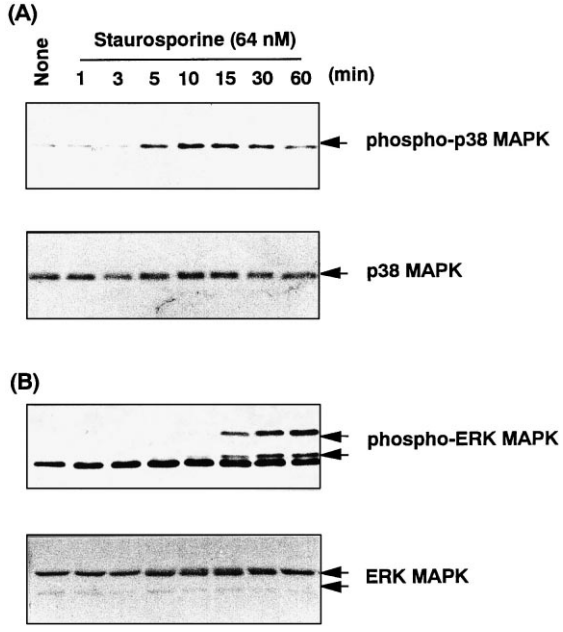

Fig. 2. Activation of p38 MAPK and ERK/MAPK by staurosporine in rat peritoneal neutrophils. (A) Protein phosphorylation of p38 MAPK. Rat peritoneal neutrophils (5×10^6 cells) were stimulated at 37°C for the periods indicated in 0.5 ml of medium in the presence of staurosporine (64 nM). Proteins in the cell lysate were separated on 8% SDS-PAGE and transferred onto a nitrocellulose membrane. Western blot was performed using phospho-p38 MAPK (Thr180/Tyr182) antibody (upper panel), and the sample was reprobed with p38 (C-20) antibody (lower panel). (B) Protein phosphorylation of ERK/MAPK. The same samples as described above were subjected to Western blot analysis using phospho-p44/42 MAPK antibody (upper panel), and reprobed with anti-rat MAPK R2. The results shown are representative of three separate experiments.

3.4. Effects of the p38 MAPK inhibitor SB 203580 and the MEK-1 inhibitor PD 98059 on the stability of MIP-2 mRNA

We next examined whether SB 203580 and PD 98059 affect the stability of MIP-2 mRNA. Because Northern blot analysis demonstrated each specific band for MIP-2 and β-actin (Figs. 1B and 4A), we

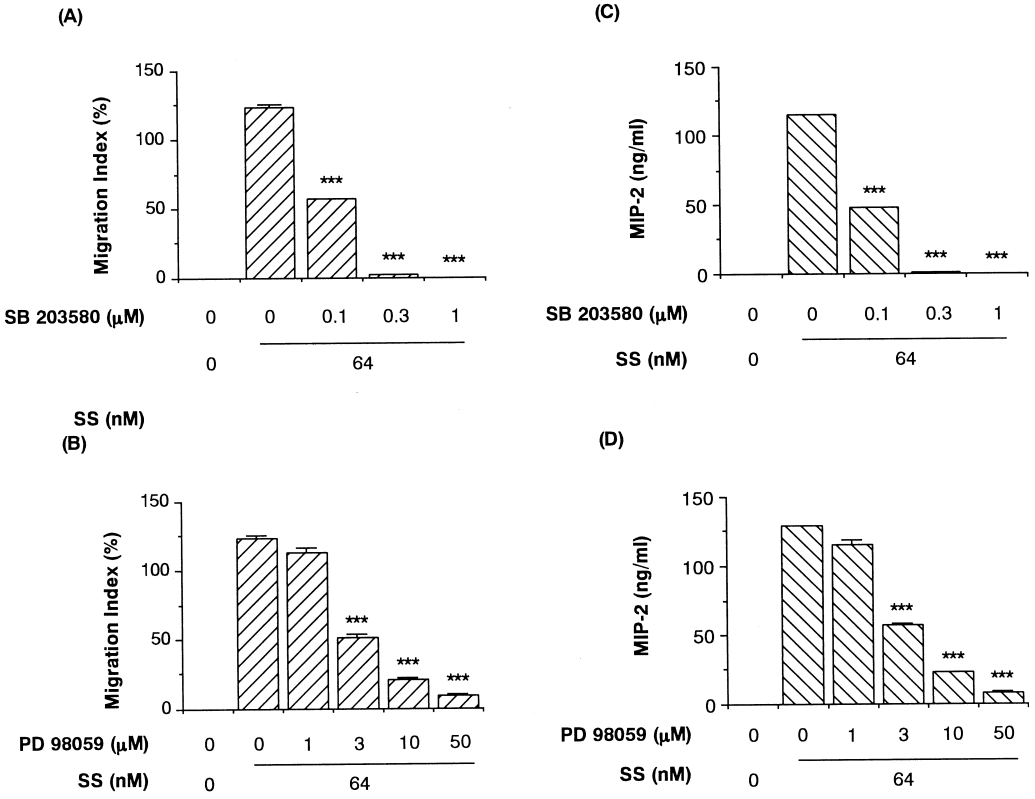

Fig. 3. Effects of SB 203580 and PD 98059 on staurosporine-induced MIP-2 production by neutrophils. Rat peritoneal neutrophils (1×10^7 cells) were incubated at 37°C for 4 h in 1 ml of medium in the presence of staurosporine (SS, 64 nM) and the indicated concentrations of SB 203580 (A,C) or PD 98059 (B,D). Neutrophil chemotactic activity expressed as migration index in the conditioned medium was determined after 10-fold dilution (A,B). Chemotaxis induced by 10 nM platelet-activating factor is expressed as 100%. Concentrations of MIP-2 were determined by ELISA (C,D). Values are the means ± S.E.M. from four samples. Statistical significance: $***P<0.001$ vs. corresponding control. The results were confirmed by three separate experiments.

performed dot blot analysis in subsequent experiments. Neutrophils were incubated for 2 h in medium containing staurosporine (64 nM) in the presence or absence of SB 203580 (1 μM) or PD 98059 (50 μM). Actinomycin D (8 μM) was then added and the cells were further incubated for 1, 2 and 3 h. As shown in Fig. 5A, levels of β-actin mRNA were not affected markedly by SB 203580 (1 μM) or PD 98059 (50 μM). However, levels of MIP-2 mRNA decreased time-dependently in the presence of SB 203580 (1 μM) or PD 98059 (50 μM) (Fig. 5B). These findings suggested that the decrease in the levels of MIP-2 mRNA by SB 203580 (1 μM) and PD 98059 (50 μM) (Fig. 4) is partly due to the decrease in the stability of MIP-2 mRNA. Therefore, it was also suggested that p38 MAPK and ERK/MAPK promote the stabilization of the staurosporine-induced MIP-2 mRNA in neutrophils.

4. Discussion

Staurosporine was originally considered to be a potent non-selective inhibitor of PKC [17]. It is reported that staurosporine promotes neurite outgrowth by specific activation of JNK isoform in PC-12 cells [18]. We have reported that staurosporine induces MIP-2 production in rat peritoneal neutrophils with a maximal effect at 64 nM, and the staurosporine-induced MIP-2 production is inhibited by the inhibitors of PKC and tyrosine kinase [10]. In the present work, we have found that staurosporine causes phosphorylation of p38 MAPK and ERK/MAPK in rat neutrophils (Fig. 2). To evaluate the biologic significance of the activation of p38 MAPK and ERK/MAPK by staurosporine, we examined the effects of SB 203580, a highly specific inhibitor of p38 MAPK, and PD 98059, a specific inhibitor of

MEK-1, on staurosporine-induced MIP-2 production. Incubation of neutrophils with staurosporine (64 nM) in the presence of 1 μM SB 203580 or 50 μM PD 98059 almost completely inhibited MIP-2 production (Fig. 3). Hobbie et al. [11] reported that SB 203580 specifically inhibits p38 MAPK with an

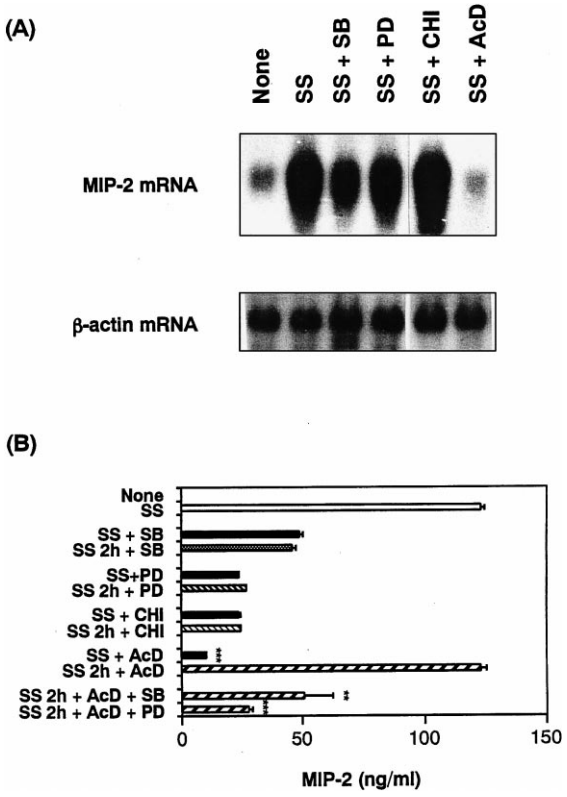

Fig. 4. Effects of SB 203580 and PD 98059 on MIP-2 production in neutrophils stimulated with staurosporine. (A) Rat peritoneal neutrophils (8×10^7 cells) were incubated at 37°C for 2 h in 8 ml of medium containing staurosporine (SS, 64 nM) alone or in combination with SB 203580 (SB, 1 μM), PD 98059 (PD, 50 μM), cycloheximide (CHI, 3.6 μM), or actinomycin D (AcD, 8 μM). Total RNA (15 μg) was extracted and subjected to Northern blot analysis. Similar results were obtained in three separate experiments. (B) Rat peritoneal neutrophils (1×10^7 cells) were incubated at 37°C for 2 h in 1 ml of medium containing staurosporine (SS, 64 nM), and further incubated for 2 h in the presence of staurosporine (SS, 64 nM) with SB 203580 (SB, 0.1 μM), PD 98059 (PD, 10 μM), cycloheximide (CHI, 0.108 μM) or actinomycin D (AcD, 0.8 μM). For comparison, each set of neutrophils (1×10^7 cells) was incubated at 37°C for 4 h in 1 ml of medium containing staurosporine (SS, 64 nM) and each drug (closed bar). Concentrations of MIP-2 in the conditioned medium were determined by ELISA. Values are the means ± S.E.M. from four samples. Statistical significance: $^{**}P<0.01$, $^{***}P<0.001$ vs. SS 2h+AcD. The results were confirmed by three separate experiments.

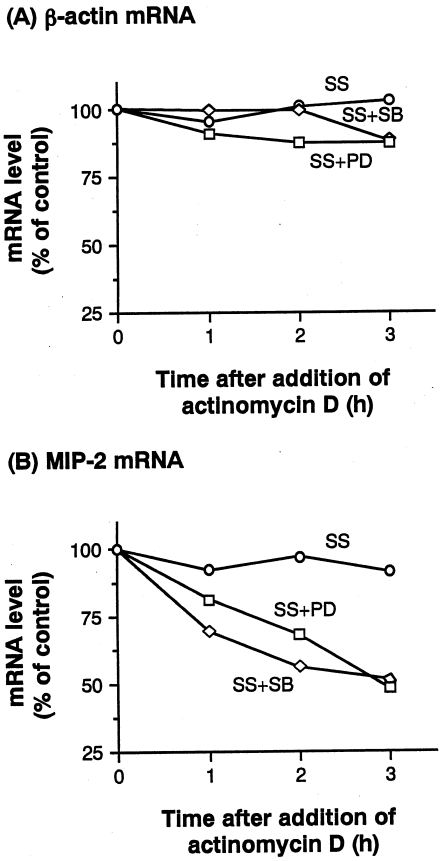

Fig. 5. Effects of SB 203580 and PD 98059 on the stability of MIP-2 mRNA in staurosporine-stimulated neutrophils. Rat peritoneal neutrophils (8×10^7 cells) were incubated at 37°C for 2 h in 8 ml of medium containing 64 nM of staurosporine in the presence and absence of SB 203580 (1 μM) or PD 98059 (50 μM). Actinomycin D (8 μM) was then added to the cultures, and total RNA was extracted at 0, 1, 2 and 3 h after incubation. Dot blot was performed using 10 μg of extracted RNA and β-actin probe (A) or MIP-2 probe (B). After densitometric scanning, levels of mRNA for β-actin and MIP-2 at time 0 after addition of actinomycin D were expressed as 100%, respectively. The results shown are representative of three separate experiments.

IC_{50} of 0.6 μM. Furthermore, maximal inhibition by SB 203580 of tumor necrosis factor-α (TNF-α)-induced p38 MAPK activation and IL-8 production was observed at a concentration of 0.15 μM in human neutrophils [13]. In our previous studies, MIP-2-induced phosphorylation of ERK/MAPK in rat neutrophils was completely inhibited by PD 98059 at 50 μM [19]. Taken together, the present findings suggested that p38 MAPK and ERK/MAPK play key roles in the staurosporine-induced MIP-2 production.

In human neutrophils, p38 MAPK activation is required for IL-8 production stimulated by granulocyte-macrophage colony-stimulating factor (GM-CSF), lipopolysaccharide (LPS) or TNF-α [13]. It is also demonstrated that p38 MAPK- and ERK/MAPK-mediated activation of NF-κB and AP-1 is involved in *Salmonella typhimurium*-induced IL-8 production [11]. Recently, it has been reported that the p38 MAPK pathway is necessary for transcriptional activation of the IL-6 promoter [12]. However, we found that the inhibition of p38 MAPK and ERK/MAPK only partially lowered the levels of staurosporine-induced increase in MIP-2 mRNA (Fig. 4A). In spite of the complete inhibition of MIP-2 protein production by the p38 MAPK inhibitor SB 203580 (1 μM) or the MEK-1 inhibitor PD 98059 (50 μM), the levels of MIP-2 mRNA were not completely lowered by SB 203580 (1 μM) or PD 98059 (50 μM). These findings suggest that the post-transcriptional regulation of MIP-2 gene is involved in the staurosporine-induced MIP-2 production. As reported by Lee et al. [20], p38 MAPK is critical for the production of IL-1 and TNF-α. In addition, in insulin-stimulated murine 3T3-L1 adipocytes, when PHAS-1, an initiation factor 4E (eIF-4E) binding protein, is phosphorylated by ERK2, eIF-4E is released and participates in the initiation of translation [21].

As shown in Fig. 4B, almost the same inhibition of MIP-2 production at 4 h was obtained when SB 203580 (0.1 μM) or PD 98059 (10 μM) was added 2 h after staurosporine (64 nM) stimulation or when these drugs were added simultaneously with staurosporine. The protein synthesis inhibitor cycloheximide (0.108 μM) also showed no difference in the inhibition of MIP-2 production at 4 h when cycloheximide was added simultaneously with staurosporine or 2 h after staurosporine stimulation (Fig. 4B). In contrast, addition of actinomycin D (0.8 μM), an inhibitor of DNA-dependent RNA polymerase, 2 h after staurosporine (64 nM) stimulation failed to inhibit MIP-2 production at 4 h. These findings indicated that MIP-2 mRNA accumulated during the first 2 h after staurosporine stimulation (Figs. 1B and 4A) participates in MIP-2 protein production during the next 2 h, and p38 MAPK and ERK/MAPK are required for MIP-2 protein production at the post-transcription level.

The levels of MIP-2 mRNA was decreased partially by SB 203580 or PD 98059 (Fig. 4A). But this might be due to the decrease in the stabilization of MIP-2 mRNA, because the MIP-2 mRNA levels were decreased much faster in the presence of SB 203580 (1 μM) or PD 98059 (50 μM) than on stimulation with staurosporine (64 nM) alone after the transcription was disrupted by actinomycin D (8 μM) (Fig. 5). It is reported that the AUUUA sequence in the 3′-untranslated region is involved in the modulation of mRNA stability [22]. mRNAs of many cytokines, including, but not limited to GM-CSF, TNF-α, interferon-γ, IL-1, IL-2, IL-3, IL-8, CINC-1 and MIP-2, contain multiple AUUUA motifs [22–24]. Because the c-Jun NH_2-terminal kinase (JNK) pathway is involved in the stabilization of IL-2 mRNA through the 3′-untranslated region [23], it remains to be clarified whether the stabilization of MIP-2 mRNA by p38 MAPK and ERK/MAPK pathways is also through the 3′-untranslated region.

As shown in this paper, staurosporine activates MAPK and ERK/MAPK and induces MIP-2 production in rat peritoneal macrophages as physiologic inflammatory stimuli including TNF-α, GM-CSF, and LPS stimulate IL-8 production in human neutrophils [13]. Therefore, the staurosporine-induced MIP-2 expression system in cell culture might be useful for the screening of inhibitors of chemokine production.

In summary, we first demonstrated that p38 MAPK and ERK/MAPK signaling pathways are involved in the regulation of staurosporine-induced MIP-2 production in rat peritoneal neutrophils at the level of post-transcription; they stimulate translation of MIP-2 mRNA and stabilize its mRNA. Further investigation is necessary to clarify whether inhibition of p38 MAPK and ERK/MAPK is useful for the suppression of chemokine production by neutrophils activated by physiologic inflammatory stimuli.

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