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# Midazolam Suppresses Thrombin-Induced Heat Shock Protein 27 Phosphorylation Through Inhibition of p38 Mitogen-Activated Protein Kinase in Cardiac Myocytes

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**Abstract** It has been shown that anesthetics have effects of cardiac preconditioning. Heat shock proteins (HSPs) function as molecular chaperone. Among them, HSP27, a low-molecular-weight HSP, abundantly exist in heart. However, the relationship between anesthetics and HSP27 in heart is not yet clarified. We investigated whether thrombin induces or phosphorylates HSP27 in primary cultured mouse myocytes and the effect of midazolam on the thrombin-stimulated HSP27 phosphorylation and the mechanism behind it. Thrombin time dependently phosphorylated HSP27 at Ser-15 and Ser-85 while having no effect on the levels of HSP27. Midazolam markedly suppressed the thrombin-induced phosphorylation of HSP27 at both Ser-15 and Ser-85. Thrombin induced the phosphorylation of p44/p42 MAP kinase and p38 MAP kinase without affecting stress-activated protein kinase/c-*Jun N*-terminal kinase. In addition, midazolam attenuated the phosphorylation of thrombin-induced p38 MAP kinase but not that of p44/p42 MAP kinase. SB203580 and PD169316, inhibitors of p38 MAP kinase, suppressed the thrombin-induced phosphorylation of HSP27 at both Ser-15 and Ser-85. These results strongly suggest that thrombin induces the HSP27 phosphorylation at least through the p38 MAP kinase activation in cardiac myocytes and that midazolam inhibits the thrombin-induced HSP27 phosphorylation via suppression of p38 MAP kinase activation. J. Cell. Biochem. 96: 56–64, 2005. © 2005 Wiley-Liss, Inc.

**Key words:** cardiac myocyte; heat shock protein 27; midazolam; preconditioning; p38 mitogen-activated protein kinase; thrombin

Myocardial ischemia results in reversible or irreversible damage of myocytes. It is generally known that some conditions such as hypothermia and ischemia and agents modulate or delay the ischemic damage [Chen et al., 2002; Zaugg et al., 2003a,b]. It has been shown that both volatile and intravenous anesthetics have cardiac protective effect against ischemic damage [Zaugg et al., 2003a,b]. Intravenous anesthetics such as midazolam are administered in the patients with cardiac ischemic diseases and undergoing coronary artery bypass graft [Shafer, 1998; Barvais et al., 2000]. It has been reported that midazolam has direct effects on cardiac myocytes and vascular smooth muscle cells, resulting in modulation of cardiac function and decreasing systemic blood pressure [Shafer, 1998; Kanaya et al., 2002; Tanabe et al., 2002, 2003]. However, the effects of anesthetics on cardiac myocytes and the exact mechanism against cardiac ischemic damage have not yet been clarified.

Thrombin is a serine protease formed at the site of vascular injury by the cleavage of prothrombin [Mann et al., 1990; Sabri et al., 2000]. Thrombin plays well-characterized roles in not only thrombosis and hemostasis but also

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inflammation and proliferative processes [Mann et al., 1990; Sabri et al., 2000]. Thrombin signaling is mediated at least in part through the GTP-binding protein-coupled protease-activated receptors [Mann et al., 1990; Sabri et al., 2000]. Recent studies indicate cardiac myocytes also as targets for the action of thrombin [Sabri et al., 2000, 2003]. In cardiac myocytes, it has been reported that thrombin stimulates phosphoinositide hydrolysis, activates p44/p42 MAP kinase, induces arterial natriuretic factor expression, modulates calcium homeostasis, and increases automaticity [Sabri et al., 2000, 2003]. It is speculated that these intracellular signaling relates to contractile function, myocardial hypertrophy, and dysfunction during ischemia and reperfusion [Sabri et al., 2000; Avkiran and Haworth, 2003]. It is currently recognized that thrombin has a crucial roles in heart.

Cells produce heat shock proteins (HSPs), when exposed to the biological stress such as heat stress and chemical stress [Benjamin and McMillan, 1998]. HSPs are classified into high-molecular-weight HSPs such as HSP90 and low-molecular-weight HSPs with molecular masses from 10 to 30 kDa based on apparent molecular sizes. HSP27 is a member of low-molecular-weight HSPs. In a previous study, we have shown that HSP27 is present in various tissues and cells, especially in skeletal muscle and smooth muscle cells [Inaguma et al., 1993]. HSP27 is constitutively expressed in several unstressed cells including myocytes where they may have essential functions [Inaguma et al., 1993]. It is well known that the high-molecular-weight HSPs act as molecular chaperones in protein folding, oligomerization, and translocation [Benjamin and McMillan, 1998]. Though the functions of the low-molecular-weight HSPs are known less than those of the high-molecular-weight HSPs, it is recognized that they may have chaperoning functions like the high-molecular-weight HSPs [Benjamin and McMillan, 1998]. It has been reported that increased expression of HSP27 provides cardioprotection against hypoxic injury in cultured cardiac myocytes [Martin et al., 1997; Heide, 2002]. In addition, HSP27 is regulated by post-translational modification such as phosphorylation [Welch, 1985; Benjamin and McMillan, 1998]. It has been shown that the mitogen-activated protein (MAP) kinase superfamily is involved in HSP27 phosphorylation in several type cells [Benjamin and McMillan, 1998]. However, the exact role and regulatory mechanism of HSP27 in heart have not yet been precisely clarified.

In the present study, we first investigated whether thrombin stimulates the phosphorylation of HSP27 or the induction in mouse primary cultured ventricular myocytes. Next, we investigated the effect of midazolam on the thrombin-induced HSP27 phosphorylation and the mechanism underlying it.

## MATERIALS AND METHODS

### Materials

Thrombin was obtained from Sigma Chemical (St. Louis, MO). Midazolam was kindly provided by Yamanouchi Pharmacy (Tokyo, Japan). SB203580 and PD169316 were obtained from Calbiochem-Novabiochem Co. (La Jolla, CA). Antibodies against phospho-specific p44/p42 MAP kinase, p44/p42 MAP kinase, phospho-specific p38 MAP kinase, p38 MAP kinase, phospho-specific stress-activated protein kinase (SAPK)/c-*Jun N*-terminal kinase (JNK), and SAPK/JNK were obtained from New England BioLabs (Beverly, MA). An ECL Western blotting detection system was obtained from Amersham Japan (Tokyo, Japan). Other materials and chemicals were obtained from commercial sources. SB203580 or PD169316 were dissolved in dimethyl sulfoxide. The maximum concentration of dimethyl sulfoxide was 0.1%, which did not affect Western blot analysis.

### Cell Culture

Mouse neonatal ventricular myocytes were prepared as previously described [Harada et al., 1997], with a minor modification. The preparation was performed in accordance with institutional guidelines. Apical halves of cardiac ventricles from 1- to 2-day-old mice were separated and minced in a chilled balanced salt solution (116 mM NaCl, 20 mM HEPES, pH 7.35, 12.5 mM $NaH_2PO_4$, 5.6 mM glucose, 5.4 mM KCl, 0.8 mM $MgSO_4$). Ventricular cardiomyocytes were dispersed in the balanced salt solution containing with 80 U/ml collagenase IV and 0.6 mg/ml pancreatin. The digestion steps were repeated four times and the collected cell suspensions were mixed with 1/10 vol of chilled fetal calf serum (FCS) and centrifuged at 3,000 rpm for 30 min. The pellets were combined in chilled FCS and kept at 4°C.

The cells were then incubated on uncoated 100-mm-diameter dishes for 30 min to remove any nonmyocytes such as fibroblasts, and the non-attached viable cells as cardiomyocytes were seeded into gelatin-coated 60-mm-diameter dishes ($2 \times 10^6$/dish) in Dulbecco's modified Eagle's medium (DMEM) containing 10% FCS. The cells were cultured at 37°C under a humidified atmosphere of 5% carbon dioxide and 95% air. After 5 days, the medium was exchanged for serum-free DMEM. The cells were used for experiments 24 h thereafter.

### Western Blot Analysis of HSP27, p44/p42 MAP Kinase, p38 MAP Kinase, and SAPK/JNK

Cultured cells were stimulated by thrombin (0.3 U/ml) in serum-free DMEM for the indicated periods. Cells were washed twice with phosphate-buffered saline and then lysed, homogenized, and sonicated in a lysis buffer containing 62.5 mM Tris/Cl, pH 6.8, 2% sodium dodecyl sulfate (SDS), 50 mM dithiothreitol, and 10% glycerol. The cytosolic fraction was collected as a supernatant after centrifugation at 125,000*g* for 10 min at 4°C. The supernatant was used for the analysis of HSP27 or each MAP kinase by Western blotting. SDS–polyacrylamide gel electrophoresis (PAGE) was performed by the method of Laemmli [1970] in 10% polyacrylamide gel. Western blot analysis was performed as described previously [Kato et al., 1996], using HSP27 antibodies, phospho-specific HSP27 antibodies or each of the MAP kinase 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. When indicated, the cells were pretreated with SB203580, PD169316, or midazolam for 60 min prior to stimulation by thrombin. The densitometric analysis was performed using Molecular Analyst for Macintosh (Bio-Rad, Hercules, CA).

### Immunoassay of HSP27

Cultured cells were stimulated by thrombin (0.3 U/ml) for 48 h in serum-free DMEM. The concentrations of HSP27 in soluble extracts of the cells were determined by means of a sandwich-type enzyme immunoassay, as described previously [Tanabe et al., 2002]. The cells were washed twice with phosphate-buffered saline, then frozen at −80°C for a few days before analysis. The frozen cells on each dish were collected and suspended in 0.3 ml of phosphate-buffered saline, then each suspension was sonicated and centrifuged at 125,000*g* for 20 min at 4°C. The supernatant was used for the immunoassay of HSP27. In brief, we used an enzyme immunoassay system that employs 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 one 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 0.3M NaCl, 0.5% hydrolyzed gelatin, 0.1% bovine serum albumin, 1 mM $MgCl_2$, and 0.1% $NaN_3$. After washing, each ball was incubated at 4°C overnight with 1.5 milliunits of galactosidase-labeled antibodies in a volume of 0.2 ml with 10 mM sodium phosphate buffer, pH 7.0, containing 0.1M 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.

### Other Methods

Antibodies specifically recognizing phosphorylated serine residues at Ser-15 and Ser-85 in HSP27 were prepared as previously described [Kato et al., 2002]. Protein concentrations in soluble extracts were determined using a protein assay kit (Bio-Rad), with bovine serum albumin as the standard protein. HSP27 was purified from skeletal muscle as previously described [Inaguma et al., 1993].

### Statistical Analysis

The data were analyzed by ANOVA followed by the Student–Newman–Keuls test, $P < 0.05$ being considered significant. The data are presented as the mean ± SD of triplicate determinations. Similar results were obtained in two other cell preparations.

## RESULTS

### Effects of Thrombin on the Phosphorylation of HSP27and the Levels of HSP27

It has been reported that mouse HSP27 is phosphorylated at two sites (Ser-15 and Ser-85)

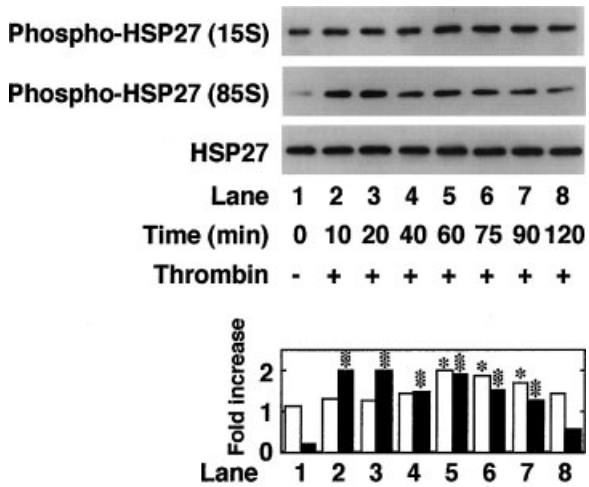


**Fig. 1.** Effects of thrombin on the phosphorylation of HSP27 at serine residues (Ser-15 and Ser-85) in cardiac myocytes. The cultured cells were stimulated by 0.3 U/ml thrombin for the indicated periods. The extracts of cells were subjected to SDS–PAGE using antibodies against phosphorylated Ser-15 (15S), Ser-85 (85S) in HSP27 or HSP27. The histogram shows quantitative representations of the phosphorylation level obtained from laser densitometric analysis (open bar or closed bar for the phosphorylation of HSP27 at Ser-15 or Ser-85, respectively). Each value represents the mean ± SD of triplicate determinations. Similar results were obtained in two other cell preparations. **P* (15S) or ***P* (85S) <0.05 compared with the value of unstimulated cells.

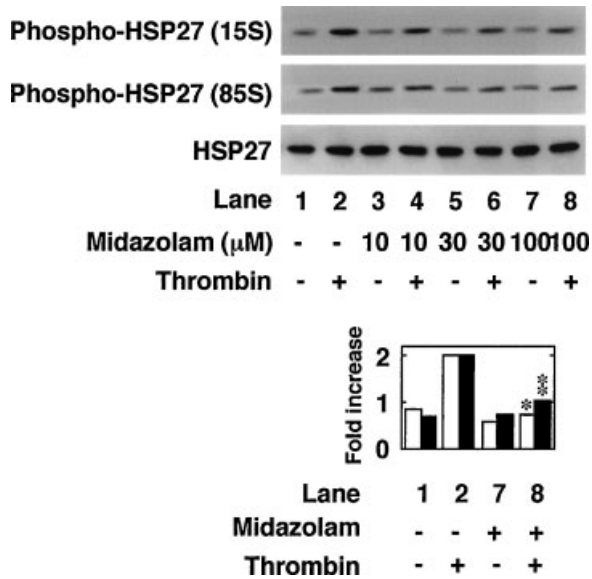


**Fig. 2.** Effects of midazolam on the thrombin-induced phosphorylation of HSP27 at serine residues (Ser-15 and Ser-85) in cardiac myocytes. The cultured cells were pretreated with various doses of midazolam for 60 min. The cells were then stimulated by 0.3 U/ml thrombin or vehicle for 75 min or 20 min for the analysis of phosphorylation of Ser-15 (15S) or Ser-85 (85S), respectively. The extracts of cells were subjected to SDS–PAGE using antibodies against phosphorylated Ser-15 (15S), Ser-85 (85S) in HSP27 or HSP27. The histogram shows quantitative representations of the phosphorylation level obtained from laser densitometric analysis (open bar or closed bar for the phosphorylation of HSP27 at Ser-15 or Ser-85, respectively). Each value represents the mean ± SD of triplicate determinations. Similar results were obtained in two other cell preparations. **P* (15S) or ***P* (85S) <0.05 compared with the value of thrombin alone.

of serine [Gaestel et al., 1991]. Thrombin (0.3 U/ml) time dependently stimulated the phosphorylation of HSP27 at Ser-15 (15S) and Ser-85 (85S) in HSP27 in cardiac myocytes (Fig. 1). The maximum effect of thrombin on the HSP27 phosphorylation at Ser-15 was observed at 75 min after the stimulation. On the other hand, the maximum effect on the phosphorylation at Ser-85 was observed at 20 min. The concentration of HSP27 was 1,099.1 ± 125.5 ng/mg protein in unstimulated cardiac myocytes. On the contrary, thrombin did not affect the levels of HSP27 (913.3 ± 81.9 ng/mg protein for vehicle, 930.6 ± 42.3 ng/mg protein for 0.3 U/ml thrombin, as measured during a stimulation of 48 h).

### Effect of Midazolam on the Thrombin-Induced Phosphorylation of HSP27

Midazolam, while alone had little effect on the HSP27 phosphorylation, significantly attenuated the thrombin (0.3 U/ml)-induced phosphorylation of HSP27 at both Ser-15 and Ser-85 (Fig. 2). The inhibitory effect of midazolam on the HSP27 phosphorylation by thrombin was dose dependent in the range between 10 and 100 μM. The inhibitory effect is significant at 10 μM or more. According to the densitometric analysis, midazolam (100 μM) caused about 80% reduction in the thrombin-effect.

### Effects of Thrombin on the Phosphorylation of p44/p42 MAP Kinase, p38 MAP Kinase, or SAPK/JNK

To investigate the possible involvement of the MAP kinase superfamily in the thrombin-induced HSP27 phosphorylation in cardiac myocytes, we first examined the effects of thrombin on the phosphorylation of p44/p42 MAP kinase, p38MAP kinase, and SAPK/JNK. Stimulation by thrombin (0.3 U/ml) markedly induced the phosphorylation of p44/p42 MAP kinase and p38 MAP kinase, but not that of SAPK/JNK at any time up to 120 min (Fig. 3). The phosphorylation of both MAP kinases

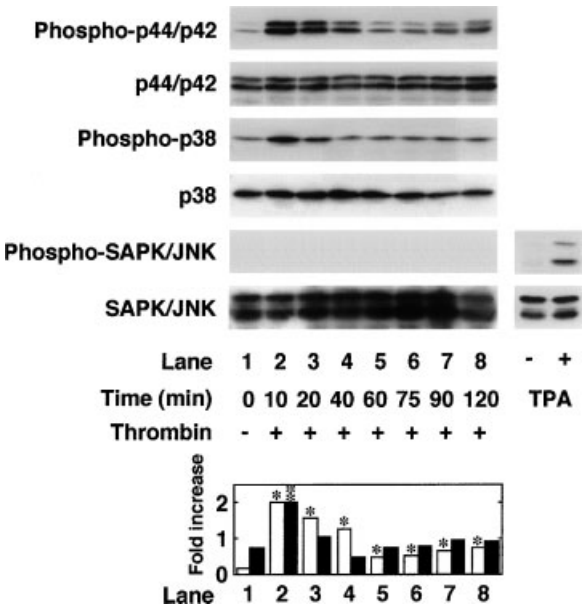


**Fig. 3.** Effects of thrombin on the phosphorylation of p44/p42 MAP kinase, p38 MAP kinase, SAPK/JNK in cardiac myocytes. The cultured cells were stimulated by 0.3 U/ml thrombin for the indicated periods. The extracts of cells were subjected to SDS–PAGE using antibodies against phospho-specific p44/p42 MAP kinase, p44/p42 MAP kinase, phospho-specific p38 MAP kinase, p38 MAP kinase, phsopho-specific SAPK/JNK, or SAPK/JNK. Lower right blot shows the positive control for 12-*O*-tetradecanoylphorbol 13-acetate (TPA)-induced SAPK/JNK. The histogram shows quantitative representations of the phosphorylation level obtained from laser densitometric analysis (open bar or closed bar for the phosphorylation of p44/p42 MAP kinase or p38 MAP kinase, respectively). Each value represents the mean ± SD of triplicate determinations. Similar results were obtained in two other cell preparations. **P* (phospho-p44/p42) or ***P* (phospho-p38) <0.05 compared with the value of unstimulated cells.

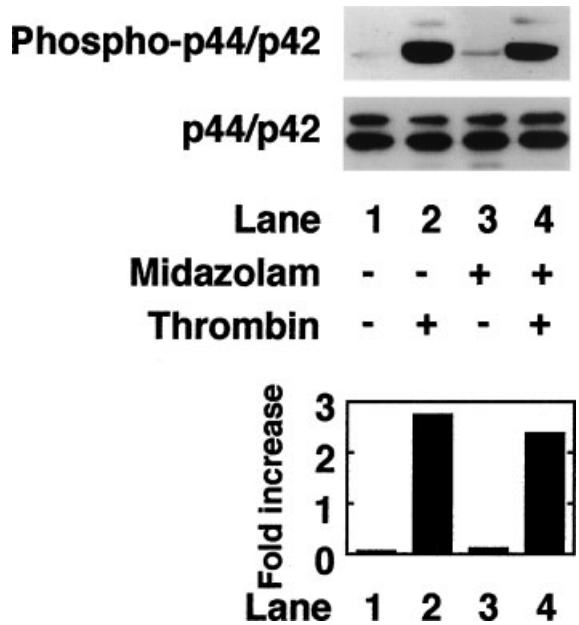


**Fig. 4.** Effect of midazolam on the thrombin-induced phosphorylation of p44/p42 MAP kinase in cardiac myocytes. The cultured cells were pretreated with 100 μM of midazolam or vehicle for 60 min. The cells were then stimulated by 0.3 U/ml thrombin or vehicle for 10 min. The extracts of cells were subjected to SDS–PAGE using antibodies against phospho-specific p44/p42 MAP kinase or p44/p42 MAP kinase. The histogram shows quantitative representations of the phosphorylation level obtained from laser densitometric analysis. Each value represents the mean ± SD of triplicate determinations. Similar results were obtained in two other cell preparations.

peaked at 10 min after the stimulation of thrombin, then showed a decline.

### Effects of Midazolam on the Thrombin-Induced Phosphorylation of p44/p42 MAP Kinase or p38 MAP Kinase

In order to clarify the mechanism behind midazolam-suppressed HSP27 phosphorylation, we examined the effect of midazolam on the thrombin-induced p44/p42 MAP kinase phosphorylation. However, midazolam (100 μM) had little effect on the thrombin (0.3 U/ml)-induced p44/p42 MAP kinase phosphorylation (Fig. 4). On the other hand, midazolam (30 μM) markedly reduced the thrombin (0.3 U/ml)-induced phosphorylation of p38 MAP kinase (Fig. 5). According to the densitometric analysis, midazolam caused about 80% reduction in the thrombin-effect.

### Effect of SB203580 or PD169316 on the Thrombin-Induced Phosphorylation of HSP27

In order to clarify whether p38 MAP kinase is involved in the thrombin-stimulated HSP27 phosphorylation in cardiac myocytes, we next examined the effect of SB203580, a specific inhibitor of p38 MAP kinase [Cuenda et al., 1995], on the thrombin-induced phosphorylation of HSP27. SB203580 markedly suppressed the thrombin-stimulated phosphorylation of HSP27 at both Ser-15 and Ser-85 (Fig. 6A). According to the densitometric analysis, SB203580 caused about complete reduction in the thrombin-effect. Furthermore, PD169316, another inhibitor of p38 MAP kinase [Kummer et al., 1997], had an inhibitory effect on the HSP27 phosphorylation as well as SB203580 (Fig. 6B). According to the densitometric analysis, PD169316 caused about complete reduction in the thrombin-effect. In addition, we found that the thrombin-stimulated phosphorylation of p38 MAP kinase was significantly reduced by SB203580 (Fig. 7).

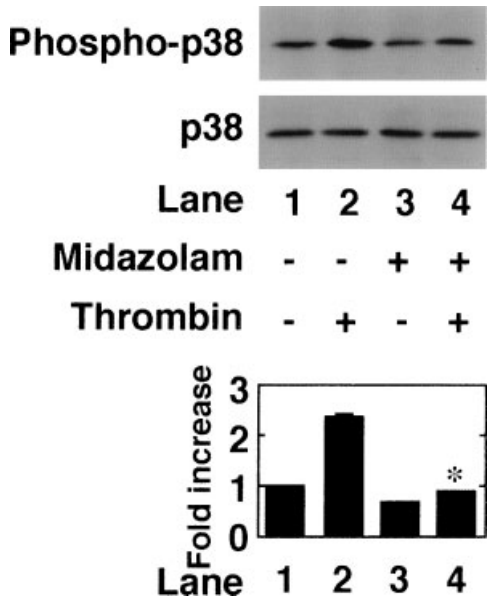


**Fig. 5.** Effect of midazolam on the thrombin-induced phosphorylation of p38 MAP kinase in cardiac myocytes. The cultured cells were pretreated with 30 μM of midazolam or vehicle for 60 min. The cells were then stimulated by 0.3 U/ml thrombin or vehicle for 10 min. The extracts of cells were subjected to SDS–PAGE using antibodies against phospho-specific p38 MAP kinase or p38 MAP kinase. The histogram shows quantitative representations of the phosphorylation level obtained from laser densitometric analysis. Each value represents the mean ± SD of triplicate determinations. Similar results were obtained in two other cell preparations. $*P < 0.05$ compared with the value of thrombin alone.

## DISCUSSION

In the present study, we demonstrated that thrombin stimulated the phosphorylation of HSP27 at two sites (Ser-15 and Ser-85) of serine in mouse primary cultured cardiac myocytes while thrombin did not affect the levels of HSP27. It has previously been reported that hypoxia and platelet derived growth factor induce HSP27 phosphorylation in cardiac myocytes [Kacimi et al., 2000; Takenaka et al., 2004]. In addition, we demonstrated that midazolam, an intravenous anesthetic, markedly attenuated the thrombin-induced phosphorylation of HSP27 at two sites (Ser-15 and Ser-85) in mouse primary cultured cardiac myocytes. To the best of our knowledge, this is probably the first report showing that an anesthetic modulate HSP27 phosphorylation.

We next investigated the mechanism behind the thrombin-induced HSP27 phosphorylation in these cardiac myocytes. The MAP kinase superfamily plays an important role in transdu-

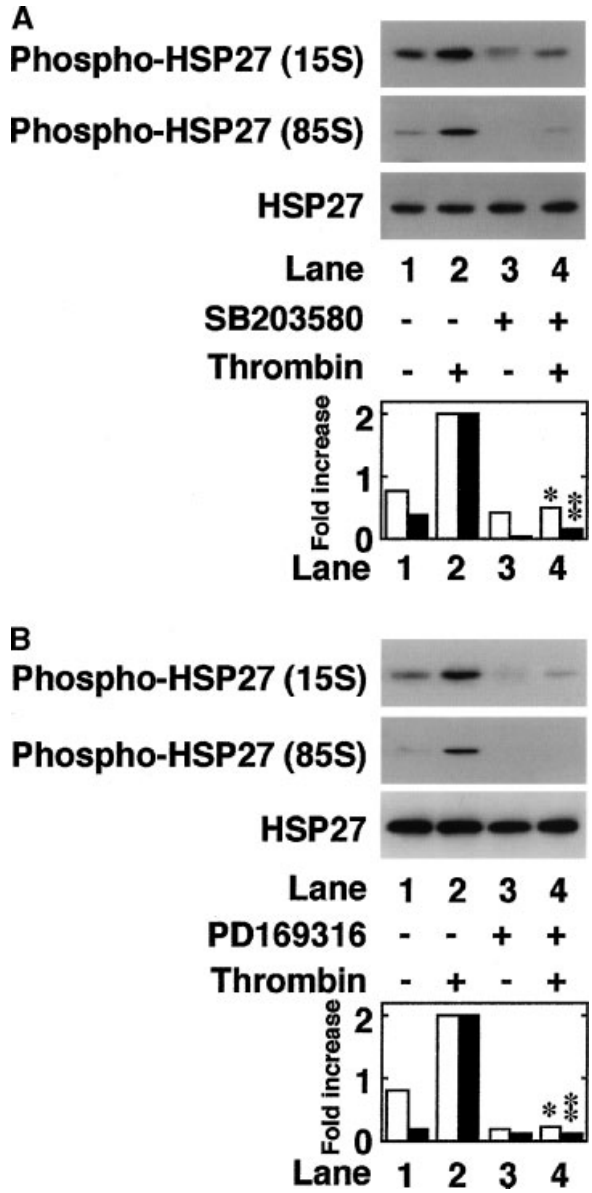


**Fig. 6.** Effect of SB203580 or PD169316 on the thrombin-induced phosphorylation of HSP27 at serine residues (Ser-15 and Ser-85) in cardiac myocytes. The cultured cells were pretreated with 30 μM SB203580 (**A**), 30 μM PD169316 (**B**) or vehicle for 60 min. The cells were then stimulated by 0.3 U/ml thrombin or vehicle for 75 min or 20 min for the analysis of phosphorylation of Ser-15 (15S) or Ser-85 (85S), respectively. The extracts of cells were subjected to SDS–PAGE using antibodies against phosphorylated Ser-15 (15S), Ser-85 (85S) in HSP27 or HSP27. The histogram shows quantitative representations of the phosphorylation level obtained from laser densitometric analysis (open bar or closed bar for the phosphorylation of HSP27 at Ser-15 or Ser-85, respectively). Each value represents the mean ± SD of triplicate determinations. Similar results were obtained in two other cell preparations. $*P$ (15S) or $**P$ (85S) $<0.05$ compared with the value of thrombin alone.

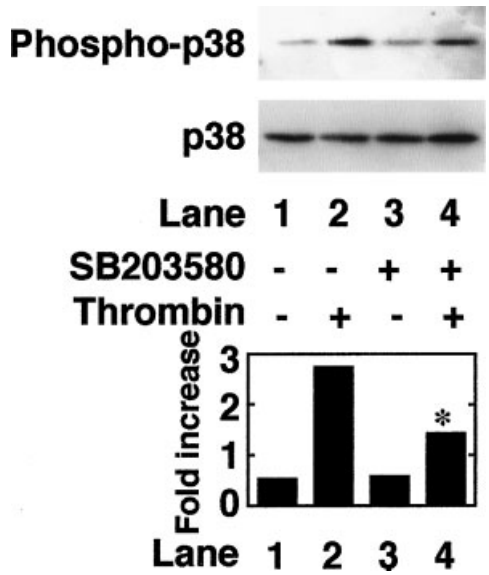


**Fig. 7.** Effect of SB203580 on the thrombin-induced phosphorylation of p38 MAP kinase in cardiac myocytes. The cultured cells were pretreated with 30 μM SB203580 or vehicle for 60 min. The cells were then stimulated by 0.3 U/ml thrombin or vehicle for 10 min. The extracts of cells were subjected to SDS–PAGE using antibodies against phospho-specific p38 MAP kinase or p38 MAP kinase. The histogram shows quantitative representations of the phosphorylation level obtained from laser densitometric analysis. Each value represents the mean ± SD of triplicate determinations. Similar results were obtained in two other cell preparations. $*P < 0.05$ compared with the value of thrombin alone.

cing extracellular signaling into a cellular response [Widmann et al., 1999; Chang and Karin, 2001]. Three major MAP kinases, p44/p42 MAP kinase, p38 MAP kinase, and SAPK/JNK, are generally recognized as being the central elements used by mammalian cells to transduce such diverse messages [Widmann et al., 1999; Chang and Karin, 2001]. In the present study, we demonstrated that thrombin induced phosphorylation of both p44/p42 MAP kinase and p38 MAP kinase, while having little effect on the phosphorylation of SAPK/JNK in cardiac myocytes. It is well known that MAP kinases are activated by phosphorylation on tyrosine and threonine by dual-specificity MAP kinase kinase [Raingeaud et al., 1995]. Thus, our findings suggest that thrombin activates both p44/p42 MAP kinase and p38 MAP kinase, but not SAPK/JNK in mouse cardiac myocytes. In addition, the thrombin-induced phosphorylation of p38 MAP kinase, not p44/42 MAP kinase, was reduced by midazolam. It is well recognized that the specificity of the cellular response to a given stimulus determines by the activation of a particular MAP kinase pathway [Widmann et al., 1999; Chang and Karin, 2001]. These findings prompted us to speculate that midazolam suppresses the thrombin-stimulated HSP27 phosphorylation through the attenuation of p38 MAP kinase activity in ventricular myocytes. We next investigated whether p38 MAP kinase is involved in the pathway by which thrombin stimulates the phosphorylation of HSP27 in these cells. We showed that the thrombin-induced HSP27 phosphorylations at two sites were markedly suppressed by SB203580, a specific p38 MAP kinase inhibitor [Cuenda et al., 1995]. We found that SB203580 truly did suppress the phosphorylation of p38 MAP kinase induced by thrombin. Thus, it is possible that p38 MAP kinase is involved in thrombin-stimulated HSP27 phosphorylation in these myocytes. In addition, we showed that PD169316, an another p38 MAP kinase inhibitor [Kummer et al., 1997], also suppressed the thrombin-induced HSP27 phosphorylations at two sites and that PD169316 actually did suppress the phosphorylation of p38 MAP kinase induced by thrombin. Based on these findings, our results suggest that thrombin stimulates HSP27 phosphorylation at two sites (Ser-15 and Ser-85) via p38 MAP kinase in ventricular myocytes. Taken together, it is most likely that midazolam inhibits thrombin-activated p38 MAP kinase, resulting in suppression of HSP27 phosphorylation in these cells consistent with our premise. The time course of the Ser-15 and Ser-85 phosphorylation of HSP27 induced by thrombin was different. Although, p38 MAP kinase mediates at least in part the thrombin-induced HSP27 phosphorylation on both Ser-15 and Ser-85, it is possible that each phosphorylation is differently regulated by additionally unknown mechanism. We previously reported that midazolam stimulates vascular endothelial growth factor release from aortic smooth muscle cells through the activation of p44/p42 MAP kinase and SAPK/JNK [Tanabe et al., 2003]. It is probable that the contrasting effects of midazolam on MAP kinases are due to the difference of species (rat or mouse) or cells (vascular smooth muscle cells or cardiac myocytes).

It is recognized that some situations or agents modulate the onset of irreversible cardiac myocyte injury in experimental model systems [Chen et al., 2002; Zaugg et al., 2003a,b]. The

phenomenon is termed "preconditioning." Trials of the effect of ischemic preconditioning against the human heart are accepted but yet conflicted [Zaugg et al., 2003b]. It has been shown hypoxia induces HSP27 phosphorylation in cardiac myocytes [Kacimi et al., 2000]. HSPs are well known to function as chaperone proteins [Benjamin and McMillan, 1998]. It has been reported that the increased expression of HSP27 provides cardioprotection against hypoxic injury in cultured cardiac myocytes [Martin et al., 1997]. It is suggested that the brief episodes of sublethal cardiac ischemia protect myocytes against subsequent prolonged ischemia through the phosphorylation of HSP27. It is generally recognized that HSP27 is present at two forms, an aggregated form and a dissociated form, in unstressed cells [Kato et al., 1994]. We have previously demonstrated that HSP27 is dissociated concomitantly with the phosphorylation of the aggregated form of HSP27 and the dephosphorylation of the dissociated HSP27 causes aggregation [Kato et al., 1994]. It has been reported that the dissociation of HSP27 from aggregated form decreases chaperoning function of HSP27 [Benjamin and McMillan, 1998]. Additionally, we have reported that conversion of the non-phosphorylated, aggregated form to the phosphorylated, dissociated form of HSP27 results in decreased tolerance to heat stress [Kato et al., 1994]. In the present study, midazolam suppressed the thrombin-induced HSP27 phosphorylation in cardiac myocytes. Taking these findings into account, it is probable that midazolam protects cardiac myocyte function through the suppression of the HSP27 phosphorylation. However, the effect of midazolam in the present study was observed at concentrations higher than those used clinically. On the other hand, it has been shown that an increased p38 MAP kinase activity is important for conferring beneficial effects of preconditioning in not only heart but also brain, liver, and intestine [Schulz et al., 2002; Teoh et al., 2002; Fu et al., 2003; Zheng and Zuo, 2004]. So, clinical relevance of the midazolam-effect on HSP27 phosphorylation which we found in this study is still unclear and remains to be clarified. Further investigations regarding about the HSP27 phosphorylation and the mechanism in cardiac myocytes will be required.

In conclusion, these results strongly suggest that thrombin induces the HSP27 phosphorylation at least through the p38 MAP kinase activation in cardiac myocytes and that midazolam inhibits the thrombin-induced HSP27 phosphorylation via suppression of p38 MAP kinase activation.

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