Journal of Cellular Biochemistry 94:573–584 (2005)

Adenylyl Cyclase-cAMP System Inhibits Thrombin-Induced HSP27 in Vascular Smooth Muscle Cells

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Abstract We previously reported that thrombin stimulates the induction of heat shock protein (HSP) 27 via p38 mitogen-activated protein (MAP) kinase activation in aortic smooth muscle A10 cells. In the present study, we investigated the effect of the adenylyl cyclase-cAMP system on the thrombin-stimulated induction of HSP27 in A10 cells. Forskolin, a direct activator of adenylyl cyclase, reduced the thrombin-induced p38 MAP kinase phosphorylation, and significantly suppressed the thrombin-stimulated accumulation of HSP27. However, dideoxyforskolin, a forskolin derivative that does not activate cAMP, failed to suppress the HSP27 accumulation. Furthermore, dibutyryl-cAMP (DBcAMP), a permeable analog of cAMP, significantly suppressed the accumulation of HSP27. On the other hand, calphostin C, an inhibitor of protein kinase C (PKC), reduced the thrombin-induced p38 MAP kinase phosphorylation, and significantly suppressed the thrombin-stimulated accumulation of HSP27. Moreover, forskolin reduced the p38 MAP kinase phosphorylation induced by the 12-*O*-tetradecanoylphorbol-13-acetate (TPA), a PKC-activating phorbol ester, and significantly suppressed the TPA-stimulated accumulation of HSP27. These results indicate that adenylyl cyclase-cAMP system has an inhibitory role in thrombin-stimulated HSP27 induction in aortic smooth muscle cells, and the effect seems to be exerted on the thrombin-induced PKC- p38 MAP kinase signaling pathway. J. Cell. Biochem. 94: 573–584, 2005.

Key words: adenylyl cyclase-cAMP system; heat shock protein 27; protein kinase C; p38 MAP kinase; thrombin

Various stressors such as heat stress and chemical stress stimulate the induction of heat shock proteins (HSPs) in mammalian cells [Nover, 1991; Nover and Scharf, 1991]. HSPs are generally divided into two groups, high-molecular-weight HSPs and low-molecular-weight HSPs according to their apparent molecular sizes. High-molecular-weight HSPs including HSP90 and HSP70 are well known to act as molecular chaperones in protein folding, oligomerization, and translocation [Ellis and van der Vies, 1991; Benjamin and McMillan, 1998]. HSP27 belongs to low-molecular-weight HSPs. It is speculated that HSP27 may act as a chaperone like high-molecular-weight HSPs [Cooper and Uoshima, 1994]. HSP27 is known to be constitutively expressed in various tissues and cells, especially in skeletal muscle cells and smooth muscle cells [Inaguma et al., 1993]. In addition, it has been shown that HSP27 is related with cellular dynamics such as modulation of actin filament and stability, growth, and secretion in several types of cells [Zhu et al., 1994; Landry and Huot, 1995; Kindas-Mugge et al., 1996]. These findings lead us to speculate that HSP27 may have crucial roles in various

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Received 7 December 2003; Accepted 20 July 2004
DOI 10.1002/jcb.20309

cellular functions. In addition, it is well known that HSP27 exists in two forms, an aggregated form and a dissociated form that has apparent molecular masses of ~500 and <70 KDa, respectively [Kato et al., 1994]. In vascular smooth muscle cells, it has been reported that HSP27 protects cell necrosis but not apoptosis and that HSP27 is involved in angiotensin II-induced vasoconstriction via p38 mitogen-activated protein (MAP) kinase [Champagne et al., 1999; Meloche et al., 2000]. However, the detailed mechanism behind HSP27 induction and the roles remain to be clarified.

Thrombin is a serine protease that is generated in circulating plasma by the cleavage of prothrombin [Mann et al., 1990]. It is generally recognized that thrombin has multiple functions implicated in not only thrombosis and homeostasis but also cell proliferation and differentiation [Mann et al., 1990; Davie et al., 1991]. The effects of thrombin on various cells such as vascular smooth muscle cells and platelets are mediated via specific receptors [Vu et al., 1991; Zhong et al., 1992]. Thrombin reportedly stimulates vasoconstriction, cell migration, and proliferation in vascular smooth muscle cells [Chen and Buchanan, 1975; McNamara et al., 1993]. As for the intracellular signaling of thrombin in these cells, it has been reported that thrombin induces the activation of protein kinase C (PKC) and the MAP kinase superfamily [Nakajima et al., 1994; Dery et al., 1998]. We have previously reported that thrombin stimulates both the dissociation and the induction of HSP27 via p38 MAP kinase activation in aortic smooth muscle cell line, A10 cells [Hirade et al., 2002].

cAMP is produced from ATP by adenylyl cyclase and then causes cAMP-dependent protein kinase activation [Morgan et al., 1991]. The adenylyl cyclase-cAMP system plays an important role in various cell functions such as differentiation and proliferation [Defer et al., 2000]. It is well recognized that the adenylyl cyclase-cAMP system has vasodilation effect through activation of cAMP-dependent protein kinase on vascular smooth muscle cells [Morgan et al., 1991]. In vascular smooth muscle cells, it has been reported as follows: cAMP inhibits thrombin-induced cell growth [Rao and Runge, 1996], and plasminogen activator inhibitor-1 production [Shen et al., 1998], thrombin induces interleukin-6 expression through the cAMP response element [Tokunou et al., 2001a], and cAMP response element-binding protein mediates thrombin-induced cell proliferation [Tokunou et al., 2001b]. In the present study, we investigated the effect of the adenylyl cyclase-cAMP system on the induction of HSP27 stimulated by thrombin in an aortic smooth muscle cell line, A10 cells. We here show that adenylyl cyclase-cAMP system has an inhibitory role in thrombin-stimulated HSP27 induction in aortic smooth muscle cells, and the effect seems to be exerted on the thrombin-induced PKC-p38 MAP kinase signaling pathway.

MATERIALS AND METHODS

Materials

Thrombin, forskolin, dideoxyforskolin, dibutyryl-cAMP (DBcAMP), SQ22536, staurosporine, and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) were purchased from Sigma Chemical Co. (St. Louis, MO). Calphostin C was obtained from Biomol (Plymouth Meeting, PA). Phospho-specific p38 MAP kinase antibodies and p38 MAP kinase antibodies were purchased from New England BioLabs, Inc. (Beverly, MA). The phospho-PKC antibodies sampler kit was obtained from Cell Signaling (Beverly, MA). Anti-HSP27 affinity-purified polyclonal antibody was generated as previously described [Inaguma et al., 1993]. An ECL Western blotting detection system and *myo*-[^{3}H]inositol (81.5 Ci/mmol) were obtained from Amersham Japan (Tokyo, Japan). Other materials and chemicals were obtained from commercial sources. Forskolin and dideoxyforskolin were dissolved in ethanol. TPA, staurosporine, calphostin C, and SQ22536 were dissolved in dimethyl sulfoxide (DMSO). The maximum concentration of ethanol or DMSO were 0.1%, which did not affect the immunoassay of HSP27 or Western blot analysis.

Cell Culture

An aortic smooth muscle cell line, A10 cells, derived originally from thoracic aorta of embryonic rats [Kimes and Brandt, 1976], were obtained from the American Type Culture Collection (Rockville, MD). The cells were seeded into 35-mm (1×10^5) or 90-mm (5×10^5) diameter dishes and maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (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 pre-treated with forskolin, dideoxyforskolin, DBcAMP, staurosporine, or calphostin C for 20 min prior to the stimulation of thrombin or TPA. Cells were pre-treated with SQ22536 for 60 min.

Immunoassay of HSP27

Cultured cells were stimulated by thrombin or TPA in serum-free DMEM for 48 h. The cells were washed twice with phosphate-buffered saline (PBS) and then frozen at −80°C for a few days before analysis. The frozen cells in each dish were collected and suspended in 0.3 ml of PBS, and 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. The concentration of HSP27 in soluble extracts of the cells was determined by sandwich-type enzyme immunoassays, as described previously [Kato et al., 1996]. 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 mmol/L sodium phosphate buffer, pH 7.0, containing 0.3 mol/L NaCl, 0.5% hydrolyzed gelatin, 0.1% bovine serum albumin (BSA), 1 mmol/L $MgCl_2$, and 0.1% NaN_3. After washing, each ball was incubated at 4°C overnight with 1.5 mU of galactosidase-labeled antibodies in a volume of 0.2 ml with 10 mmol/L sodium phosphate buffer, pH 7.0, containing 0.1 mol/L NaCl, 1 mmol/L $MgCl_2$, 0.1% BSA, and 0.1% NaN_3. The galactosidase activity bound to the ball was assayed using a fluorogenic substrate, 4-methylumbelliferyl-β-D-galactoside.

Semiquantitative Analysis of mRNA Expression

Cultured cells were stimulated by thrombin or TPA in serum-free DMEM for 6 h. Total RNA was isolated from cells using Isogen (Nippon Gene, Tokyo, Japan) after which 1-μg sample was reverse-transcribed using M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. Using 4% of the reverse-transcribed mix, cDNA fragments of test genes were amplified within the linear range by PCR using Expand High Fidelity PCR System (Roche Diagnostics, Mannheim, Germany) according to manufacturer's instructions. The specific primers were synthesized according to motifs: ATGACCGAGCGCCGCGTGCCCTTCTCG (HSP27, sense), CTACTTGGCTCCAGACTGTTCCGAC (HSP27, antisense), TGAACCCTCAGTGGAATGAGT (PKCα, sense), GGCTGCTTCCTGTCTTCTGAA (PKCα, antisense), CACCATCTTCCAGAAAGAACG (PKCδ, sense), CTTGCCATAGGTCCCGTTGTTG (PKCδ, antisense), TTCATTGACCTCAACTACATG (glyceraldehyde-3-phosphate dehydrogenase (GAPDH), sense), and GTGGCAGTGATGGCATGGAC (GAPDH, antisense). PCR amplification of HSP27 cDNA for 28 cycles was 94°C denaturation (60 s), 58°C annealing (60 s), and 72°C extension (60 s). PCR amplifications of PKCα and PKCδ cDNAs for 30 cycles were 94°C denaturation (60 s), 57°C annealing (60 s), and 72°C extension (60 s). PCR amplification of GAPDH cDNA for 20 cycles was 94°C denaturation (60 s), 60°C annealing (60 s), and 72°C extension (60 s). Following these cycles of PCR amplifications, the amplified cDNAs were further extended by additional incubation at 72°C for 10 min. The equal amount of each reaction was fractionated on 1% agarose gel in 1 × TAE buffer, then the agarose gel was soaked in 1 × TAE buffer containing ethidium bromide for 15 min with gentle agitation. The amplified cDNA fragments in the agarose gel were then visualized on UV trasnilluminator and photographed.

Analysis of p38 MAP kinase, HSP27, and PKCs by Western Blotting

Cultured cells were stimulated by thrombin or TPA in serum-free DMEM for 10 min. The cells were washed twice with PBS and then lysed, homogenized, and sonicated in a lysis buffer containing 62.5 mmol/L Tris/Cl, pH 6.8, 2% sodium dodecyl sulfate (SDS), 50 mmol/L 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 electrophoresis (PAGE) was performed by the method of Laemmli in 10% polyacrylamide gel [Laemmli, 1970]. Western blot analysis was performed as described previously [Inaguma et al., 1993] by using antibodies against phospho-specific p38 MAP

kinase, p38 MAP kinase, HSP27, phospho-specific PKCα/βII (Thr638/641), phospho-specific PKCδ (Thr505), phospho-specific PKCδ (Ser643), phospho-specific PKCθ (Thr538), phospho-specific PKCμ(Ser744/748), or phospho-specific PKCμ (Ser916), 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.

Measurement of the Formation of Inositol Phosohates

Cultured cells were labeled with *myo*-[^{3}H]inositol (2 μCi/dish) for 48 h in inositol-free DMEM. The labeled cells were pre-incubated with 10 mmol/L LiCl for 10 min at 37°C in 1 ml of the assay buffer (5 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4, 150 mmol/L NaCl, 5 mmol/L KCl, 0.9 mmol/L $MgSO_4$, 1 mmol/L $CaCl_2$, 5.5 mmol/L glucose) containing 0.01% BSA. The cells were stimulated by thrombin at 37°C for 30 min. The reaction was terminated by addition of 1 ml of 30% trichloroacetic acid. The acidic supernatant was applied to an anion exchange column containing 1 ml of Dowex AG1-X8 (100–200 mesh, formate form; Bio-Rad, Hercules, CA). The radioactive inositol phosphates were eluted with 8 ml of 0.1 mol/L formic acid containing 1 mol/L ammonium formate [Berridge et al., 1983, 1984]. The radioactivity of ^{3}H samples was determined using a Beckman LS6500IC liquid scintillation spectrometer.

Other Methods

Protein concentrations in soluble extracts were determined using a protein assay kit (Bio-Rad) with BSA as the standard protein. Rat HSP27, which was used as the standard for the immunoassay, was purified from skeletal muscle [Inaguma et al., 1993]. The densitometric analysis was performed using Molecular Analyst/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 Forskolin, Dideoxyforskolin, or DBcAMP on Thrombin-Stimulated Accumulation of HSP27 in A10 Cells

To clarify the role of the adenylyl cyclase-cAMP system in thrombin-stimulated HSP27 induction in aortic smooth muscle A10 cells, we examined the effects of each of the direct activator of the adenylyl cyclase-cAMP system on HSP27 accumulation stimulated by thrombin. We have shown that the level of HSP27 reaches a maximum at 48 h after thrombin stimulation in A10 cells [Hirade et al., 2002]. Forskolin, a direct activator of adenylyl cyclase [Seamon and Daly, 1981], which by itself did not affect the basal level of HSP27 (data not shown), significantly suppressed thrombin-stimulated HSP27 accumulation (Fig. 1). The inhibitory effect of forskolin on HSP27 accumulation induced by thrombin was dose dependent in the range between 1 and 50 μmol/L. The maximum effect of forskolin was observed at 50 μmol/L and it caused about a 90% reduction in the effect of thrombin. However, dideoxyforskolin (30 μmol/L),

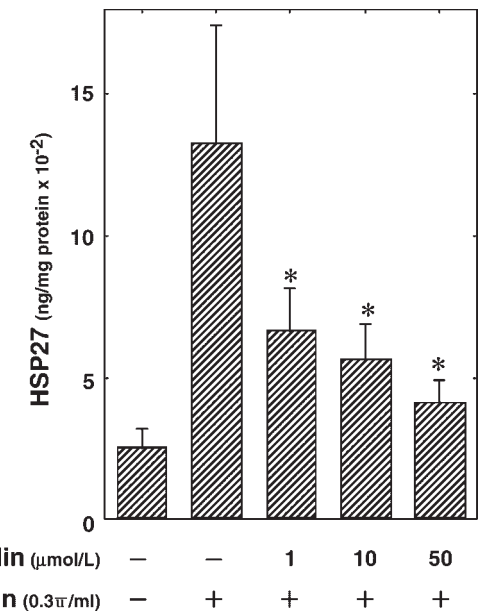

Fig. 1. Effect of forskolin on the thrombin-stimulated accumulation of HSP27 in A10 cells. Cultured cells were pre-treated with or without various doses of forskolin for 20 min, and then stimulated by 0.3 U/ml thrombin for 48 h. Equal amount of ethanol and PBS for the solvent of forskolin and thrombin, respectively, was added to any culture. Each value represents the means ± SD of triplicate determinations from three independent experiments. *$P < 0.05$ compared with the value in thrombin alone.

a forskolin derivative that does not activate cAMP [Seamon et al., 1984], failed to reduce HSP27 accumulation induced by thrombin while forskolin (30 μmol/L) significantly suppressed the accumulation of HSP27 (Table I). In addition, DBcAMP (0.3 mmol/L), a permeable analog of cAMP [Rasmussen et al., 1968], which alone had little effect on the basal level of HSP27, significantly reduced HSP27 accumulation stimulated by thrombin (data not shown).

Effect of DBcAMP on the Thrombin-Increased mRNA Level for HSP27 in A10 Cells

We previously reported that the expression level of mRNA for HSP27 increased 6 h after stimulation of thrombin in aortic smooth muscle A10 cells [Hirade et al., 2002]. We next examined the effect of DBcAMP on the thrombin-increased level of mRNA for HSP27. DBcAMP (1 mmol/L), which alone had little effect on the basal level of HSP27 mRNA, markedly suppressed the thrombin-increased level of mRNA for HSP27 (Fig. 2). In contrast, the levels of PKCα, and PKCδ mRNA were downregulated by thrombin- or DBcAMP-treatment (Fig. 2). Therefore, it is likely that the level of HSP27 mRNA expression is not in parallel with that of PKC mRNA expression in A10 cells.

Effect of Forskolin on Thrombin-Induced Phosphorylation of p38 MAP Kinase in A10 Cells

We have previously reported that thrombin stimulates the induction of HSP27 via p38 MAP kinase activation in A10 cells [Hirade et al., 2002]. To investigate whether the adenylyl cyclase-cAMP system affects thrombin-induced p38 MAP kinase activation in these cells, we examined the effect of forskolin on thrombin-induced phosphorylation of p38 MAP kinase. Forskolin (30 μmol/L) markedly suppressed the thrombin-stimulated phosphorylaryion of p38 MAP kinase (Fig. 3).

TABLE I. Effects of Forskolin or Dideoxyforskolin on the Thrombin-Stimulated Accumulation of HSP27 in A10 Cells

	HSP27 (ng/mg protein)
Control	267.5 ± 126.4
Thrombin	1,338.8 ± 331.1
Forskolin	204.5 ± 10.5
Forskolin + thrombin	293.0 ± 22.3[a]
Dideoxyforskolin	231.2 ± 42.6
Dideoxyforskolin + thrombin	1,289.2 ± 67.2

Cultured cells were pre-treated with 30 μmol/L forskolin, 30 μmol/L dideoxyforskolin or vehicle for 20 min, and then stimulated by 0.3 U/ml thrombin or vehicle for 48 h. Each value represents the means ± SD of triplicate determinations from three independent experiments.
[a]$P<0.05$ compared with the value in thrombin alone.

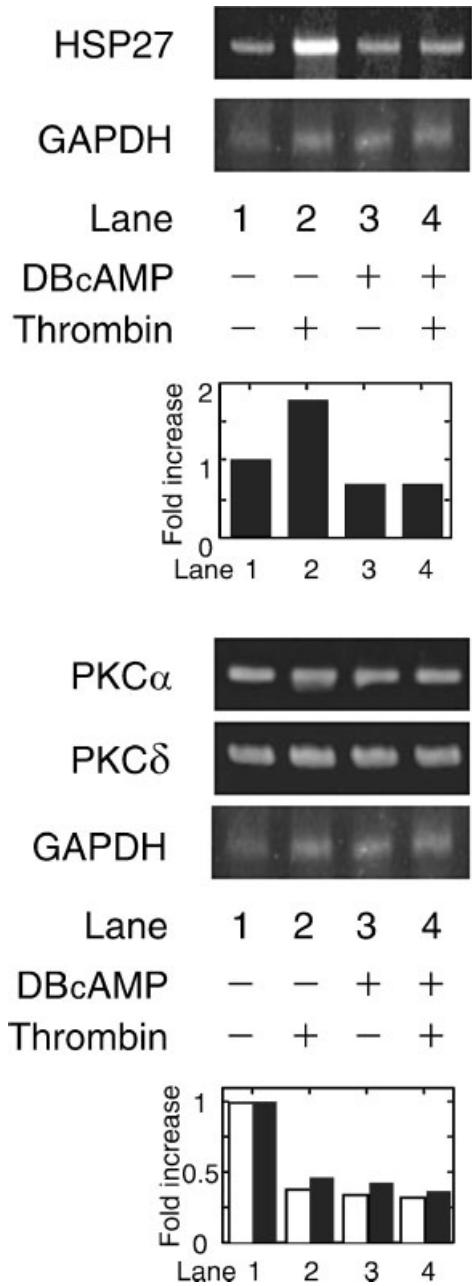

Fig. 2. Effect of DBcAMP on the thrombin-increased mRNA level for HSP27 in A10 cells. Cultured cells were pre-treated with 1 mmol/L DBcAMP or vehicle for 20 min, and then stimulated by 0.3 U/ml thrombin for 6 h. After which, expressions of mRNA encoding HSP27, PKCα, PKCδ, and GAPDH were examined by semiquantitative reverse transcription-PCR (RT-PCR). The histograms show quantitative representations of mRNA of HSP27 (**upper panel**) or PKCs (**lower panel**) obtained from laser densitometric analyses. In lower panel, open bars and closed bars mean levels of PKCα and PKCδ mRNA, respectively.

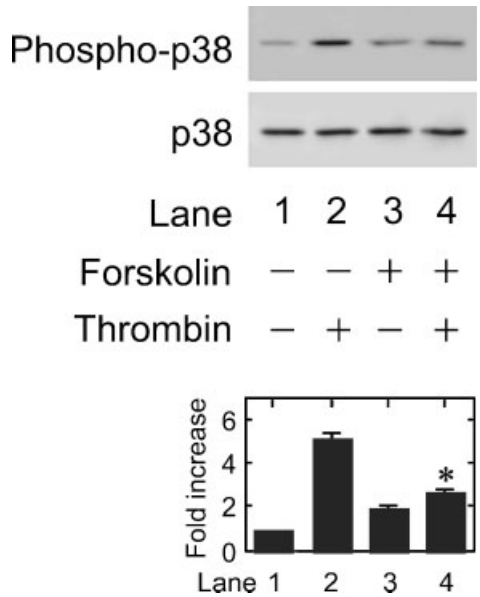

Fig. 3. Effect of forskolin on the thrombin-induced phosphorylation of p38 MAP kinase in A10 cells. Cultured cells were pretreated with 30 μmol/L forskolin or vehicle for 20 min, and then stimulated by 0.1 U/ml thrombin or vehicle for 10 min. The extracts of cells were subjected to SDS–PAGE with subsequent Western blot analysis using antibodies against phospho-specific p38 MAP kinase or p38 MAP kinase. The histogram shows quantitative representations of the phosphorylation level of p38 MAP kinase obtained from laser densitometric analysis. Each value represents the means ± SD of triplicate determinations. Similar results were obtained in two other cell preparations. *$P < 0.05$ compared with the value in thrombin alone.

Effect of Staurosporine on the Thrombin-Stimulated Accumulation of HSP27 in A10 Cells

It has also been reported that thrombin activates PKC in vascular smooth muscle cells [Dery et al., 1998]. Phosphoinositide (PI) hydrolysis by phospholipase C is well known to lead to the formation of inositol 1,4,5-trisphosphate and diacylglycerol, which mobilizes Ca^{2+} and activates PKC, respectively [Nishizuka, 1992; Berridge, 1993]. We found that thrombin-induced the formation of inositol phosphates in aortic smooth muscle A10 cells (data not shown). To investigate whether PKC is involved in thrombin-stimulated HSP27 induction in A10 cells, we examined the effect of staurosporine, a PKC inhibitor [Tamaoki et al., 1986], on HSP27 accumulation. Staurosporine (3 nmol/L), which alone had little effect on the basal level of HSP27, significantly reduced HSP27 accumulation stimulated by thrombin (Table II). Thus, we suggested that PKC activation is involved in thrombin-induced HSP27 accumulation in A10 cells.

TABLE II. Effect of Staurosporine on the Thrombin-Induced Accumulation of HSP27 in A10 Cells

	HSP27 (ng/mg protein)
Control	270.9 ± 45.2
Thrombin	1,342.2 ± 167.3
Staurosporine	238.4 ± 51.3
Staurosporine + thrombin	373.6 ± 58.8[a]

Cultured cells were pre-treated with 3 nmol/L staurosporine or vehicle for 20 min, and then stimulated by 0.3 U/ml thrombin or vehicle for 48 h. Each value represents the means ± SD of triplicate determinations from three independent experiments. [a]$P < 0.05$ compared with the value in thrombin alone.

Effect of Calphostin C on Thrombin-Induced Phosphorylation of p38 MAP Kinase and HSP27 Accumulation in A10 Cells

To clarify whether PKC acts at a point upstream of p38 MAP kinase in thrombin-induced HSP27 in A10 cells, we examined the effect of calphostin C, a PKC inhibitor [Kobayashi et al., 1989], on p38 MAP kinase phosphorylation. Calphostin C (0.7 μmol/L) markedly reduced the thrombin-induced phosphorylation of p38 MAP kinase (Fig. 4A). In addition, calphostin C significantly suppressed thrombin-induced HSP27 accumulation (Fig. 4B).

Effect of Forskolin on TPA-Stimulated Accumulation of HSP27 in A10 Cells

We previously reported that TPA, a PKC direct-activating phorbol ester [Nishizuka, 1986], induces accumulation of HSP27 in aortic smooth muscle A10 cells [Kaida et al., 1999]. Forskolin, which by itself did not affect the basal level of HSP27 (data not shown), significantly reduced TPA-stimulated HSP27 accumulation (Fig. 5). The inhibitory effect of forskolin on HSP27 accumulation induced by TPA was dose dependent in the range between 1 and 50 μmol/L. The maximum effect of forskolin was observed at 50 μmol/L, which caused about a 90% reduction in the effect of TPA.

Effect of DBcAMP on the TPA-Increased mRNA Level for HSP27 in A10 Cells

We next examined the effect of DBcAMP on the TPA-increased level of mRNA for HSP27. DBcAMP (1 mmol/L), which alone had little effect on the basal level of HSP27 mRNA, markedly suppressed the TPA-increased level of

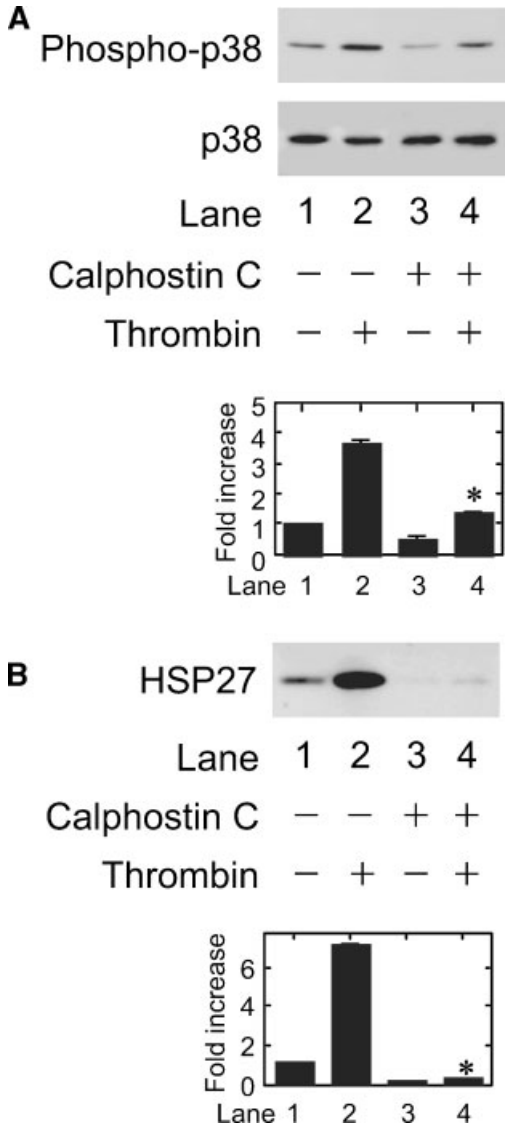

Fig. 4. Effect of calphostin C on the thrombin-induced phosphorylation of p38 MAP kinase (**A**) and HSP27 accumulation (**B**) in A10 cells. Cultured cells were pre-treated with 0.7 μmol/L calphostin C or vehicle for 20 min, and then stimulated by 0.1 U/ml thrombin or vehicle for 10 min (A). Cultured cells were pre-treated with 0.7 μmol/L calphostin C or vehicle for 20 min, and then stimulated by 0.3 U/ml thrombin or vehicle for 48 h (B). The extracts of cells were subjected to SDS–PAGE with subsequent Western blot analysis using antibodies against phospho-specific p38 MAP kinase or p38 MAP kinase (A) or those against HSP27 (B). The histograms show quantitative representations of the phosphorylation level of p38 MAP kinase or the basal level of HSP27 obtained from laser densitometric analyses. Each value represents the means ± SD of triplicate determinations. Similar results were obtained in two other cell preparations. $*P < 0.05$ compared with the value in thrombin alone.

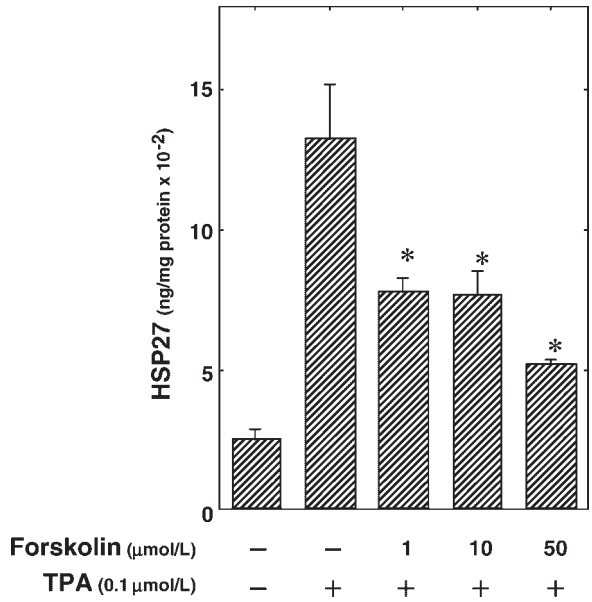

Fig. 5. Effect of forskolin on the TPA-stimulated accumulation of HSP27 in A10 cells. Cells were cultured with or without various doses of forskolin for 20 min, and then stimulated by 0.1 μmol/L TPA for 48 h. Equal amount of ethanol and DMSO for the solvent of forskolin and TPA, respectively, was added to any culture. Each value represents the means ± SD of triplicate determinations from three independent experiments. $*P < 0.05$ compared with the value in TPA alone.

mRNA for HSP27 (Fig. 6). In contrast, the levels of PKCα and PKCδ mRNA were downregulated by TPA- or DBcAMP-treatment (Fig. 6). Therefore, it is likely that the level of HSP27 mRNA expression is not in parallel with that of PKC mRNA expression in A10 cells.

Effect of Forskolin on TPA-Induced Phosphorylation of p38 MAP Kinase in A10 Cells

In addition, we investigated whether the adenylyl cyclase-cAMP system affects p38 MAP kinase activation at a point upstream or downstream from PKC in A10 cells. Forskolin (30 μmol/L) significantly suppressed the TPA-induced p38 MAP kinase phosphorylation (Fig. 7).

Effects of Forskolin or DBcAMP on TPA-Induced Phosphorylation of PKCs in A10 Cells

To clarify whether the adenylyl cyclase-cAMP system directly affects PKC activity in A10 cells, the phosphorylation status of PKC isoenzyme in forskolin- or DBcAMP-treated A10 cells was analyzed by immunoblot analysis using anti-phospho-PKC antibodies (Fig. 8). PKC activity is controlled by three distinct phosphorylation events (specifically, the threonine 500 in the activation loop, the threonine 641 autophosphorylation site, and the serine 660 hydrophobic site at the carboxy terminus of PKCβII are

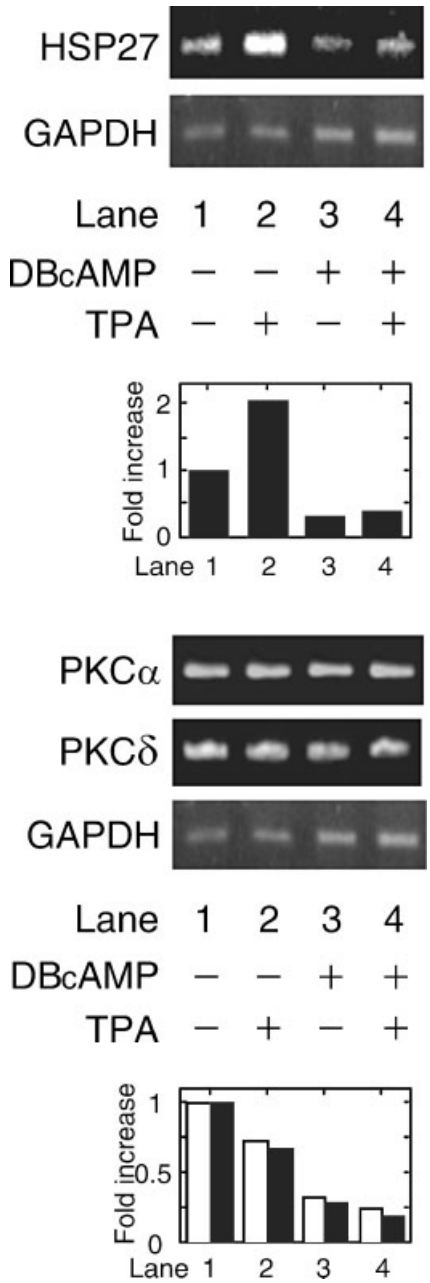

Fig. 6. Effect of DBcAMP on the TPA-increased mRNA level for HSP27 in A10 cells. Cultured cells were pre-treated with 1 mmol/L DBcAMP or vehicle for 20 min, and then stimulated by 0.1 μmol/L TPA for 6 h. After which, expressions of mRNA encoding HSP27, PKCα, PKCδ, and GAPDH were examined by semiquantitative RT-PCR. The histograms show quantitative representations of mRNA of HSP27 (upper **panel**) or PKCs (lower **panel**) obtained from laser densitometric analyses. In lower panel, open bars and closed bars mean levels of PKCα and PKCδ mRNA, respectively.

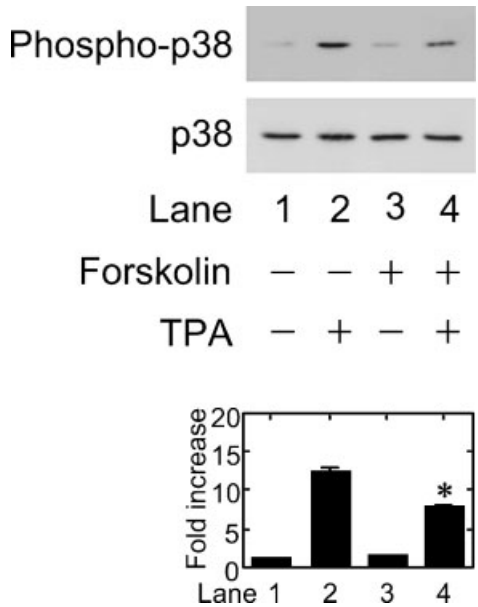

Fig. 7. Effect of forskolin on the TPA-induced phosphorylation of p38 MAP kinase in A10 cells. Cultured cells were pre-treated with 10 μmol/L forskolin or vehicle for 20 min, and then stimulated by 0.1 μmol/L TPA or vehicle for 10 min. The extracts of cells were subjected to SDS–PAGE with subsequent Western blot analysis using antibodies against phospho-specific p38 MAP kinase or p38 MAP kinase. The histogram shows quantitative representations of the phosphorylation level of p38 MAP kinase obtained from laser densitometric analysis. Each value represents the means ± SD of triplicate determinations. Similar results were obtained in two other cell preparations. $*P < 0.05$ compared with the value in TPA alone.

phosphorylated in vivo) [Keranen et al., 1995]. The conservation of each of these residues in PKCs emphasizes the essential roles they play in regulating the PKC family [Keranen et al., 1995]. To examine the phosphorylation status of these residues (Thr638/641 in PKCα/βII; Thr505 and Ser643 in PKCδ; Thr538 in PKCθ; and Ser744/748 and Ser916 in PKCμ), immunoblot analysis was performed using anti-phospho-PKC antibodies. Phosphorylation of PKCα/βII (Thr638/641) and PKCθ (Thr538) were not influenced by either forskolin- or DBcAMP-treatment (data not shown). In contrast, TPA-induced phosphorylation of PKCμ (Ser744/748 and Ser916) and PKCδ (Ser643 and Thr505) was suppressed by either forskolin- or DBcAMP-treatment (Fig. 8). These results suggest that the cAMP system directly inhibits some PKC-isoenzyme activities.

Effect of SQ22536, an Adenylyl Cyclase Inhibitor, on Inhibitory Activity of Forskorin on Thrombin-Induced HSP27 Accumulation in A10 Cells

We examined whether a specific inhibitor of adenylyl cyclase suppresses forskolin-induced

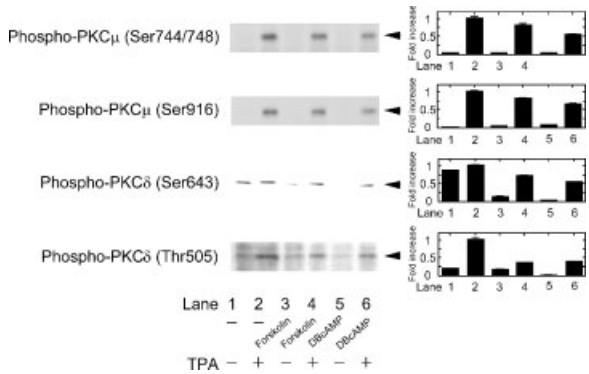

Fig. 8. Effects of forskolin or DBcAMP on the TPA-induced phosphorylation of PKCs in A10 cells. Cultured cells were pretreated with 30 μmol/L forskolin, 1 mmol/L DBcAMP or vehicle for 20 min, and then stimulated by 0.1 μmol/L TPA or vehicle for 30 min. The extracts of cells were subjected to SDS–PAGE with subsequent Western blot analysis using anti-phospho-PKC antibodies. Arrowheads indicate the phospho-PKCs bands. The histograms show quantitative representations of the phosphorylation level of PKCs obtained from laser densitometric analysis. Similar results were obtained in two other cell preparations.

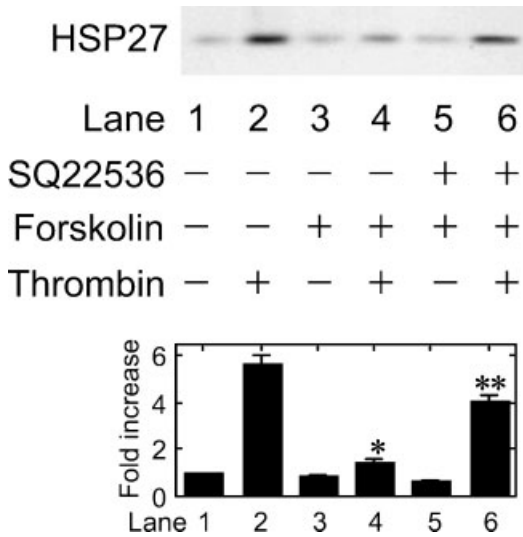

Fig. 9. Effect of SQ22536, an adenylyl cyclase inhibitor, on inhibitory activity of forskolin on the thrombin-induced HSP27 accumulation in A10 cells. Firstly, cells were treated with SQ22536 (10 μmol/L), or vehicle for 60 min. Then, cells were treated with 30 μmol/L forskolin or vehicle for 20 min, and then stimulated by thrombin (0.3 U/ml) or vehicle for 48 h. The extracts of cells were subjected to SDS–PAGE with subsequent Western blot analysis using anti-HSP27 affinity-purified polyclonal antibodies. The histogram shows quantitative representations of the basal level of HSP27 obtained from laser densitometric analysis. Similar results were obtained in two other cell preparations. $*P < 0.05$ compared with the value in thrombin alone, $**P < 0.05$ compared with the value in thrombin with forskolin pre-treatment.

inhibition of thrombin-induced HSP27 accumulation in A10 cells. As shown in Figure 9, SQ22536 (10 μmol/L), a specific inhibitor of adenylyl cyclase [Goldsmith and Abrams, 1991], indeed reversed forskolin-induced inhibition of thrombin-stimulated HSP27 accumulation. These results justify our statement that the adenylyl cyclase-cAMP system inhibits thrombin-induced HSP27 in vascular smooth muscle cells.

DISCUSSION

We previously reported that thrombin stimulates HSP27 induction in vascular smooth muscle A10 cells [Hirade et al., 2002]. It is well known that forskolin, a direct activator of adenylyl cyclase [Seamon and Daly, 1981], induces cAMP production. In the present study, we showed that forskolin reduced thrombin-stimulated HSP27 accumulation, and that dideoxyforskolin, a forskolin derivative that does not activate cAMP [Seamon et al., 1984], did not affect HSP27 accumulation induced by thrombin. Thus, our findings suggest that forskolin suppresses HSP27 induction stimulated by thrombin via cAMP production in aortic smooth muscle A10 cells. In addition, we showed that DBcAMP, a permeable analog of cAMP [Rasmussen et al., 1968], significantly reduced HSP27 accumulation stimulated by thrombin and that DBcAMP significantly suppressed the thrombin-increased level of mRNA for HSP27. Therefore, based on our findings, it is most likely that the adenylyl cyclase-cAMP system has an inhibitory role on thrombin-stimulated HSP27 induction in aortic smooth muscle A10 cells.

We have demonstrated that p38 MAP kinase activation is involved in the thrombin-stimulated HSP27 induction in aortic smooth muscle A10 cells [Hirade et al., 2002]. Therefore, we investigated the exact mechanism underlying the inhibitory effect of the adenylyl cyclase-cAMP system on thrombin-induced HSP27. To clarify whether the adenylyl cyclase-cAMP system affects thrombin-induced p38 MAP kinase phosphorylation in these cells, we next investigated the effect of forskolin on the p38 MAP kinase phosphorylation stimulated by thrombin. Forskolin markedly suppressed thrombin-stimulated phosphorylaryion of p38 MAP kinase. Thus our result suggests that the adenylyl cyclase-cAMP system suppresses thrombin-stimulated HSP27 induction at a point upstream from p38 MAP kinase in aortic

smooth muscle A10 cells. It has been reported that thrombin binds to its specific receptors, which are coupled to PI-hydrolyzing phospholipase C via a pertussis toxin-sensitive heterotrimeric GTP-binding protein in vascular smooth muscle cells [Grand et al., 1996]. It is well known that phospholipase C hydrolyzes PI, resulting in the formation of diacylglycerol and inositol phosphates [Nishizuka, 1992]. Among these products, diacylglycerol and inositol 1,4,5-trisphosphate serve as messengers for PKC activation and mobilization of Ca^{2+} from the intracellular stores, respectively [Nishizuka, 1992; Berridge, 1993]. We also confirmed that thrombin-stimulated the formation of inositol phosphates in A10 cells. Thus, it is probable that thrombin activates PKC in A10 cells. In addition, staurosporine, an inhibitor of PKC [Tamaoki et al., 1986], significantly reduced HSP27 accumulation stimulated by thrombin. Furthermore, calphostin C, another inhibitor of PKC [Kobayashi et al., 1989], markedly suppressed thrombin-induced phosphorylation of p38 MAP kinase and accumulation of HSP27. These results indicate that thrombin stimulates p38 MAP kinase activation in a PKC-dependent manner to induce HSP27 expression in aortic smooth muscle A10 cells. Moreover, SQ22536, a specific inhibitor of adenylyl cyclase, indeed reversed forskolin-induced inhibition of thrombin-stimulated HSP27 accumulation. These results justify our statement that the adenylyl cyclase-cAMP system inhibits thrombin-induced HSP27 in vascular smooth muscle cells.

We next examined whether the adenylyl cyclase-cAMP system affects activation of p38 MAP kinase induced by TPA, a direct PKC activator [Nishizuka, 1986]. We showed that forskolin inhibited TPA-stimulated HSP27 accumulation and p38 MAP kinase phosphorylation. In addition, DBcAMP significantly suppressed the TPA-increased level of mRNA for HSP27. However, it is unclear whether the suppression is at the transcriptional level or at the post-transcriptional level. We need further investigation to clarify how DBcAMP suppresses the thrombin- or TPA-increased level of mRNA for HSP27. Taking our findings into account, it is probable that the adenylyl cyclase-cAMP system may act as a suppressor in HSP27 induction stimulated by thrombin. The inhibitory effect is exerted at a point around PKC and p38 MAP kinase in aortic smooth muscle A10 cells.

Next, to clarify whether the cAMP system directly affects PKC activity in A10 cells, the phosphorylation status of PKC isoenzyme in forskolin- or DBcAMP-treated A10 cells was analyzed by immunoblot analysis using anti-phospho-PKC antibodies (Fig. 8). Phosphorylations of PKCα/βII (Thr638/641) and PKCθ (Thr538) were not influenced by either forskolin or DBcAMP treatment (data not shown). In contrast, TPA-induced phosphorylation of PKCμ (Ser744/748 and Ser916) and PKCδ (Ser643 and Thr505) was suppressed by either forskolin or DBcAMP treatment (Fig. 8). These results suggest that the cAMP system directly inhibits some PKC- isoenzyme activities. Taken together, these results indicate that the adenylyl cyclase-cAMP system has an inhibitory role in thrombin-stimulated HSP27 induction in aortic smooth muscle cells and the effect seems to be exerted on the thrombin-induced PKC-p38 MAP kinase signaling pathway.

Furthermore, we previously showed that a p38 MAP kinase inhibitor, PD169316, which indeed suppresses thrombin-induced p38 MAP kinase phosphorylation in A10 cells, significantly, but not completely, inhibits thrombin-induced HSP27 expression, indicating that thrombin induces expression of HSP27 at least in part through the p38 MAP kinase pathway [Hirade et al., 2002]. These results suggest that thrombin may activate another intracellular signaling pathway in addition to the p38 MAP kinase pathway for the induction of HSP27. On the other hand, Figure 7 indicates that forskolin partially inhibits TPA-induced p38 MAP kinase phosphorylation, whereas DBcAMP completely inhibits thrombin- or TPA-induced HSP27 mRNA expression, as shown in Figures 2 and 6. Even if the cAMP system inhibits the TPA-induced HSP27 mRNA expression more extensively than the TPA-induced p38 MAP kinase phosphorylation, it is probable for the cAMP system to inhibit TPA- or thrombin-induced HSP27 expression by inhibiting p38 MAP kinase activation. However, it is also possible that the cAMP system may inhibit other thrombin-induced signals than the p38 MAP kinase pathway for HSP27 induction. In addition, Figure 4 indicates that even in the presence of calphostin C, p38 MAP kinase could be activated by thrombin, suggesting that thrombin could induce p38 MAP kinase phosphorylation through both the PKC-dependent- and the PKC-independent-pathway. Therefore,

the detailed mechanism of HSP27 induction by thrombin in vascular smooth muscle A10 cells remains to be clarified to understand how the adenylyl cyclase system inhibits thrombin-stimulated HSP27 induction.

Recently, it has been reported that HSP27 has been identified as an actin binding protein and that HSP27 is involved in the regulation of the actin assembly [Zhu et al., 1994; Landry and Huot, 1995; Kindas-Mugge et al., 1996], leading us to speculate that HSP27 plays an important role in regulation of vascular smooth muscle contraction and vascular tone. Thrombin is a potent vasoactive agent [Walz et al., 1985; Grand et al., 1996]. It has been reported that thrombin induces contraction of vascular smooth muscle through phosphorylation of HSP27 [Brophy et al., 1997, 1998]. On the other hand, it is generally recognized that smooth muscle relaxation results from activation of cAMP-dependent protein kinase and cGMP-dependent protein kinase in the continued presence of the contractile agent [Morgan et al., 1991]. In the present study, the adenylyl cyclase-cAMP system inhibited thrombin-stimulated HSP27 induction. These findings lead us to speculate that the adenylyl cyclase-cAMP system might have a vasodilation effect through suppression of thrombin-stimulated HSP27 induction in vascular smooth muscle cells. Furthur investigation is necessary to clarify the exact role of HSP27 in vascular smooth muscle cells.

In conclusion, the adenylyl cyclase-cAMP system has an inhibitory role in thrombin-stimulated HSP27 induction in aortic smooth muscle cells. The effect seems to be exerted on the thrombin-induced PKC-p38 MAP kinase signaling pathway.

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