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Involvement of p38 mitogen-activated protein kinase in heat shock protein 27 induction in human neutrophils

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Abstract

We investigated whether tumor necrosis factor-α (TNF-α) stimulates the induction of heat shock protein 27 (HSP27) in human neutrophils and the mechanism underlying this induction. In intact neutrophils, almost no HSP27 was detected. Stimulation of neutrophils by TNF-α increased the levels of HSP27 in the presence, but not in the absence, of cycloheximide. Reverse transcription-polymerase chain reaction (RT-PCR) experiments showed that TNF-α also induced HSP27 mRNA in the presence of cycloheximide. TNF-α induced the phosphorylation of p44/p42 mitogen-activated protein (MAP) kinase and p38 MAP kinase. The HSP27 accumulation induced by TNF-α was significantly suppressed by 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1*H*-imidazole (SB203580) or 4-(4-fluorophenyl)-2-(4-nitrophenyl)-5-(4-pyridyl)-1*H*-imidazole (PD169316); both are specific inhibitors of p38 MAP kinase, but not by 2′-amino-3′-methoxyflavone (PD098059, a specific inhibitor of the upstream kinase that activates p44/p42 MAP kinase). The accumulation of HSP27 induced by TNF-α plus cycloheximide was also suppressed by pretreatment with a specific protein kinase C (PKC) inhibitor. Furthermore, phorbol myristate acetate (PMA), a PKC stimulant, but not dibutyryl cyclic AMP, a protein kinase A stimulant, stimulated the accumulation of HSP27. Interestingly, SB203580 did not inhibit PMA-stimulated HSP27 induction. These results strongly suggest that TNF-α may act as the regulator of HSP27 induction in neutrophils. p38 MAP kinase (but not p44/p42 MAP kinase) and PKC take part in TNF-α-stimulated HSP27 induction in human neutrophils.

Keywords: Heat shock protein; MAP (mitogen-activated protein) kinase; Neutrophil; TNF-α (tumor necrosis factor-α)

1. Introduction

Cells produce heat shock proteins (HSPs) when exposed to biological stress such as heat (Nover, 1991). HSPs are classified into high-molecular-weight HSPs and low-molecular-weight HSPs according to apparent molecular sizes. High-molecular-weight HSPs, such as HSP110, HSP90 and HSP70, are well recognized as acting as molecular chaperones in protein folding, oligome lization and translocation (Benjamin and McMillan, 1998). Low-molecular-weight HSPs with molecular masses from 15 to 30 kDa, such as HSP27 and αB-crystallin, have high amino acid sequence homology (Benjamin and McMillan, 1998). Although the functions of low-molecular-weight HSPs are less known than those of high-molecular-weight HSPs, they may act as molecular chaperones such as the high-molecular-weight HSPs (Nover, 1991).

Neutrophils take part in host defense mechanisms against infection and in inflammatory and allergic reactions such as asthma. To fulfill this role, neutrophils migrate from blood to various tissues. The number of neutrophils in circulating blood is maintained within a narrow range by the balance between the constant production of cells by bone marrow (McConkey et al., 1990) and their death following spontaneous apoptotic processes (Grigg et al., 1991; Savill et al.,

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1989). Apoptotic senescent neutrophils in tissue are recognized and phagocytosed by macrophages. This apoptotic process has been suggested to represent an in vivo mechanism to limit the tissue injury caused by neutrophils at sites of inflammation.

Tumor necrosis factor-α (TNF-α), a 17-kDa mammalian cell macrophage/monocyte-derived lymphokine, was originally defined by its anti-tumor activity, binds to specific receptors on most mammalian cells, including neutrophils to produce various effects on target cells (Tracey and Cerami, 1994), and plays a major role in host defense such as during inflammation, cancer and infection (Carswell et al., 1975; Fong and Lowry, 1990).

Recent evidence indicates that the expression of small stress proteins, especially HSP27, regulates mammalian cell survival (Mehlen et al., 1996; Samali and Cotter, 1996). However, it has not been clearly established whether the low-molecular-weight HSP, HSP27, is expressed in human neutrophils although intracellular oxidative stress has been reported to induce cell surface expression of HSP27 in neutrophils (Camins et al., 1995). In this report, we show that TNF-α induced HSP27 expression in neutrophils and suggest a regulatory mechanism via p38 mitogen-activated protein (MAP) kinase activation.

2. Materials and methods

2.1. Materials

Recombinant human TNF-α was a kind gift from Dainippon Pharmaceutical (Osaka, Japan). Cycloheximide, actinomycin D, GF109203X, phorbol myristate acetate (PMA) and histopaque were purchased from Sigma (St. Louis, MO, USA). 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1*H*-imidazole (SB203580), 4-(4-fluorophenyl)-2-(4-nitrophenyl)-5-(4-pyridyl)-1*H*-imidazole (PD169316), 2′-amino-3′-methoxyflavone (PD098059), anthral [1,9-cd]pyrazol-6(2*H*)-one (SP600125) and dibutyryl cAMP were obtained from Calbiochem-Novabiochem (La Jolla, CA, USA). An antibody raised in rabbits against human HSP27 was extracted as previously described (Kato et al., 1992). This anti-human HSP27 antibody is specific to human HSP27, showing only one band on Western blotting. The antibody also shows no reactivity with other small HSPs such as rat HSP27 and αB-crystallin. Phospho-specific p38 MAP kinase antibody (rabbit polyclonal immunoglobulinG (IgG), affinity-purified), and p38 MAP kinase antibody (rabbit polyclonal IgG, affinity-purified), phospho-specific p44/p42 MAP kinase antibody (rabbit polyclonal IgG, affinity-purified) and p44/p42 MAP kinase antibody (rabbit polyclonal IgG, affinity-purified) were purchased from Cell Signalling Technology (Beverly, MA, USA). An ECL Western blotting detection system was obtained from Amersham Japan (Tokyo, Japan). Other materials and chemicals were obtained from commercial sources. SB203580, PD169316, PD098059 and GF109203X were dissolved in dimethylsulfoxide (DMSO). The maximum concentration of DMSO was 0.1%, which did not affect immunoassay of HSP27 or Western blot analysis.

2.2. Preparation of neutrophils

Human neutrophils in blood were isolated by using mono–poly resolving medium (Dainippon Pharmaceutical) (Boyum, 1968; Ting and Morris, 1971). Purification of neutrophils was performed to minimize exposure of the cells to bacterial endotoxin. The purity of neutrophils was greater than 95%. Cell number was counted with a Coulter counter model Z1 (Coulter Electronics, Beds, England). Cells were diluted in RPMI 1640 medium supplemented with 10% fetal calf serum, 300 mg/ml L-glutamate, 100 units/ml penicillin and 100 μg/ml streptomycin (RPMI 1640 medium) to the final required concentrations and were kept on ice until used.

2.3. Western blot analysis of HSP27, p38 MAP kinase and p44/p42 MAP kinase

Neutrophils were stimulated for the indicated periods; then cells were lysed, homogenized and sonicated in a lysis buffer containing 62.5 mM Tris–HCl, pH 6.8, 4% sodium dodecylsulfate (SDS), 50 mM dithiothreitol and 10% glycerol. The cytosolic fraction was collected as the supernatant after centrifugation at 125,000 × *g* for 10 min at 4 °C. SDS-polyacrylamide gel electropholesis (SDS-PAGE) was performed by the method of Laemmli (1970) with 10% polyacrylamide gel. After SDS-PAGE, the samples were electrophoretically transferred to 0.2 μm polyvinylidene difluoride (PVDF) membranes (Bio-Rad Lab., Hercules, CA, USA) for 30 min. Western blotting analysis was performed as described previously (Kato et al., 1996) by using HSP27 antibody, phospho-specific p38 MAP kinase antibody, p38 MAP kinase antibody, phospho-specific p44/p42 MAP kinase antibody or p44/p42 MAP kinase antibody with peroxidase-labeled antibodies raised in goat against rabbit IgG being used as second antibody (Chemicon International, Temecula, CA, USA). Peroxidase activity on the nitrocellulose sheet was visualized on X-ray film via the ECL Western blotting detection system (Amersham Pharmacia Biotech, Tokyo, Japan).

2.4. Immunoassay for HSP27

The concentration of HSP27 in soluble extracts of cells was determined by sandwich-type enzyme immunoassays as described previously (Inaguma et al., 1993). Neutrophils were stimulated for the indicated periods. The cells were then washed twice with 1 ml of phosphate-buffered saline (PBS) and frozen at −80 °C for a few days before analysis. The frozen cells were collected and suspended in 0.2 ml of

PBS containing protease inhibitor cocktail—Sigma; each suspension was sonicated and centrifuged at 125,000 × *g* for 20 min at 4 °C. The supernatant was used for the specific immunoassay of HSP27. In brief, we used an enzyme immunoassay system that uses polystyrene balls (3.2 mm in diameter, Immuno Chemicals, Okayama, Japan) carrying immobilized $F(ab')_2$ fragments of antibody and the same Fab′ fragments labeled with β-D-galactosidase from *Escherichia coli*. A polystyrene ball-carrying antibodies was incubated either with the purified standard for HSP27 or with an aliquot of the samples. This incubation was carried out at 30 °C for 5 h in a final volume of 0.5 ml of 10 mM sodium phosphate buffer, pH 7.0, containing 0.3 M NaCl, 0.5% hydrolyzed gelatin, 0.1% bovine serum albumin, 1 mM $MgCl_2$ and 0.1% NaN_3. After being washed, each ball was incubated at 4 °C overnight with 1.5 munits of galactosidase-labeled antibodies in a volume of 0.2 ml with 10 mM sodium phosphate buffer, pH 7.0, containing 0.1 M NaCl, 1 mM $MgCl_2$, 0.1% bovine serum albumin and 0.1% NaN_3. The galactosidase activity bound to the ball was assayed using a fluorogenic substrate, 4-methylumbelliferyl-β-D-galactoside.

2.5. Reverse transcription-polymerase chain reaction (RT-PCR)

First-strand cDNA was synthesized as follows: 1 μg of DNase-treated total RNA together with 0.5 μg of oligo dT12-18 (Pharmacia, Uppsala, Sweden) in a total volume of 11 μl were heated to 70 °C for 10 min and then chilled on ice. A mix consisting of 4 μl of five times first strand cDNA buffer (Gibco-BRL, Gaithersburg, MD), 2 μl of 100 mM dithiothreitol, 1 μl of 10 mM dNTPs and 1 μl of RNase block (40 units/μl, Stratagene, La Jolla, CA) was added to the tube and heated at 42 °C for 2 min. SuperScript™ II Rnase H-Reverse Transcriptase (Gibco-BRL) (200 units) was then added and the reaction was continued at 42 °C for 50 min. After a 15-min inactivation step at 70 °C, the cDNA was stored at −20 °C until use.

RT-PCR was performed by coamplification of the gene in question using cDNA template generated as described. The primer sequences were as follows: 5′-CACGAGGAGCGGCAGGACGAG-3′ (sense) and 5′-CAGTGGCGGCAGCAGGGGTGG-3′ (antisense). PCR was carried out in a total volume of 10 μl containing 0.5 μl of cDNA solution, 0.5 U of Taq DNA polymerase (Sigma), 1 mM MgCl2, 0.2 mM of each dNTP, 0.1 μM of sense and antisense primers, 10 mM Tris–HCl, pH 8.3, and 50 mM KCl in a Robot Thermal Cycler (Stratagene) as follows: initial denaturation for 5 min at 94 °C, 29–35 cycles with denaturation at 94 °C for 30 s, annealing at 56–61 °C for 1 min and extension at 72 °C for 1 min, followed by a final extension step for 5 min at 72 °C. After PCR, 7 μl of products was run on an agarose gel, stained with ethidium bromide and the intensity of bands was quantified using 1-D Image Analysis Software (Kodak Digital Science™, USA).

2.6. Evaluation of apoptosis

For morphological assessments, neutrophils were suspended at 2×10^6/ml in RPMI 1640 medium and then incubated with TNF-α and/or cycloheximide at 37 °C for 3 h, dried in cool air and stained with May–Grünwald–Giemsa solution (Merck, Germany) for light microscopic evaluation. The percentage of apoptotic cells was assessed by counting at least 500 cells/slide (Niwa et al., 2000).

2.7. Protein concentration

Protein concentrations in soluble extracts were determined using a protein assay kit (Pierce, Rockford, IL, USA) with BSA as standard.

2.8. Statistical analysis

The data were analyzed by analysis of variance (ANOVA) followed by Fisher's Protected Least Significant Difference (PLSD) test for multiple comparisons between pairs; $P < 0.05$ was considered significant. All data are presented as the means ± S.D. from three separate determinations in triplicate.

3. Results

3.1. Effects of TNF-α on HSP27 induction in human neutrophils

In unstimulated neutrophils, the levels of HSP27 were quite low. TNF-α (100 ng/ml) time dependently increased the levels of HSP27 in the presence of 1 μg/ml of cycloheximide, and maximum levels of HSP27 were observed 24–36 h after stimulation and, thereafter, HSP27 levels decreased (Fig. 1A). The TNF-α-induced stimulation of HSP27 accumulation was dose-dependent in the range of 0.1 to 1000 ng/ml in the presence of 1 μg/ml cycloheximide (Fig. 1B). Saturation of HSP27 accumulation was observed at 10 ng/ml of TNF-α. TNF-α and cycloheximide by themselves did not produce accumulation of HSP27 in neutrophils (data not shown). To confirm that TNF-α induced HSP27 accumulation in human neutrophils in the presence of cycloheximide, Western blotting analysis was used for detection of HSP27. As shown in Fig. 2, TNF-α plus cycloheximide caused a time-dependent increase in HSP27 (Fig. 2).

3.2. Effects of protein or RNA synthesis inhibitor on HSP27 induction in human neutrophils

Next, we determined the dose dependency of cycloheximide on TNF-α-stimulated HSP27 induction. In the absence of cycloheximide, TNF-α did not show significant induction of HSP27 in human neutrophils. In the presence

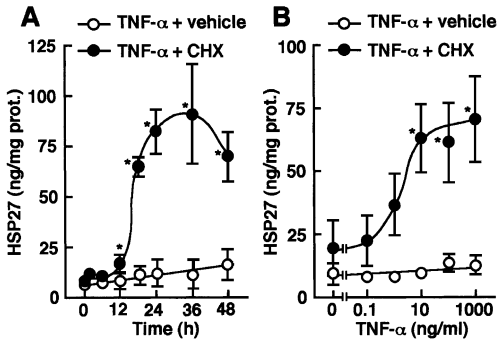

Fig. 1. Effect of TNF-α on HSP27 induction in human neutrophils. (A) Neutrophils were stimulated with 100 ng/ml TNF-α plus 1 μg/ml cycloheximide or vehicle for the indicated periods. (B) Neutrophils were stimulated with various doses of TNF-α in the presence or the absence of 1 μg/ml cycloheximide for 24 h. Then HSP27 was measured by EIA as described in Materials and methods. Each value represents the mean ± S.D. of three separate experiments in triplicate. *$P < 0.05$, compared to the value of control.

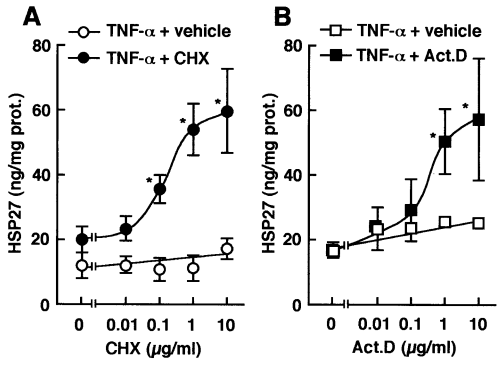

Fig. 3. Effect of cycloheximide (A) and actinomycin D (B) on TNF-α-stimulated HSP27 induction in human neutrophils. (A) Neutrophils were stimulated with various doses of cycloheximide in the presence or the absence of 100 ng/ml TNF-α for 24 h. (B) Neutrophils were stimulated with various doses of actinomycin D in the presence or the absence of 100 ng/ml TNF-α for 24 h. HSP27 was measured by EIA as described in Materials and methods. Each value represents the mean ± S.D. of three separate experiments in triplicate. *$P < 0.05$, compared to the value of control.

of cycloheximide, TNF-α stimulated HSP27 induction in a cycloheximide dose-dependent manner between 0.01 and 10 μg/ml. Cycloheximide alone showed no significant induction of HSP27 at the concentrations used (Fig. 3A). Similar to cycloheximide, the RNA synthesis inhibitor actinomycin D also enhanced TNF-α-stimulated HSP27 induction in human neutrophils (Fig. 3B).

3.3. TNF-α-induced gene expression of HSP27 in neutrophils

It is clear that TNF-α stimulated the accumulation of HSP27 in the presence of cycloheximide as determined with a specific immunoassay and Western blotting analysis. To clarify whether this accumulation is dependent on the gene expression of HSP27, we measured HSP27 mRNA by RT-PCR analysis. Although almost no expression of the HSP27 gene was observed in the absence of stimulation, TNF-α plus cycloheximide induced HSP27 mRNA (Fig. 4).

3.4. Effects of TNF-α on the phosphorylation of p38 MAP kinase and p44/p42 MAP kinase in human neutrophils

To investigate whether or not TNF-α activates p38 MAP kinase and p44/p42 MAP kinase in neutrophils, we next examined the effect of TNF-α on the phosphorylation of p38 MAP kinase and p44/p42 MAP kinase. TNF-α markedly induced the phosphorylation of p38 MAP kinase and p44/p42MAP kinase (Fig. 5).

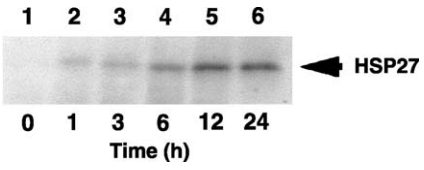

Fig. 2. Time-dependent HSP27 induction by TNF-α in the presence of cycloheximide in human neutrophils by Western blotting analysis. Neutrophils were stimulated with 100 ng/ml TNF-α and 1 μg/ml cycloheximide for the indicated periods. The extracts of cells were subjected to SDS-PAGE with HSP27 antibodies.

3.5. Effects of MAP kinase inhibitors, SB203580, PD169316, PD098059 or SP600125 on the TNF-α plus cycloheximide-stimulated induction of HSP27 in human neutrophils

In order to investigate whether or not p38 MAP kinase is involved in the TNF-α-stimulation of HSP27 induction in neutrophils, we examined the effect of SB203580, a highly specific inhibitor of p38 MAP kinase (Cuenda et al., 1995), which by itself did not affect the basal levels of HSP27. SB203580 markedly suppressed the HSP27 accumulation induced by TNF-α in a dose-dependent manner between 0.01 and 30 μM in the presence of 1 μg/ml of cycloheximide. The maximum effect of SB203580 was observed at 30 μM, a dose that caused an about 80% reduction of the

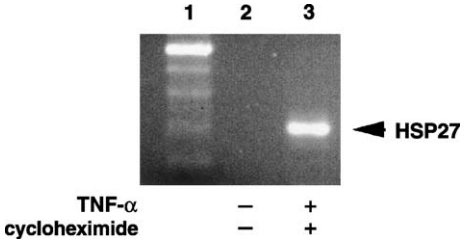

Fig. 4. Gene expression of HSP27 induced by TNF-α in the presence of cycloheximide in human neutrophils by RT-PCR analysis. Neutrophils were stimulated with 100 ng/ml TNF-α and 1 μg/ml cycloheximide for 6 h. RT-PCR, using specific primers, assessed HSP27 mRNA expression as described in Materials and methods. The data are for unstimulated neutrophils (lane 1) and TNF-α plus cycloheximide-stimulated neutrophils (lane 2) for 6 h. The figure shows three representative experiments performed with similar results.

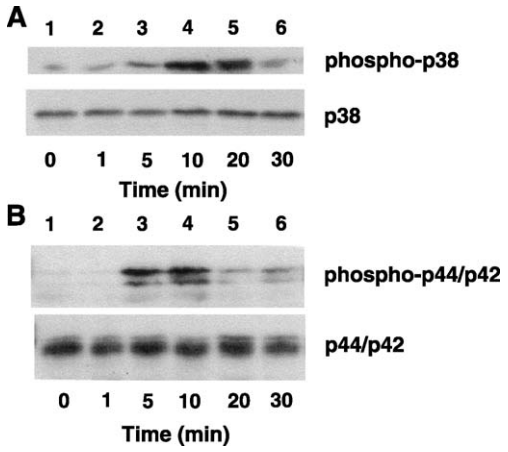

Fig. 5. Effects of TNF-α on the phosphorylation of (A) p38MAP kinase and (B) p44/p42 MAP kinase in human neutrophils. Neutrophils were stimulated with 100 ng/ml TNF-α for the indicated periods. The extracts of cells were subjected to SDS-PAGE with phospho-specific p38 MAP kinase antibody, phospho-specific p44/p42 MAP kinase antibody, p38 MAP kinase antibody or p44/p42 MAP kinase antibody.

effect of TNF-α plus cycloheximide (Fig. 6A). PD169316, which is also a specific inhibitor of p38 MAP kinase (Kummer et al., 1997), produced an inhibition of HSP27 accumulation similar to that produced by SB203580. This inhibition by p38 MAP kinase inhibitors was also observed at all time points after TNF-α stimulation (Fig. 6B).

We next investigated the involvement of p44/p42 MAP kinase in TNF-α-stimulated HSP27 induction in human neutrophils. PD098059, a specific inhibitor of the upstream kinase that activates p44/p42 MAP kinase (Alessi et al., 1995), had no significant effect on HSP27 accumulation stimulated by TNF-α between 0.1 and 50 μM in the presence of 1 μg/ml of cycloheximide (Fig. 6A).

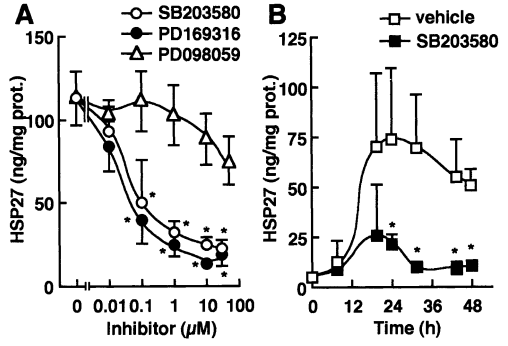

Fig. 6. Effects of MAP kinase inhibitor on TNF-α and cycloheximide-stimulated HSP27 induction in human neutrophils. (A) Neutrophils were pretreated with various doses of SB203580, PD169316 or PD098059 for 15 min, and then stimulated with 100 ng/ml TNF-α plus 1 μg/ml cycloheximide for 24 h. (B) Neutrophils were pretreated with vehicle or 10 μM of SB203580 for 15 min, then stimulated with 100 ng/ml TNF-α plus 1 μg/ml cycloheximide for indicated periods. Then HSP27 was measured by EIA as described in Materials and methods. Each value represents the mean ± S.D. of three separate experiments in triplicate. * indicates significant difference from control at $P<0.05$.

Furthermore, we investigated the involvement of JNK kinase in TNF-α-stimulated HSP27 induction in human neutrophils. SP600125, a highly specific inhibitor of JNK kinase (Bennett et al., 2001), had no significant effect on HSP27 accumulation stimulated by TNF-α between 0.1 and 10 μM in the presence of 1 μg/ml of cycloheximide (data not shown).

3.6. Effects of SB203580, PD169316 or PD098059 on the TNF-α-induced phosphorylation of MAP kinases in neutrophils

We found that PD098059 actually inhibited the TNF-α-induced phosphorylation of p44/p42 MAP kinase (Fig. 7B). Interestingly, both SB203580 and PD169316 enhanced the phosphorylation of p44/p42 MAP kinase. SB203580 and PD169316, but not PD098059, partially suppressed the phosphorylation of p38 MAP kinase stimulated by TNF-α (Fig. 7A).

3.7. Effects of cycloheximide on the TNF-α-induced phosphorylation of p38 MAP kinases in neutrophils

Next, we determined the effect of cycloheximide on the TNF-α-induced phosphorylation of p44/p42 MAP kinase. Cycloheximide by itself had almost no effect on the phosphorylation of p38 MAP kinase in neutrophils. (Fig. 8). Furthermore, pretreatment of neutrophils with cycloheximide did not affect the TNF-α-induced phosphorylation of p38 MAP kinase (Fig. 8).

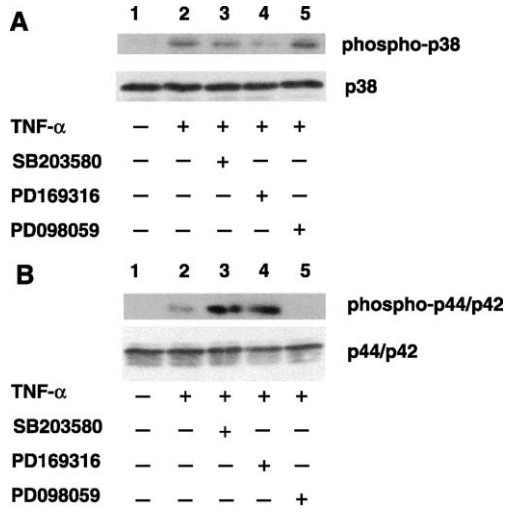

Fig. 7. Effects of MAP kinase inhibitors on the TNF-α-induced phosphorylation of p38 MAP kinase or p44/p42 MAP kinase in human neutrophils. Neutrophils were pretreated with 10 μM SB203580, 10 μM PD169316 or 50 μM PD098059 for 15 min, and then stimulated with 100 ng/ml TNF-α or vehicle for 10 min. (A) Extracts of cells were subjected to SDS-PAGE with phospho-specific p38 MAP kinase antibody or p38 MAP kinase antibody. (B) Extracts of cells were subjected to SDS-PAGE with phospho-specific p44/p42 MAP kinase antibodies or p44/p42 MAP kinase antibody.

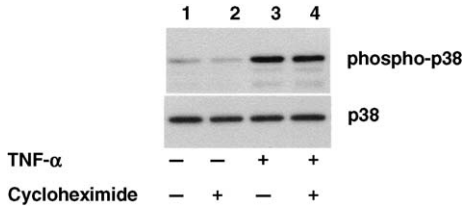

Fig. 8. Effects of cycloheximide on the TNF-α-induced phosphorylation of p38 MAP kinase in human neutrophils. Neutrophils were pretreated with 1 μg/ml cycloheximide for 10 min, and then stimulated with 100 ng/ml TNF-α or vehicle for 10 min. Extracts of cells were subjected to SDS-PAGE with phospho-specific p38 MAP kinase antibody or p38 MAP kinase antibody.

3.8. Involvement of protein kinase C (PKC) in TNF-α and cycloheximide-stimulated HSP27 induction in human neutrophils

To determine whether PKC is involved in the TNF-α plus cycloheximide-stimulated induction of HSP27 in human neutrophils, neutrophils were pretreated with the specific PKC inhibitor, GF-109203X (Toullec et al., 1991), before TNF-α plus cycloheximide stimulation. Accumulation of HSP27 was significantly inhibited by pretreatment with GF-109203X in a dose-dependent manner (Fig. 9A). The result indicates that PKC activation affects TNF-α plus cycloheximide-stimulated HSP27 induction. Thus, next, we evaluated whether direct PKC activation could induce HSP27. PMA induced in a dose-dependent manner (Fig. 9B). The stable cAMP analog, dibutyryl cAMP, did not cause an accumulation of HSP27 (data not shown). These results strongly suggest that PKC activation is involved in the TNF-α plus cycloheximide-stimulated induction of HSP27.

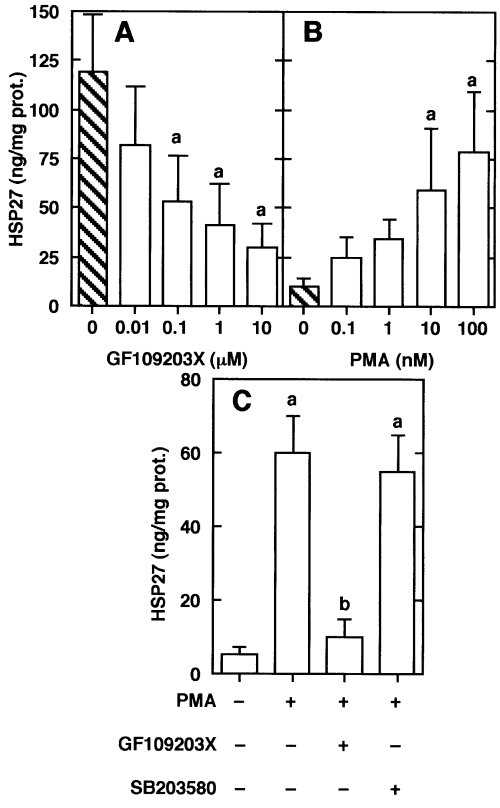

Fig. 9. Involvement of PKC in the TNF-α plus cycloheximide-stimulated induction of HSP27 in human neutrophils. (A) Neutrophils were pretreated with various doses of GF109203X for 15 min, and then stimulated with 100 ng/ml TNF-α plus 1 μg/ml cycloheximide for 24 h. (B) Neutrophils were stimulated with PMA for 24 h. (C) Neutrophils were pretreated with 10 μM of GF109203X or 10 μM of SB203580 for 15 min, and then stimulated with 10 nM of PMA for 24 h. HSP27 was measured by EIA as described in Materials and methods. Each value represents the mean ± S.D. of three separate experiments in duplicate. "a" in A and B indicates significant difference from control at $P<0.05$. "b" in B indicates significant different from PMA control.

3.9. Effects of PDTC on TNF-α plus cycloheximide-stimulated HSP27 induction in human neutrophils

Next, to investigate the role of nuclear factor (NF)-κB in the TNF-α-stimulated induction of HSP27 in neutrophils, the effect of pyrrolidine dithiocarbamate (PDTC), an inhibitor of NF-κB (Niwa et al., 2000), was examined. Although PDTC by itself did not affect the basal levels of HSP27, it markedly elevated the HSP27 accumulation induced by TNF-α plus cycloheximide in a dose-dependent manner (Fig. 10).

3.10. Apoptosis induced by TNF-α plus cycloheximide in human neutrophils

We evaluated apoptosis by morphological evaluation in human neutrophils. Treatment of neutrophils with either 100 ng/ml TNF-α or 1 μg/ml cycloheximide resulted in less than 5% neutrophils appearing apoptotic. However, when neutrophils were pretreated with 1 μg/ml cycloheximide,

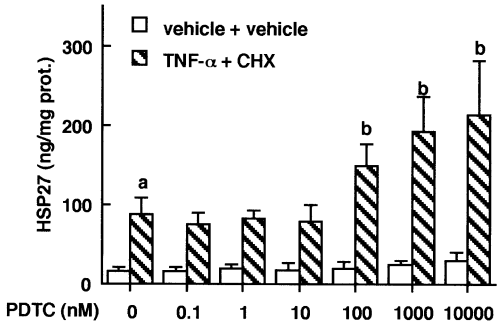

Fig. 10. Effects of NF-κB inhibitor on the TNF-α plus cycloheximide-stimulated induction of HSP27 in human neutrophils. Neutrophils were pretreated with various doses of PDTC for 2 h, and then stimulated with 100 ng/ml TNF-α plus 1 μg/ml cycloheximide or vehicle for 24 h. Then HSP27 was measured by EIA as described in Materials and methods. Each value represents the mean ± S.D. of three separate experiments in duplicate. "a" and "b" indicate significant differences from the value without TNF-α, cycloheximide or PDTC and the value without PDTC and with TNF-α and cycloheximide, respectively, at $P<0.05$.

Table 1
Neutrophil apoptosis induced by TNF-α and/or cycloheximide

Treatment	Percentage of apoptotic cells
Control	2.4 ± 1.9
TNF-α	3.4 ± 2.1
Cycloheximide	4.6 ± 3.9
TNF-α + cycloheximide	75.1 ± 14.7*

Neutrophils were treated by TNF-α (100 ng/ml) and/or cycloheximide (1 μg/ml) for 3 h at 37 °C. Then neutrophil apoptosis was morphologically evaluated by microscopic analysis as described in Materials and methods. Each value represents the mean ± S.D. of three separate experiments.

* $P < 0.05$, compared to the value of control.

TNF-α treatment produced apoptosis in about 75% of cells (Table 1).

4. Discussion

In the present study, we showed that TNF-α induced HSP27 in the presence, but not in the absence, of cycloheximide in human neutrophils. This accumulation of HSP27 was accompanied by an elevation of HSP27 gene expression. Specific inhibitors of p38 MAP kinase, but not a specific inhibitor of the upstream kinase that activates p44/p42 MAP kinase, significantly inhibited this induction of HSP27. Additionally, a specific PKC inhibitor also suppressed the TNF-α plus cycloheximide-stimulated induction of HSP27. These findings suggest that TNF-α acts as a regulatory factor of HSP27, a low-molecular-weight HSP, induction in human neutrophils, and that p38 MAP kinase and PKC are involved in this regulatory system.

It is possible that cycloheximide, in the presence of TNF-α, can stimulate HSP27 production; however, we showed that cycloheximide by itself did not affect HSP27 production at the doses we used. Interestingly, Kato et al. (1999) also had shown that cycloheximide enhanced stress-stimulated HSP27 induction in C6 rat glioma cells. Our observation that the induction of HSP27 induced by TNF-α plus cycloheximide was significantly enhanced by pretreatment of neutrophils with the NF-κB inhibitor, PDTC suggests that TNF-α stimulation may produce some protein(s) which negatively regulates HSP27 induction. Furthermore, HSP27 is produced physiologically when cells become apoptotic to protect cells from further damage (Concannon et al., 2003). From our results, TNF-α induced neutrophil apoptosis in the presence, but not in the absence, of cycloheximide. This evidence suggests that apoptotic neutrophils might induce HSP27 for their defense mechanism. Further investigation would be necessary to resolve the exact role of cycloheximide in TNF-α-stimulated HSP27 induction.

The MAP kinase superfamily has key roles in the intracellular signaling of a variety of agonists (Keyse, 2000). It is well known that MAP kinase is activated by phosphorylation on both threonine and tyrosine residues by a dual-specificity kinase (Raingeaud et al., 1995). In the present study, we showed that TNF-α phosphorylates both p38 and p44/p42 MAP kinase in human neutrophils. It has also been reported that TNF-α phosphorylates p38 MAP kinase and p44/p42 MAP kinase (Suzuki et al., 1999; McLeish et al., 1998). Thus, it is most likely that TNF-α activates p38 MAP kinase in neutrophils. To clarify whether p38 MAP kinase activation is involved in the induction of HSP27 stimulated by TNF-α plus cycloheximide in human neutrophils, the effect of specific p38 MAP kinase inhibitors, SB203580 and PD162316, on HSP27 accumulation was investigated. Both inhibitors significantly reduced HSP27 induction by TNF-α in the presence of cycloheximide. Furthermore, SB203580 and PD169316 partially inhibited the TNF-α-induced phosphorylation of p38 MAP kinase. SB203580 and PD169316 act on p38 MAP kinase directly to inhibit its kinase activity; therefore, this may be the reason for the partial inhibition. Our findings suggest that p38 MAP kinase activation is involved in the TNF-α-stimulated induction of HSP27 in human neutrophils.

p44/p42 MAP kinase is another member of the MAP kinase superfamily (Keyse, 2000). In neutrophils, it has been reported that TNF-α tyrosine phosphorylates both p38 MAP kinase and p44/p42 MAP kinase (Suzuki et al., 1999). We also found that TNF-α phoshorylated p44/p42 MAP kinase and that this phosphorylation was abolished by PD098059, but not by either SB203580 or PD162316. However, PD098059 did not inhibit TNF-α-stimulated HSP27 induction in the presence of cycloheximide. These results strongly indicate that p44/p42 MAP kinase is not involved in the HSP27 induction triggered by TNF-α plus cycloheximide. c-*Jun* amino-terminal kinase (JNK) is also a member of MAP kinase subfamily; however, it is not tyrosine phosphorylated by TNF-α in human neutrophils (Suzuki et al., 1999). In our experiment, SP600125, a highly specific inhibitor of the JNK (Bennett et al., 2001), did not affect the TNF-α plus cycloheximide-stimulated induction of HSP27in neutrophils. These observations suggest that p44/p42 MAP kinases, including JNK, are not involved in the induction of HSP27 triggered by TNF-α plus cycloheximide in human neutrophils.

PKC activation also phosphorylates both p38 MAP kinase and p44/p42 MAP kinase (Zhang et al., 1998) as well as TNF-α stimulation. We also showed that PKC activation is involved in TNF-α plus cycloheximide-stimulated HSP27 induction because a specific inhibitor of PKC significantly suppressed this induction. These results strongly suggest that PKC is involved in TNF-α-stimulated HSP27 induction.

Cycloheximide stimulates the induction of the immediately early genes c-*fos* and c-*Jun* by activating p38 MAP kinase (Cano et al., 1994). Furthermore, Kato et al. (1999) reported that cycloheximide enhanced stress-induced HSP27 production in C6 rat glioma cells. These results suggest that cycloheximide stimulates HSP27 induction via activation of p38 MAP kinase. However, from our results,

cycloheximide by itself did not phosphorylate p38 MAP kinase, and cycloheximide did not affect TNF-α-induced phosphorylation of p38 MAP kinase in neutrophils. These results suggest that the target of cycloheximide is not TNF-α-stimulated HSP27 production (i.e. via p38 MAP kinase activation).

TNF-α induces neutrophil apoptosis in the presence, but not in the absence, of the protein synthase inhibitor cycloheximide. This correlates with the HSP27 production in neutrophils. Furthermore, it has been reported that TNF-α can promote both cell apoptosis and cell survival (Niwa et al., 2000). As an NF-κB inhibitor enhanced TNF-α + cycloheximide-induced HSP27 production, this suggests that induction might be part of a TNF-α-stimulated apoptotic pathway. Therefore, this suggests that apoptotic cells upregulate their HSP27 gene or HSP27 function.

In conclusion, TNF-α stimulates HSP27 induction through p38 MAP kinase in human neutrophils although the target(s) of p38 MAP kinase for the induction of HSP27 remains to be clarified.

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