Thrombin stimulates dissociation and induction of HSP27 via p38 MAPK in vascular smooth muscle cells

KOSEKI HIRADE,1,3 OSAMU KOZAWA,1 KUMIKO TANABE,1,2 MASAYUKI NIWA,1 HIROYUKI MATSUNO,1 YUTAKA OISO,4 SHIGERU AKAMATSU,2 HIDENORI ITO,5 KANEFUSA KATO,5 YOSHIHIRO KATAGIRI,3 AND TOSHIHIKO UEYAMASU1

Departments of 1Pharmacology and 2Anesthesiology and Critical Care Medicine, Gifu University School of Medicine, Gifu 500-8705; 3Department of Pharmacy, Gifu University Hospital, Gifu 500-8705; 4First Department of Internal Medicine, Nagoya University School of Medicine, Nagoya 466-8560; and 5Department of Biochemistry, Institute for Developmental Research, Aichi Human Service Center, Kasugai, Aichi 480-0392, Japan

Received 31 January 2001; accepted in final form 13 May 2002


—We investigated the effects of thrombin on the induction of heat shock proteins (HSP) 70 and 27, and the mechanism behind the induction in aortic smooth muscle A10 cells. Thrombin increased the level of HSP27 but had little effect on the level of HSP70. Thrombin stimulated the accumulation of HSP27 dose dependently between 0.01 and 1 U/ml and cycloheximide reduced the accumulation. Thrombin stimulated an increase in the level of HSP27 mRNA and actinomycin D suppressed the thrombin-increased mRNA level. Thrombin induced the phosphorylation of p38 mitogen-activated protein kinase (MAPK). The HSP27 accumulation by thrombin was reduced by SB-203580 and PD-169316 but not by SB-202474. SB-203580 and PD-169316 suppressed the thrombin-induced phosphorylation of p38 MAPK. SB-203580 reduced the thrombin-increased level of HSP27 mRNA. Dissociation of the aggregated HSP27 to the dissociated HSP27 was induced by thrombin. Dissociation was inhibited by SB-203580. Thrombin induced the phosphorylation of HSP27 and the phosphorylation was suppressed by SB-203580. These results indicate that thrombin stimulates not only the dissociation of HSP27 but also the induction of HSP27 via p38 MAPK activation in aortic smooth muscle cells.

Address for reprint requests and other correspondence: O. Kozawa, Dept. of Pharmacology, Gifu Univ. School of Medicine, Gifu 500-8705, Japan (E-mail: okozawa@cc.gifu-u.ac.jp).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
MATERIALS AND METHODS

Materials. Thrombin and cycloheximide were purchased from Sigma (St. Louis, MO). SB-203580, PD-169316, and SB-202474 were obtained from Calbiochem-Novabiochem (La Jolla, CA). Actinomycin D was purchased from Nacalai Tesque (Kyoto, Japan). Phospho-specific p38 MAPK antibodies (rabbit polyclonal IgG, affinity purified) and p38 MAPK antibodies (rabbit polyclonal IgG, affinity purified) were obtained from New England BioLabs (Beverly, MA). HSP70 antibodies (affinity purified goat polyclonal IgG) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). An enhanced chemiluminescence (ECL) Western blotting detection system was obtained from Amersham Japan (Tokyo, Japan). Other materials and chemicals were obtained from commercial sources. SB-203580, PD-168316, and SB-202474 were dissolved in dimethyl sulfoxide. Cycloheximide and actinomycin D were dissolved in methanol. The maximum concentration of dimethyl sulfoxide or methanol was 0.1%, and this did not affect the immunoblot assays of HSP27, Northern blot analysis, or Western blot analysis.

Cell culture. An aortic smooth muscle cell line, A10 cells, derived originally from thoracic aorta of embryonic rats (18), were obtained from the American Type Culture Collection (Rockville, MD). The cells were seeded into 35-mm (1 × 10^4) or 90-mm (5 × 10^4) diameter dishes and maintained in DMEM containing 10% FCS at 37°C in a humidified atmosphere of 5% CO_2-95% air. After 5 days, the medium was exchanged for serum-free DMEM. The cells were used for experiments after 48 h. When indicated, the cells were pretreated with SB-203580, PD-168316, or SB-202474 for 60 min before the stimulation of thrombin. Pretreatment of cycloheximide was performed for 20 min before the stimulation of thrombin. Cells were pretreated with actinomycin D for 30 min. For heat treatment, dishes with cells were coated with peptide-coupled Sepharose by the procedures described previously (17). Antisera were raised in rabbits by injection of conjugate (0.5 mg of peptide/animal), and antibodies were purified with peptide-coupled Sepharose by the procedures described previously (17).

Preparation and purification of phospho-specific HSP27 antibodies. We produced affinity-purified antibodies that specifically recognize phosphorylated Ser^86 in rat HSP27 and Ser^82 in human HSP27 in Western blot analysis as described previously (13). In brief, peptide corresponding to internal sequence of rat HSP27, containing phosphorylated Ser^86 (residues 83–93, RQLSSGVSEIR, which is identical with residues 79–89 in human HSP27) was synthesized. Peptide was conjugated with hemocyanin (Sigma) using N-(4-carboxycyclohexylmethyl) maleimide (Zeiblen Chemicals, Tokyo, Japan) (43). Antisera were raised in rabbits by injection of conjugate (0.5 mg of peptide/animal), and antibodies were purified with peptide-coupled Sepharose by the procedures described previously (17).

Analysis of HSP27, HSP70, and p38 MAPK by Western blotting. Cultured cells were stimulated by thrombin in serum-free DMEM for the indicated periods. Cells were washed twice with PBS and then lysed, homogenized, and sonicated in a lysis buffer containing 62.5 mM Tris-HCl, pH 6.8, 2% 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-PAGE was performed by the method of Laemmli (22) in 10% polyacrylamide gel. Western blot analysis was performed as described previously (11) by using HSP27 antibodies, HSP70 antibodies, phospho-specific p38 MAPK antibodies, p38 MAPK antibodies, or phosphospecific HSP27 antibodies, with peroxidase-labeled antibodies raised in goat against rabbit IgG being used as secondary antibodies. Peroxidase activity on the nitrocellulose sheet was visualized on X-ray film by means of the ECL Western blotting detection system.
Hitachi). After centrifugation, each sample was fractionated into 15 test tubes, in which had been placed 0.25 ml of 10 mM sodium phosphate buffer, pH 7.0, containing 0.1% (wt/vol) BSA to prevent an absorption of the fractionated protein to the tubes. For calibration, 5 mg of BSA and 50 mg of -D-galactosidase were subjected to sucrose density gradient centrifugation and subsequent fractionation as described above. The position at which BSA was sedimented was determined by quantitation of absorbance at 280 nm, and the position at which -D-galactosidase was sedimented was determined by the assay of its activity as described in the section of immunoassay.

Gel mobility shift assays. Cultured cells were stimulated by thrombin in serum-free DMEM for 60 min. Then the cells were suspended at 0°C in 20 mM HEPES-KOH buffer, pH 7.9, that contained 25% (vol/vol) glycerol, 0.5 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.5 mg/ml pepstatin A, and 20 mg/ml Pefablock SC (Merck; Darmstadt, Germany). The suspension was sonicated at 0°C for 20 s and then centrifuged at 125,000 g for 10 min at 4°C. Supernatants were collected and aliquots were frozen in liquid N₂ and stored at -80°C. Gel mobility shift assays were performed as described previously (12).

Other methods. Protein concentrations in soluble extracts were determined using a protein assay kit (Bio-Rad, Hercules, CA) with BSA as the standard protein. Rat HSP27, which was used as the standard for the immunoassay, was purified from skeletal muscle (11). The densitometric analysis was performed using Molecular Analyst for Macintosh (Bio-Rad).

Statistical analysis. The data were analyzed by ANOVA followed by the Bonferroni method for multiple comparisons between pairs. P < 0.05 was considered significant. Except otherwise noted, data are presented as the means ± SD of triplicate determinations from three independent experiments.

RESULTS

Effects of thrombin on the induction of HSP27 and HSP70 in A10 cells. Thrombin (0.3 U/ml) significantly increased the level of HSP27 in a time-dependent manner by Western blot analysis. On the contrary, thrombin had little effect on the level of HSP70 (Fig. 1). According to the densitometric analysis, thrombin caused ~200% induction of HSP27 at 72 h after stimulation but had no significant effect on HSP70. In addition, we showed the levels of HSP27 and HSP70 induced by heat stress (43°C, treated for 30 min) as positive control proteins (Fig. 1).

Effect of thrombin on the accumulation of HSP27 in A10 cells. We then investigated the effect of thrombin on the level of HSP27 by a specific immunoassay. Thrombin significantly stimulated the HSP27 accumulation in a time-dependent manner (Fig. 2). The thrombin-induced level of HSP27 reached a maximum at 48 h after the stimulation. The stimulatory effect of thrombin on the accumulation of HSP27 was dose dependent in the range between 0.01 and 1 U/ml (Fig. 3). The maximum effect of thrombin was observed at 0.3 U/ml. To clarify whether the thrombin-induced HSP27 is de novo or not, we examined the effect of cycloheximide, an inhibitor of protein synthesis (32) on the thrombin-stimulated HSP27 accumulation. The thrombin-stimulated accumulation of HSP27 was significantly reduced by 1 μM cycloheximide (Table 1).

![Fig. 1. Effects of thrombin on the induction of heat shock protein (HSP)27 and HSP70 in A10 cells. Cultured cells were stimulated by 0.3 U/ml thrombin for the indicated periods. Extracts of cells were subjected to SDS-PAGE with subsequent Western blot analysis using antibodies against HSP27 or HSP70. The histogram shows quantitative representations of HSP27 or HSP70 level obtained from laser densitometric analysis. Each value represents the mean ± SD of triplicate determinations from three independent experiments. *P < 0.05 vs. control value.](http://ajpheart.physiology.org/)

![Fig. 2. Effect of thrombin on the accumulation of HSP27 in A10 cells. Cultured cells were stimulated by 0.3 U/ml thrombin (●) or vehicle (○) for the indicated periods. Each value represents the mean ± SD of triplicate determinations from three independent experiments. *P < 0.05 vs. vehicle alone.](http://ajpheart.physiology.org/)
Northern blot analysis of the mRNA for HSP27 in response to thrombin in A10 cells. Expression level of the mRNA for HSP27 was markedly increased by 0.3 U/ml thrombin (Fig. 4). The stimulatory effect of thrombin on the level of HSP27 mRNA appeared at 2 h after the stimulation of thrombin. The level of the mRNA for HSP27 at 6 h after the stimulation was upregulated more than that at 2 h. Actinomycin D (1 μg/ml), an inhibitor of transcription (35), significantly suppressed the thrombin-increased level of HSP27 mRNA (Fig. 5).

Effect of thrombin on the phosphorylation of p38 MAPK in A10 cells. To investigate whether or not thrombin activates p38 MAPK in A10 cells, we examined the effect of thrombin on the phosphorylation of p38 MAPK (Fig. 6). The phosphorylation of p38 MAPK by thrombin reached the peak at 10 min after the stimulation of thrombin.

Effects of SB-203580, PD-169316, or SB-202474 on the thrombin-induced accumulation of HSP27 in A10 cells. To clarify the involvement of p38 MAPK in the thrombin-stimulated HSP27 induction in A10 cells, we examined the effect of SB-203580, a specific inhibitor of p38 MAPK (6), on the accumulation of HSP27 stimulated by thrombin. The thrombin-induced accumulation of HSP27 was significantly reduced by SB-203580, which alone had little effect on the level of HSP27 (Fig. 7). The inhibitory effect of SB-203580 on the thrombin-stimulated HSP27 accumulation was dose dependent in the range between 1 and 30 μM and it was significant at doses of 10 μM or more. The maximum effect of SB-203580 was observed at 30 μM, a dose that caused ~75% reduction in the effect of thrombin (Fig. 7). In addition, we examined the effect of PD-169316, another inhibitor of p38 MAPK (21) on the thrombin-induced HSP27 accumulation. The thrombin-induced accumulation of HSP27 was markedly suppressed by PD-169316, which by itself did not affect the basal level of HSP27 (Fig. 8). The inhibitory effect of PD-169316 on the HSP27 accumulation was dose dependent in the range between 1 and 30 μM and it was significant at doses of 1 μM or more. PD-169316 (30 μM) caused ~70% reduction in the thrombin effect. However, 15 μM SB-202474, a negative control for p38 MAPK inhibitor (25), had little effect on the thrombin-stimu-

Table 1. Effect of cycloheximide on the accumulation of HSP27 induced by thrombin in A10 cells

<table>
<thead>
<tr>
<th></th>
<th>HSP27, ng/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>252.9 ± 13.1</td>
</tr>
<tr>
<td>Thrombin</td>
<td>1,324.2 ± 18.2</td>
</tr>
<tr>
<td>Cycloheximide</td>
<td>298.4 ± 47.3</td>
</tr>
<tr>
<td>Cycloheximide + Thrombin</td>
<td>344.9 ± 2.0</td>
</tr>
</tbody>
</table>

Each value represents mean ± SD of triplicate determinations from three independent experiments. Cultured cells were pretreated with 1 μM cycloheximide or vehicle for 20 min and then stimulated by 0.3 U/ml thrombin or vehicle for 48 h. *P < 0.05 vs. thrombin alone. HSP, heat shock protein.

Fig. 5. Effect of actinomycin D on the thrombin-increased level of the HSP27 mRNA in A10 cells. Cultured cells were pretreated with 1 μM actinomycin D or vehicle for 30 min, and then stimulated by 0.3 U/ml thrombin for 2 h. Cells were then harvested and total RNA was isolated. Twenty micrograms of RNA from each sample were subjected to electrophoresis and blotted onto a nitrocellulose membrane. The membrane was then allowed to hybridize with the cDNA probe for HSP27. Bands of 28S RNA are shown for reference.
lated accumulation of HSP27, whereas PD-169316 (15 μM) significantly reduced the HSP27 accumulation (Table 2).

Effect of SB-203580 on the thrombin-induced level of the mRNA for HSP27 in A10 cells. To further clarify the role of p38 MAPK in the HSP27 induction stimulated by thrombin in A10 cells, we examined the effect of SB-203580 on the thrombin-increased level of HSP27 mRNA. SB-203580 significantly suppressed the thrombin-induced level of HSP27 mRNA (Fig. 9).

Effects of SB-203580 or PD-169316 on the thrombin-induced phosphorylation of p38 MAPK in A10 cells. SB-203580 actually reduced the phosphorylation of p38 MAPK by thrombin (Fig. 10A). In addition, we found that PD-169316 suppressed the phosphorylation of p38 MAPK by thrombin (Fig. 10B).

Effect of thrombin on aggregated HSP27 and effect of SB-203580 on dissociation in A10 cells. It is well-recognized that HSP27 exists in two forms, aggregated and dissociated forms that have apparent molecular masses of ~500 and ~70 kDa, respectively (15). To investigate the effect of thrombin on the oligomerization state of HSP27 in A10 cells, we performed sucrose density gradient centrifugation with subsequent fractionation and immunoassay. Extracts of unstimulated A10 cells contained both aggregated and dissociated forms of HSP27. We showed that HSP27 exists mostly in the dissociated form in the thrombin-stimulated A10 cells.

Fig. 6. Effect of thrombin on the phosphorylation of p38 MAPK in A10 cells. Cultured cells were stimulated by 0.1 U/ml thrombin for the indicated periods. The extracts of cells were subjected to SDS-PAGE with subsequent Western blot analysis using antibodies against phosphospecific p38 MAPK or p38 MAPK.

Fig. 7. Effect of SB-203580 on the accumulation of HSP27 induced by thrombin in A10 cells. Cultured cells were pretreated with various doses of SB-203580 or vehicle for 60 min and then stimulated by 0.3 U/ml thrombin (●) or vehicle (○) for 48 h. Each value represents the mean ± SD of triplicate determinations from three independent experiments. *P < 0.05 vs. thrombin alone.

Fig. 8. Effect of PD-169316 on the accumulation of HSP27 induced by thrombin in A10 cells. Cultured cells were pretreated with various doses of PD-169316 or vehicle for 60 min, and then stimulated by 0.3 U/ml thrombin (●) or vehicle (○) for 48 h. Each value represents the mean ± SD of triplicate determinations from three independent experiments. *P < 0.05 vs. thrombin alone.

Table 2. Effects of PD-169316 or SB-20474 on the accumulation of HSP27 induced by thrombin in A10 cells

<table>
<thead>
<tr>
<th></th>
<th>HSP27, ng/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>273.8 ± 48.7</td>
</tr>
<tr>
<td>Thrombin</td>
<td>1,354.3 ± 102.4</td>
</tr>
<tr>
<td>PD-169316</td>
<td>260.6 ± 20.3</td>
</tr>
<tr>
<td>PD-169316 + thrombin</td>
<td>665.4 ± 50.8*</td>
</tr>
<tr>
<td>SB-20474</td>
<td>284.6 ± 25.9</td>
</tr>
<tr>
<td>SB-20474 + thrombin</td>
<td>1,212.8 ± 115.5</td>
</tr>
</tbody>
</table>

Each value represents mean ± SD of triplicate determinations from three independent experiments. Cultured cells were pretreated with 15 μM PD-169316, 15 μM SB-20474 or vehicle for 60 min and then stimulated by 0.3 U/ml thrombin or vehicle for 48 h. *P < 0.05 vs. thrombin alone.

To clarify the role of p38 MAPK in the thrombin-induced dissociation of HSP27, we examined the effect of SB-203580 on the dissociation. SB-203580 (30 μM) significantly suppressed the thrombin-induced dissociation of HSP27 (Fig. 11).

Effect of thrombin on phosphorylation of HSP27 and effect of SB-203580 on phosphorylation in A10 cells. It has been shown that dissociation of HSP27 occurs concomitantly with phosphorylation of HSP27 as previously described (15). We examined the effect of...
thrombin on the phosphorylation of HSP27 and the role of p38 MAPK in the thrombin-induced phosphorylation of HSP27 in A10 cells. Thrombin significantly induced the phosphorylation of HSP27, and SB-203580 reduced the phosphorylation of HSP27 by thrombin (Fig. 12).

**DISCUSSION**

In the present study, we showed that thrombin stimulated the accumulation of HSP27, as detected by specific enzyme immunoassay, in a time- and dose-dependent manner in aortic smooth muscle A10 cells. The thrombin-induced accumulation of HSP27 was reduced by cycloheximide (32). Thus it is probable that thrombin stimulates de novo synthesis of HSP27 protein in A10 cells. Additionally, we demonstrated that thrombin increased the expression levels of the mRNA for HSP27 in these cells. The thrombin-increased level of HSP27 mRNA was suppressed by actinomycin D (35). Therefore, it is probable that thrombin-induced increase in HSP27 mRNA is mediated through a transcriptional event. Thus our findings suggest that thrombin as a physiological agonist for aortic smooth muscle cells stimulates the induction of HSP27, a low-molecular-weight HSP, in A10 cells.

We investigated the mechanism underlying the thrombin-stimulated HSP27 induction in aortic smooth muscle A10 cells. We showed that thrombin induced the phosphorylation of p38 MAPK in these cells. It is well recognized that p38 MAPK is activated...
by phosphorylation on tyrosine and threonine by dual-specificity MAPK kinase (33). Therefore, these findings suggest that thrombin activates p38 MAPK in A10 cells. We then investigated whether or not p38 MAPK is involved in the pathway of the thrombin-stimulated induction of HSP27 in aortic smooth muscle A10 cells. The thrombin-induced accumulation of HSP27 was reduced by SB-203580, a specific inhibitor of p38 MAPK (6). In addition, we showed that PD-169316, another inhibitor of p38 MAPK (21), suppressed the HSP27 accumulation. We found that thrombin-stimulated phosphorylation of p38 MAPK was reduced by SB-203580 or PD-169316. Furthermore, we showed that the HSP27 accumulation stimulated by thrombin was not affected by SB-202474, a negative control for p38 MAPK inhibitor (25). These results suggest that p38 MAPK is involved in the thrombin-stimulated HSP27 induction. Furthermore, the thrombin-increased level of the mRNA for HSP27 was markedly reduced by SB-203580. Taking our findings into account, it is most likely that p38 MAPK activation is necessary for the thrombin-stimulated HSP27 induction in aortic smooth muscle A10 cells.

As for the intracellular signaling system for HSP induction, it is generally recognized that activation of heat shock transcriptional factors (HSFs) occurs in response to heat stress and that these activated factors bind to specific DNA sequences, known as heat shock elements (HSEs) found in the promoter regions of the genes for HSP, with a resultant increase in the transcription of the respective genes (37). However, we found that thrombin had little effect on the HSE-binding activity using gel mobility shift assay (data not shown). Thus it seems unlikely that the induction of HSP27 by thrombin is mediated by HSFs in aortic smooth muscle A10 cells.

It is generally known that HSP27 exists in an aggregated form and a dissociated form (15). Thus, we then examined the effect of thrombin on two forms of HSP27 in thrombin-stimulated A10 cells. Extracts of unstimulated A10 cells contained both forms. We showed that HSP27 in thrombin-stimulated A10 cells mainly showed a dissociated form of HSP27. Furthermore, we demonstrated that SB-203580 suppressed this shift to a dissociation form from an aggregated form. It has been reported that HSP27 is dissociated concomitantly with the phosphorylation of HSP27 (15). We showed that thrombin-induced phosphorylation of HSP27 and the phosphorylation was reduced by SB-203580. These results suggest that thrombin induces the phosphorylation of HSP27 and the dissociation via the activation of p38 MAPK in A10 cells.

HSP70, a member of high-molecular-weight HSPs, is well known to act as molecular chaperones (1). It is recognized that low-molecular-weight HSPs, such as HSP27, may also act as chaperones (1, 14). It has been shown that heat stress induces both HSP70 and HSP27 in vascular smooth muscle cells (1). It has been reported that an expression of HSP27 is induced in aortic smooth muscle cells in restrained rat (38). In the present study, thrombin had little effect on the level of HSP70, but it stimulated the level of HSP27 in A10 cells. These findings lead us to speculate that HSP27 may play important roles in aortic smooth muscle cell function after the stimulation of thrombin.

It is well known that HSP27 was originally discovered as an inhibitor of actin polymerization (1). Accumulating evidence suggests that HSP27 acts in the regulation of the structure and dynamics of actin filaments (10, 24). Thrombin reportedly induces contraction of vascular smooth muscle (40). As for the roles played by HSP27 in the vascular system, it has recently been shown that thrombin-induced contraction of vascular smooth muscle is associated with an increased phosphorylation of HSP27 (2, 3). On the basis of these findings, it is probable that HSP27 plays an important role in aortic smooth muscle cell functions, such as contraction. Further investigations of HSP27 in vascular smooth muscle cells may contribute to clarify the exact mechanism behind vascular pathophysiology, such as hypertension.

In conclusion, our results indicate that thrombin stimulates not only the dissociation of HSP27 but also the induction of HSP27 via p38 MAPK activation in aortic smooth muscle cells.

We are very grateful to Dajiro Hatakeyama and Masaichi Miwa for skillful technical assistance.

The work was supported, in part, by Grants-in-Aid 08457405 and 12470015 for Scientific Research from the Ministry of Education, Science, Sports, and Culture of Japan.

REFERENCES


P38 MAPK AND HSP27 INDUCTION


